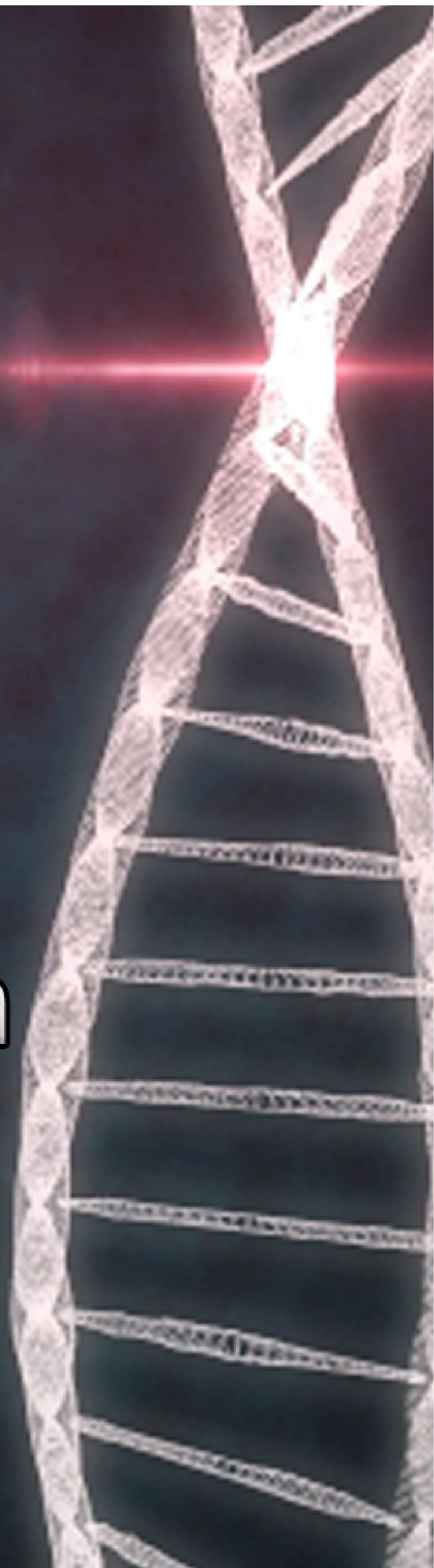
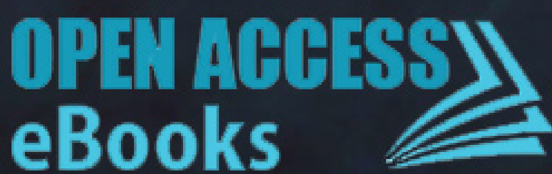


Advances in Biochemistry & Applications in Medicine



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Advances in Biochemistry & Applications in Medicine

Chapter 1

Protein-Protein Interactions as Potential Targets of Drug Designing

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Abstract

Protein–protein interactions (PPIs) control a large number of biological processes. An attempt to design novel drug molecules has led to an increasing interest in the protein surfaces. Studying interaction interfaces in PPIs has set the stage for drug discovery to identify therapeutic agents for a range of human ailments. Inhibiting or stabilizing PPIs with small molecule drugs controls cellular behaviours significantly affecting disease outcomes. On one hand inhibiting PPIs can modulate pathological conditions, while on the other hand stabilizing certain PPIs can potentially treat diseases. It is, therefore, the calculated targeting of specific PPIs, with either small molecule inhibitors or stabilizers, which will have significant pharmacological value. PPIs control regulatory pathways and have been widely studied to design novel chemotherapeutics. Examples of certain small molecule drugs targeting PPIs involved in different biological pathways are discussed here in.

Keywords: Protein–protein interactions; Drug designing; Small molecule inhibitors; Small molecule stabilizers

1. Introduction

The pathology of diseases often lies in a complex web of molecular interactions that needs to be understood both at clinical and molecular levels. Protein–protein interactions are of crucial importance in biological systems and have implications in the development of diseases. Recently, scientists have showed that certain PPIs could be targets of drugs with great significance in healthcare. The importance of the complex network of interactions between proteins and interactome is widely recognized in all biological systems. The human interactome is esti-

mated to involve approximately half a million PPIs, unravelling which, may provide answers to many baffling questions in biology. Moreover, PPIs provide a wealth of information for therapeutic intervention in a host of human diseases. Understanding the architectural interfaces of protein interactions has identified the fine structural features such as pockets, grooves, or clefts as potential docking sites for small drug molecules [1]. The structural intricacies of the interaction interfaces often poses a challenge as the binding sites of PPI surfaces are often formed by native high order protein conformations rather than the linear amino acid sequences, thus preventing the use of a linear peptide templates for designing new therapeutic agents [2]. Another impediment to alternative approach of drug design, is the lack of natural ligands which could mimic the molecular interactions. However, with the identification of "hot spots" in PPIs, it became possible to target a wider range of PPIs with small molecule drugs [3]. The small chemical mimics that modulate PPIs, can directly interfere with protein interactions at the interfaces causing disruption or stabilization of these interactions (**Figure 1**). Targeting PPIs for designing drugs has continued to be a daunting task. However, rapid advances in the understanding of disease processes and their underlying mechanisms highlight multifactorial strategies to identify and design effective drugs [45]. A large number of diseases, including Alzheimer's disease, Parkinson's disease, Huntington's disease and majority of cancers originate from aberrant interactions of specific PPIs. An increasing interest to investigate the unexplored PPIs for drug discovery is driven by the need to find novel therapeutic agents for a whole range of diseases with a high medical relevance. The disruption of PPI with small molecules for drug design has been significantly explored.

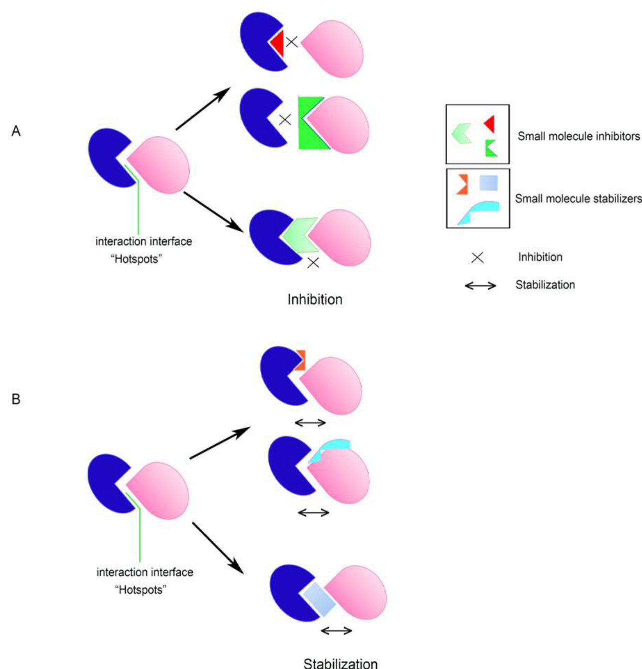

Figure 1: PPI modulators binding to interacting proteins and interaction interfaces. Small molecule inhibitors/stabilizers binds to the individual proteins or together to their binding interfaces there by preventing or reinforcing their interaction. (**A**) Inhibitors bind to interacting proteins either individually or together to cause the inhibition and modulate the interaction kinetics (**B**) Stabilizers bind to the individual interacting proteins or interaction interface together and modulate the interaction kinetics. These small molecule drugs induce a conformational change that inhibits or stabilizes the association with the proteins.

2. Targeting PPIs in Treatment of Cancers

Cancers have been extensively studied to explore PPIs involved in signalling pathways. Interaction of murine double minute 2 (MDM2) with p53 is widely studied PPIs in oncology (**Figure 2A-D**). p53 is a tumor suppressor that plays a critical role in cell cycle regulation, DNA repair and programmed cell death [6]. The mutations in p53, a transcription factor is the cause of almost 50% of human cancers [7] and in most other cancers, the function of p53 is disrupted by one of the several mechanisms. MDM2 is the inhibitor of p53 which directly binds to it and represses its activity by increasing its nuclear export and proteasomal degradation [8]. MDM2 and p53 interact with each other through hydrophobic residues in their N-terminal domains [9]. Scientists have tried to target these hydrophobic residues to identify small molecule drugs that can interrupt MDM2 and p53 interactions [10]. Such pursuits have resulted into designing of several MDM2-p53 interaction disruptors that are already in clinical phase.

Orally administrable imidazoline-based compounds that mimic p53 for binding to MDM2 have been designed. Yet another inhibitor of MDM2, RG7112 was identified by chemical screening and has found use in treatment of sarcoma, leukemia and neoplasms (Fig.2E) [11]. An chemical analogue of RG7112 is a pyrrolidine-containing compound RG7388, which is a more selective and potent p53-MDM2 inhibitor [12]. Many other small chemical molecules identified to selectively target MDM2/p53 interactions are MI-77301, AMG 232 (AM-8553), MK-8242 (SCH 900242), DS-3032b and CGM097 [13].

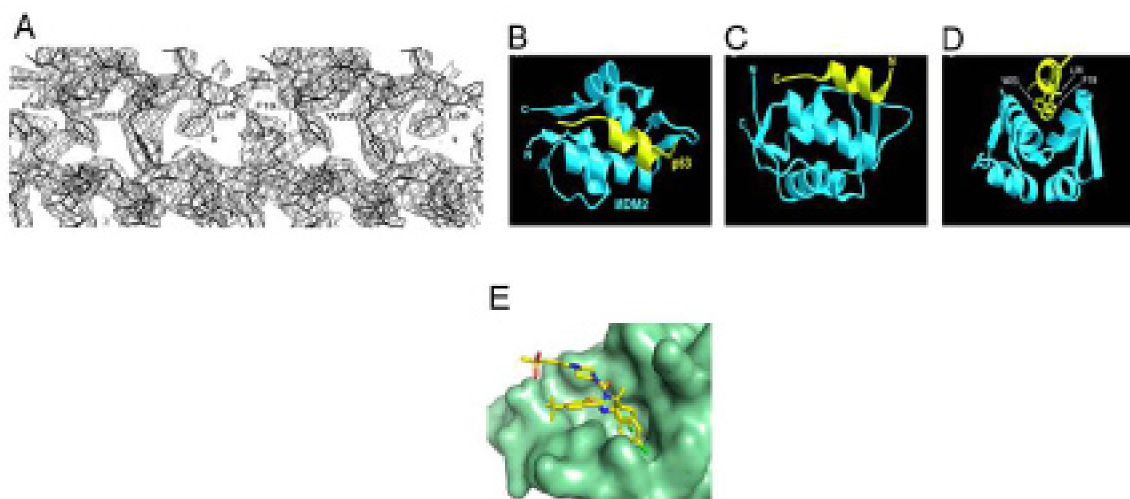

Figure 2: (**A**) Electron density map of the X. laevis MDM2-p53 interface at 3.0 Å resolution with 2.3 Å resolution represented in stick model. Stereo view shows the interactions of F19, W23, and L26 of p53 with the α2 helix of MDM2 10(**B**) The MDM2-p53 complex. MDM2 is in cyan, p53 peptide is in yellow (**C**) 90° rotation of the MDM2-p53 complex about the horizontal axis of (**B**) (**D**) The complex rotated approximately 90° about the vertical axis of (C), looking down the helix axis of p53 [10](**E**) Crystal structure of MDM2 bound to small molecule inhibitor RG7112 (carbon atoms in yellow, oxygen in red, nitrogen in blue, sulfur in orange and chlorine atoms in green) [11]. Figure 2(A-D) adapted from 10 and Figure 2(E) adapted from [12].

Targeting PPIs in apoptosis or programmed cell resulting into activation of caspases can have implications in cancer research. Caspase-9 activates procaspase-3 and procaspase-7 by dimerization into a catalytically active form [14]. The inhibitor of apoptosis proteins (IAPs) which are constitutively active in tumor cells prevent programmed cell death. The XIAP (X-linked IAP) is a target of choice for drug development in cancers. It is by far the most potent caspase inhibitor among the IAP protein family. This protein interacts with initiator caspase-9 and inhibits its dimerization, which is required for its catalytic activity. Over the years, targeting XIAP–caspase interaction for the treatment of cancers has attracted a lot scientific attention. Since the identification of the SMAC protein as a natural inhibitor of XIAP, many SMAC mimics have been designed [15].

The B-cell lymphoma 2 (BCL2) family of proteins are involved in the intrinsic apoptotic pathway [16]. By nuclear magnetic resonance (NMR)-based screening scientists identified ABT-737, a potent small molecule drug that inhibits Bcl2, Bcl-XL, and Bcl2l2 (**Figure 3**) [17]. Further research led to the development of Navitoclax (ABT-263) as a more potent inhibitor of Bcl2 family, both in terms of pharmacokinetics and efficacy. However, suppression of Bcl-xL by ABT-63 led to the development of thrombocytopenia which was further circumvented by designing Bcl2-specific drug ABT-199 (RG7601). Mcl1 has been targeted by small drugs to treat cancers. Increased expression of Mcl-1 leads to sequestration of the proapoptotic proteins Bak, Bax, Bad, and Bim. Thus, targeting Mcl-1 has been effective to disrupt the interaction of Mcl-1 with proapoptotic factors and treat cancers [18]. Many indole-carboxylic acids bind to Mcl-1 particularly at low doses and effectively interrupt Mcl-1/Bim interactions.

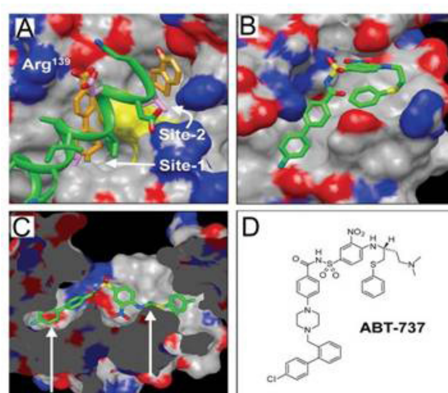

Figure 3: Generation of ABT-737. (**A**) Molecular surface of the complex of Bcl-XLwith a Bak- peptide (GQVGRQLAI-IGDDINR, green) overlaid on the ternary complex of Bcl-XL and NMR-based screening leads (orange). F97 is shown in yellow. Bak peptide residues (E83, L78, I85) critical for binding are shown in magenta. (**B**) Surface view of the NMR structure of Bcl-XL complexed with drug (**C**), Cutaway molecular surface for the NMR structure of domain III of human serum albumin bound to the thioethylamino-2,4-dimethylphenyl analogue of drug inhibitor. Arrows indicate proposed sites of modification. (**D**) Chemical structure of ABT-737. Fig 3 adapted from [17].

Targeting PPIs in the formation of complexes of transcription factors has also yielded significant results in treatment of cancers. Many researchers have effectively targeted bromodomains which mediate interaction of transcription factors to activate oncogenes [19]. These drugs have been efficient anti-cancer agents [20].

3. Drugs against PPIs Involved in Signal Transduction

Cells do not live in isolation and continuously coordinate and communicate with each other. A large variety of small molecules including peptides, proteins etc. act as signaling molecules in cell-to-cell communication. These signalling molecules called "ligands" interact with specific receptors at the surface of cells. Some signaling molecules especially steroids bind intracellular receptors. The binding of a ligand to its receptor leads to conformational changes in the receptor that eventually induces downstream signaling events to show cellular responses. Signalling proteins can act as adaptors or anchoring proteins, mediating PPIs. Owing to the cascade of signalling events, signalling proteins form complexes, either stable or transient with a number of proteins. However, these PPIs are specific and lead to precise downstream signals to affect a cellular response. Over 20 years a considerable progress in understanding of the mechanisms by which ligands bind to the receptors at the plasma membrane to transduce downstream signals has been made.

Stimulation of receptors controls cellular physiology and disruption of ligand-receptor interaction leads to pathological conditions. Targeting ligand-receptors interactions by small drugs could, therefore be important for the treatment of a variety of diseases. Cushing's disease (CD) results from elevated levels of glucocorticoids leading to endogenous hypercortisolism. The glucocorticoids bind to glucocorticoid receptor (GR) to transduce downstream signalling. When unstimulated, GRs form a complex with chaperone HSP90 which prevents the misfolding of GR. HSP90 also facilitates glucocorticoid binding and translocation of the receptor to the nucleus [21]. Silibinin, an extract of milk thistle seeds has been used to treat prostate cancer and liver toxicity and also has therapeutic value in the treatment of CD. Silibinin binds to HSP90 and inhibits its interaction with GR [22]. The CD caused by misregulation of GR sensitivity can be pharmacologically treated with silibinin.

Cytokine interleukin-17A (IL-17A) plays an important role in several immune diseases including psoriasis, psoriatic arthritis, Crohn's disease and rheumatoid arthritis. IL-17A activates proinflammatory genes, increasing production of chemokines, cytokines and other antimicrobial peptides [23]. The interaction between IL receptor and IL-17 has been under investigation to treat inflammatory diseases. Certain IL-17A inhibitors have shown promising activity in clinical studies. A series of small molecule drugs (Ensemblins) that inhibit interaction of IL-17 with its receptor have been synthesized. NMR studies have helped understand the interaction of IL-2 with the α-subunit of its receptor (IL-2Rα). This approach has also helped in designing small molecule inhibitors of this interaction [24]. Tumor necrosis factor α (TNFα) is important for many inflammatory diseases, including inflammatory bowel disease and hepatitis. It can be therapeutically explored for clinical interventions. Chemical inhibitors for TNFα have been designed by computerized systems. (E)-4-(2-(4-chloro-3-nitrophenyl) binds to TNFα and inhibits the TNFα-induced signaling cascade [25]. Many drugs that prevent the

interaction of TNF with its receptor have also been designed and many are under preclinical trials. Another drug inhibiting PPIs in immune signalling is lifitegrast (SAR1118). It inhibits the interaction between LFA-1 and ICAM1 involved in T-cell activation [26].

4. Small Molecule Drugs for Neurological Diseases Treatment

PPIs between membrane-resident receptors and signaling molecules control neuronal function and provide targets for drug design in neuro-pharmacology. Aggregation of amyloid beta (Aβ) peptides in the brain leads to severe neuropathological conditions. Aβ peptides have been considered as a potential target for Alzheimer's disease (AD), as cerebral Aβ deposition plays a key role in the AD. Blocking the aggregation of Aβ peptide with small molecule drugs would, therefore, promise the development of novel therapeutics for AD. Elevated levels of Aβ (42) peptide formation causes early-onset familial AD due to mutations in Aβ precursor protein (AβPP). Lysis of AβPP by the β- and γ-secretases releases the N- and C-termini of the Aβ peptide. L-685 and L-458 have been identified as structurally novel inhibitors of AβPP γ-secretase interaction [27]. Alternatively, benzodiazepine-containing γ-secretase inhibitors have been designed that are potentially important for treatment of AD [28]. Structure-based studies have incorporated a substituted hydrocinnamide C-3 side chain to design highly potent inhibitors of γ-secretase [29].

Parkinson's disease (PD) is another serious neurodegenerative disease associated with protein misfolding and aggregation. Synucleins are small charged proteins primarily expressed in neuronal tissues. Misfolding and aggregation of α-synuclein (α-syn) results in the formation of self-associating β-pleats called "Lewy bodies"[30]. Dopamine inhibits fibrillization of α -syn generating spherical oligomers (**Figure 4**). Mutagenesis and competition assays with synthetic peptides identified 5 amino acid residues (125-129;YEMPS) to be important for the dopamine based inhibition of α-syn fibrillization. More importantly, the oxidation product of dopamine, dopaminochrome has been identified as a more specific inhibitor of α -syn oligomerization. Dopaminochrome inhibits α-syn fibrillization by inducing conformational changes through the interaction with the YEMPS region of α-syn [31]. A study which screened a library of small molecules to identify drugs to inhibit α-syn fibrilization, identified catecholamines as fibril inhibitors [32]. This provides an explanation for the role of α-syn in PD and has implications for chemopreventive and diagnostic applications in future.

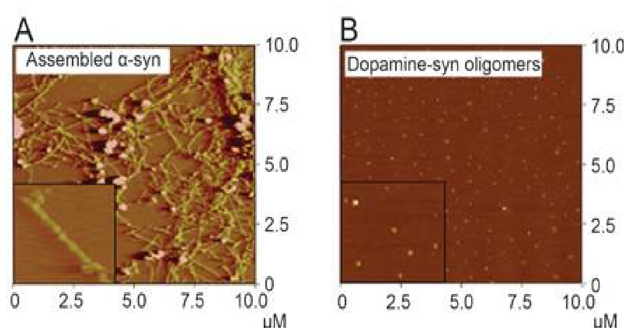

Figure 4: Dopamine inhibits α-syn fibril formation. (**A**) Atomic force microscopy (AFM) images showing the filamentous α-syn after assembly (***B***) AFM images showing spherical oligomers after α-syn is incubated with dopamine and purified (dopamine-syn oligomers). Figure 4 adapted from [31].

Table 1: List of small molecule inhibitors of PPIs discussed in the text. These molecules have been designed to inhibit specific PPIs that modulate biological processes.

PPIs	Small molecule inhibitors
MDM2/p53	RG7112
	MI-77301,
	AMG 232 (AM-8553),
	MK-8242 (SCH 900242),
	DS-3032b
	CGM097
Bcl2 family	ABT-737
	Navitoclax (ABT-263)
	ABT-199 (RG7601)
GR/HSP90	Silibinin
Amyloid beta peptides (AβP)	L-685 and L-458
	benzodiazepine-containing γ-secretase inhibitors
Synuclein	dopamine
	dopaminochrome
	catecholamines
IN/LEDGF	BI 224436
FtsZ/ZipA	indolo[2,3-a]quinolizin-7-one

5. PPIs and Immunity

Humans continually encounter microbes that are harmful and cause diseases. The pathology of these micro-organisms depends on both the pathogenicity of the organism and the robustness of the host immune system. The immune system is an interactive network of lymphoid tissues, circulating immune cells, antibodies, and cytokines. The essential function of the immune system is to recognize and effectively neutralize pathogens. The majority of viruses survive by overtaking the cellular machinery of hosts. PPIs between viral and host proteins or among viral proteins which are essential for their growth and progression in the host are important clinical targets. The retroviruses secrete the enzyme retroviral integrase (IN) which helps it to integrate the retroviral DNA into the DNA of the host. Human protein lens epithelium-derived growth factor (LEDGF) interacts with IN and helps the viral integration into the host genome, further preventing proteolytic degradation of IN. The interaction of LEDGF with IN has been targeted for effective anti-viral therapy. The small molecule inhibitors of this PPI called LEGDINs have been designed by analysing the structural interface of LEDGF-IN interactions. LEGDINs disrupt the LEDG-IN interactions and also act as the allosteric inhibitors of IN [35]. The most important LEDGINs are tert-Butoxy-(4-phenyl-quinolin-3-yl)-acetic acid

derivatives e.g BI 224436 which is an allosteric inhibitor of viral integrase of HIV-1[36,37]. (**Figure 5**).

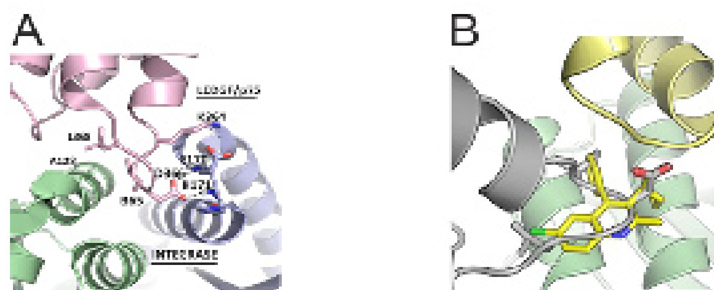

Figure 5: LEDGF/p75–IN interaction and its inhibition by LEDGINs (**A**) Representation of the LEDGF/p75-IN complex. IN molecules are shown in green and blue, whereas the LEDGF/p75 is shown in magenta. Residues critical for the interaction are represented as sticks and highlighted (**B**) Co-crystal structure of tert-Butoxy-(4-phenyl-quinolin-3-yl)-acetic acid derivative (yellow stick) bound in the LEDGF/p75 binding pocket of HIV-IN (green and yellow) . Figure 5 adapted from [35].

PPIs have also been targeted with small molecule drugs to inhibit the replication of human papilloma viruses (HPVs) in host cells. Interaction of E2 transcription factor and E1 initiation factor of human papillomaviruses (HPVs) is essential for the replication of viral DNA. A series of small molecule inhibitors that specifically bind to the N-terminal transactivation domain (TAD) of E2 (of HPV-11) and prevent its interaction with E1 have been described in [38].

In addition to viruses, bacterial PPIs have also been studied to design small molecule chemotherapeutic agents for bacterial infections. A tubulin homologue, FtsZ is involved in prokaryotic replication and is ubiquitous in eubacteria and archea. Like Tubulin, it assembles into protofilaments and mini-rings to form cytoskeletal structures. Recent advances in immunofluorescence and microscopy have showed that FtsZ is localized in septal rings in bacteria [39]. ZipA is a membrane anchored protein involved in the assembly of the septal ring to regulate cell division. X-ray crystallographic studies have showed that FtsZ and ZipA interact through C-termini and this interaction is essential for the bacterial replication [40]. Targeting the interaction of FtsZ and ZipA has become important for antibacterial therapy. A small molecule inhibitor, indolo[2,3-a]quinolizin-7-one targeting the key region of ZipA involved in binding to FtsZ has been synthesized. The crystal structure of this molecule bound to ZipA has also been solved [41]. Moreover, NMR screening coupled with structure-based analysis identified novel inhibitors of the ZipA/FtsZ complex and the X-ray crystal structures of these analogues with ZipA were also solved to gain insights into their structures for synthetic chemistry [42].

6. Stabilizing PPIs

In addition to inhibiting PPIs certain PPIs need to be stabilized for the desired molecular consequences of those interactions. Small molecule stabilizers of PPI either binds to one of the interaction partners or to the interaction interface and stabilize the interactions (**Figure 1B**).

Paclitaxel derived from Taxus brevifolia binds to β-tubulin, thereby stabilizing α-β tubulin polymerization [43,44] (**Figure 6**). This is one of the important anti-cancer drugs used clinically to stabilize monomer stabilization in microtubule assembly. Similarly other natural products like FK506 , rapamycin, fusicoccin, brefeldin and forskolin modulate disease conditions by stabilizing specific PPIs [45,46]. FK506 and rapamycin bind tightly to FKBP12, stabilizing FKBP12/calcineurin and FKBP12/mTOR interactions respectively. Recently a number of synthetic molecules have been reported to stabilize oligomeric interactions. Mizoribine (MIZ) an imidazole based nucleoside interacts with 14-3-3 proteins. 14-3-3 proteins interact with many signalling components, including the glucocorticoid receptor (GR). MIZ affects the conformation of 14-3-3 proteins and stabilizes its interaction with GR [47]. Many of the PPI stabilizers such cyclosporine, rapamycin, glycosides fusicoccin A and cotylenin A act as immunosuppressants and have been used as therapeutics for different diseases [48,49].

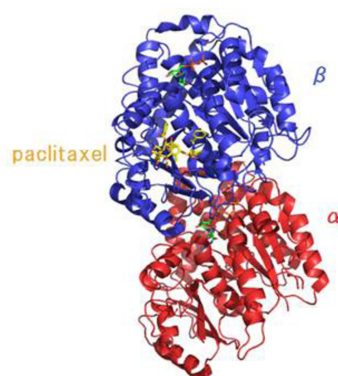

Figure 6: Binding of drug paclitaxel (yellow) to the complex of α- (red) and β-tubulin (blue) subunits. This file has been created from PDB file id= [2HXF http://www.rcsb.org/pdb/explore/explore.do?structureId= 2HXF] (Source: Takumasa)

PPIs	Small molecule stabilizers
Tubulin monomers	Paclitaxel
FKBP12/mTOR	Rapamycin
FKBP12/calcineurin	FK506
14-3-3/GR	Mizoribine
14-3-3	Cotylenin A

Table 2: List of certain small molecule stabilizers of PPIs discussed in the text. These molecules have been designed to stabilize specific PPIs to modulate cellular processes.

7. Use of Computational Methods to Design Drugs for Specific PPIs.

New approaches, driven by improved computational methodologies, have allowed the use of computer-assisted design of structure-based drugs. Novel computational tools have been used to understand PPIs and design small molecule drugs. Studying a PPI as a potential drug target requires identification and characterization of the binding interface. A number of computational methods have been used to identify PPI interfaces [50]. With faster high-throughput screening, thousands of molecules can be assayed with robotic automation. The serious drawback of using computations for analysing PPIs is that it is expensive and requires

huge resources. Computational methods often rely on the protein structure and also become useful in the cases where the structure cannot be determined by experimental methods. Several computational methods have been used for protein structure prediction including homology modeling [52] threading approaches [53], and ab initio folding [54]. Using computational methods for studying PPIs can aid in;

7.1. Predicting PPI interfaces

Studying a PPI as a potential therapeutic target requires identification and characterization of the binding interfaces. A number of computational methods have been used to identify PPI interfaces from protein structures [55,56]. Methods based on the similarity of interface regions have been successfully used to predict the topography of binding interfaces, but require a reference template [57]. However, due to protein flexibility, it is easier to identify a binding interface from an isolated PPI complex. In addition, computational methods could be very useful to identify potential allosteric binding sites modulating PPIs. Although efforts have been made in this direction, it still remains an area with the scope of significant improvement [58].

7.2. Identifying binding hotspots

Hotspots at protein surfaces can be identified by a number of methods 59 such as the alanine scanning energetics database [60], the binding interface database [61] and the HotSprint database [62]. Softwares have been generated that accurately predict the protein surface hotspots, such as the HotPoint and the ligand binding hotspots, such as SiteMap [63,64].

7.3. Modeling molecular flexibility

Protein flexibility is a very important feature of recognition kinetics in PPIs. This is also true for the interactions between proteins and small-molecule drugs. Thus conformational flexibility must be considered for the efficiency of computational methods in modeling a broad range of PPIs. Using predefined structural ensembles, where an ensemble of multiple protein structures is used for analysis rather than a single protein, has proved useful in molecular docking. For selecting the ensemble, protein structures can be determined by NMR or X-ray crystallography or computationally by molecular dynamics [65]. Ensemble selection is a critical determinant for performing the molecular docking, assessing druggbility and hotspot identification. However, this computational area needs improvement to predict the PPIs.

7.4. Virtual screening

It is used to enrich libraries for small molecules with an increased likelihood of hitting a particular target. There are, however, a number of pitfalls associated virtual screening that should be understood before using it. It might be expected that virtual screening has greater utility in identifying small molecule targets of PPIs. However, the majority of these methods

have been optimized for hidden active sites, and it is not yet clear whether these will translate to making calculations at protein surfaces.

8. Future Perspectives

Drug discovery for identifying effective therapeutic agents is target driven and PPIs have emerged as attractive molecular targets for drug designing. PPIs regulate almost all biological processes, including cell growth and development, pathological conditions and signaling pathways. The study of protein networks has provided mechanistic insights into cellular processes governed by signalling molecules. Understanding how proteins bind and communicate to initiate cellular events has revealed the molecular basis of different diseases especially cancer. Classic small molecule drugs consist mainly of planar molecules where as successful disruption of interaction requires interaction in 3D. Improved design of small drug molecules needs to consider conformational changes for the binding kinetics of interactions. Additionally, peptides that could specifically target PPIs with improved efficiency need to be designed.

Traditional drugs have mainly targeted distinct binding clepts. However, with the advent of new technologies and improved design, finer architectural features of protein-protein interfaces have been understood. The recent knowledge of how small drug molecules bind to protein interfaces has opened up new vistas for drug development. Targeting PPIs for treatment of diseases constitutes an active area of research and structural aspects of protein binding need to be studied to design novel therapeutics with clinical advantages.

As no major breakthrough has been achieved by using computational methods for optimum screening conditions and designing desirable architectural patterns of drug molecules for PPI inhibition or stabilization. Therefore developing new methodologies, for both experimental and computational strategies, to target PPIs is needed as it would lead to the design of molecules capable of modulating new, more specific and previously undruggable targets.

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10. References

1. Arkin, M. R. & Wells, J. A. Small-molecule inhibitors of protein–protein interactions: progressing towards the dream. Nat. Rev. Drug Discov. 3, 301–317 (2004).

2. Arkin, M. R., Tang, Y. & Wells, J. A. Small-molecule inhibitors of protein-protein interactions: Progressing toward the reality. Chemistry and Biology 21, 1102–1114 (2014).

3. Guo, W., Wisniewski, J. A. & Ji, H. Hot spot-based design of small-molecule inhibitors for protein-protein interac-

tions. Bioorganic Med. Chem. Lett. 24, 2546–2554 (2014).

4. Jacunski, A. & Tatonetti, N. P. Connecting the Dots: Applications of Network Medicine in Pharmacology and Disease. Clin. Pharmacol. Ther. 94, 659–669 (2013).

5. Janero, D. R. The future of drug discovery: enabling technologies for enhancing lead characterization and profiling therapeutic potential. Expert Opin. Drug Discov. 441, 1–12 (2014).

6. Brown, C. J., Lain, S., Verma, C. S., Fersht, A. R. & Lane, D. P. Awakening guardian angels: drugging the p53 pathway. Nat. Rev. Cancer 9, 862–873 (2009).

7. Feki, A. & Irminger-Finger, I. Mutational spectrum of p53 mutations in primary breast and ovarian tumors. Critical Reviews in Oncology/Hematology 52, 103–116 (2004).

8. Wu, X., Bayle, J. H., Olson, D. & Levine, A. J. The p53-mdm-2 autoregulatory feedback loop. Genes Dev. 7, 1126–1132 (1993).

9. Capoulade, C. et al. Overexpression of MDM2, due to enhanced translation, results in inactivation of wild-type p53 in Burkitt's lymphoma cells. Oncogene 16, 1603–1610 (1998).

10. Kussie, P. H. et al. Structure of the MDM2 Oncoprotein Bound to the p53 Tumor Suppressor Transactivation Domain. Science (80-.). 274, 948–953 (1996).

11. Vu, B. et al. Discovery of RG7112: A small-molecule MDM2 inhibitor in clinical development. ACS Med. Chem. Lett. 4, 466–469 (2013).

12. Ding, Q. et al. Discovery of RG7388, a potent and selective p53-MDM2 inhibitor in clinical development. J. Med. Chem. 56, 5979–5983 (2013).

13. Zhao, Y., Aguilar, A., Bernard, D. & Wang, S. Small-molecule inhibitors of the MDM2-p53 protein-protein interaction (MDM2 inhibitors) in clinical trials for cancer treatment. Journal of Medicinal Chemistry 58, 1038–1052 (2015).

14. Moore, C. D. et al. Structural and biophysical characterization of XIAP BIR3 G306E mutant: Insights in protein dynamics and application for fragment-based drug design. Chem. Biol. Drug Des. 74, 212–223 (2009).

15. Sun, H. et al. Design of small-molecule peptidic and nonpeptidic smac mimetics. Acc. Chem. Res. 41, 1264–1277 (2008).

16. Reed, J. C. Bcl-2 family proteins. Oncogene 17, 3225–3236 (1998).

17. Oltersdorf, T. et al. An inhibitor of Bcl-2 family proteins induces regression of solid tumours. Nature 435, 677–681 (2005).

18. Calcul, L. et al. NMR strategy for unraveling structures of bioactive sponge-derived oxy-polyhalogenated diphenyl ethers. J. Nat. Prod. 72, 443–449 (2009).

19. Filippakopoulos, P. & Knapp, S. Targeting bromodomains: Epigenetic readers of lysine acetylation. Nature Reviews Drug Discovery 13, 337–356 (2014).

20. Filippakopoulos, P. et al. Selective inhibition of BET bromodomains. Nature 468, 1067–1073 (2010).

21. Kirschke, E., Goswami, D., Southworth, D., Griffin, P. R. & Agard, D. A. Glucocorticoid receptor function regulated by coordinated action of the Hsp90 and Hsp70 chaperone cycles. Cell 157, 1685–1697 (2014).

22. Riebold, M. et al. A C-terminal HSP90 inhibitor restores glucocorticoid sensitivity and relieves a mouse allograft model of Cushing disease. Nat. Med. 21, 276–280 (2015).

23. Kirkham, B. W., Kavanaugh, A. & Reich, K. Interleukin-17A: A unique pathway in immune-mediated diseases:

Psoriasis, psoriatic arthritis and rheumatoid arthritis. Immunology 141, 133–142 (2014).

24. Emerson, S. D. et al. NMR characterization of interleukin-2 in complexes with the IL-2R alpha receptor component, and with low molecular weight compounds that inhibit the IL-2/IL-R alpha interaction. Protein Sci. 12, 811–822 (2003).

25. Ma, L. et al. A novel small-molecule tumor necrosis factor α inhibitor attenuates inflammation in a hepatitis mouse model. J. Biol. Chem. 289, 12457–12466 (2014).

26. Long, E. O. ICAM-1: Getting a Grip on Leukocyte Adhesion. J. Immunol. 186, 5021–5023 (2011).

27. Shearman, M. S. et al. L-685,458, an aspartyl protease transition state mimic, is a potent inhibitor of amyloid β-protein precursor γ-secretase activity. Biochemistry 39, 8698–8704 (2000).

28. Churcher, I. et al. Design and synthesis of highly potent benzodiazepine gamma-secretase inhibitors: preparation of (2S,3R)-3-(3,4-difluorophenyl)-2-(4-fluorophenyl)-4- hydroxy-N-((3S)-1-methyl-2-oxo-5- phenyl-2,3-dihydro-1H-benzo[e][1,4]-diazepin-3-yl)butyramide by use of a. J. Med. Chem. 46, 2275–2278 (2003).

29. Churcher, I. et al. A new series of potent benzodiazepine gamma-secretase inhibitors. Bioorg. Med. Chem. Lett. 13, 179–183 (2003).

30. Norris, E. H., Giasson, B. I. & Lee, V. M.-Y. Alpha-synuclein: normal function and role in neurodegenerative diseases. Curr. Top. Dev. Biol. 60, 17–54 (2004).

31. Norris, E. H. et al. Reversible inhibition of alpha -synuclein fibrillization by dopaminochrome mediated conformational alterations. J.Biol.Chem. (2005).

32. Conway, K. A., Rochet, J. C., Bieganski, R. M. & Lansbury Jr, P. T. Kinetic stabilization of the a-synuclein protofibril by a dopamine-a-synuclein adduct. Science (80-.). 294, 1346–1349 (2001).

33. Bertaso, F. et al. PICK1 uncoupling from mGluR7a causes absence-like seizures. Nat. Neurosci. 11, 940–948 (2008).

34. Sharma, S. C., Rupasinghe, C. N., Parisien, R. B. & Spaller, M. R. Design, synthesis, and evaluation of linear and cyclic peptide ligands for PDZ10 of the multi-PDZ domain protein MUPP1. Biochemistry 46, 12709–12720 (2007).

35. Christ, F. & Debyser, Z. The LEDGF/p75 integrase interaction, a novel target for anti-HIV therapy. Virology 435, 102–109 (2013).

36. Fader, L. D. et al. Discovery of BI 224436, a noncatalytic site integrase inhibitor (NCINI) of HIV-1. ACS Med. Chem. Lett. 5, 422–427 (2014).

37. Fenwick, C. et al. Preclinical profile of BI 224436, a novel hiv-1 non-catalytic-site integrase inhibitor. Antimicrob. Agents Chemother. 58, 3233–3244 (2014).

38. Wang, Y. et al. Crystal structure of the E2 transactivation domain of human papillomavirus type 11 bound to a protein interaction inhibitor. J. Biol. Chem. 279, 6976–85 (2004).

39. Erickson, H. P. FtsZ, a tubulin homologue in prokaryote cell division. Trends in Cell Biology 7, 362–367 (1997).

40. Mosyak, L. et al. The bacterial cell-division protein ZipA and its interaction with an FtsZ fragment revealed by X-ray crystallography. EMBO J. 19, 3179–3191 (2000).

41. Jennings, L. D. et al. Design and synthesis of indolo[2,3-a]quinolizin-7-one inhibitors of the ZipA-FtsZ interaction. Bioorganic Med. Chem. Lett. 14, 1427–1431 (2004).

42. Tsao, D. H. H. et al. Discovery of novel inhibitors of the ZipA/FtsZ complex by NMR fragment screening coupled with structure-based design. Bioorganic Med. Chem. 14, 7953–7961 (2006).

43. Downing, K. H. Structural Basis for the Interaction of Tubulin with Proteins and Drugs that Affect Microtubule Dynamics. Annu. Rev. Cell Dev. Biol. 16, 89–111 (2000).

44. Jordan, M. Mechanism of Action of Antitumor Drugs that Interact with Microtubules and Tubulin. Curr. Med. Chem. Agents 2, 1–17 (2012).

45. Harding, M. W., Galat, A., Uehling, D. E. & Schreiber, S. L. A receptor for the immuno-suppressant FK506 is a cis–trans peptidyl-prolyl isomerase. Nature 341, 758–760 (1989).

46. Giordanetto, F., Schäfer, A. & Ottmann, C. Stabilization of protein-protein interactions by small molecules. Drug Discovery Today 19, 1812–1821 (2014).

47. Takahashi, S., Wakui, H., Gustafsson, J.-Å., Zilliacus, J. & Itoh, H. Functional Interaction of the Immunosuppressant Mizoribine with the 14-3-3 Protein. Biochem. Biophys. Res. Commun. 274, 87–92 (2000).

48. Bier, D., Thiel, P., Briels, J. & Ottmann, C. Stabilization of Protein-Protein Interactions in chemical biology and drug discovery. Progress in Biophysics and Molecular Biology 119, 10–19 (2015).

49. Milroy, L. G., Grossmann, T. N., Hennig, S., Brunsveld, L. & Ottmann, C. Modulators of protein-protein interactions. Chemical Reviews 114, 4695–4748 (2014).

50. Fernández-Recio, J. Prediction of protein binding sites and hot spots. Wiley Interdisciplinary Reviews: Computational Molecular Science 1, 680–698 (2011).

51. Chen, H. & Zhou, H. X. Prediction of interface residues in protein-protein complexes by a consensus neural network method: Test against NMR data. Proteins Struct. Funct. Genet. 61, 21–35 (2005).

52. Zhang, C. & Lai, L. Towards structure-based protein drug design. Biochem. Soc. Trans. 39, 1382–1386 (2011).

53. Lemer, C. M., Rooman, M. J. & Wodak, S. J. Protein structure prediction by threading methods: evaluation of current techniques. Proteins 23, 337 (1995).

54. Lee, J., Wu, S. & Zhang, Y. Ab initio protein structure prediction. in From Protein Structure to Function with Bioinformatics 3–25 (2009).

55. Burgoyne, N. J. & Jackson, R. M. Predicting protein interaction sites: Binding hot-spots in protein-protein and protein-ligand interfaces. Bioinformatics 22, 1335–1342 (2006).

56. Fuller, J. C., Burgoyne, N. J. & Jackson, R. M. Predicting druggable binding sites at the protein. "protein interface. Drug Discov. Today 14, 155–161 (2009).

57. Tuncbag, N., Keskin, O., Nussinov, R. & Gursoy, A. Fast and accurate modeling of protein-protein interactions by combining template-interface-based docking with flexible refinement. Proteins Struct. Funct. Bioinforma. 80, 1239–1249 (2012).

58. Demerdash, O. N. A., Daily, M. D. & Mitchell, J. C. Structure-based predictive models for allosteric hot spots. PLoS Comput. Biol. 5, (2009).

59. Villoutreix, B. O. et al. Drug-like protein-protein interaction modulators: Challenges and opportunities for drug discovery and chemical biology. Molecular Informatics 33, 414–437 (2014).

60. Thorn, K. S. & Bogan, A. A. ASEdb: a database of alanine mutations and their effects on the free energy of binding in protein interactions. Bioinformatics 17, 284–285 (2001).

61. Fischer, T. B. et al. The binding inteference database (BID): A compilation of amino acid hot spots in protein interfaces. Bioinformatics 19, 1453–1454 (2003).

62. Guney, E., Tuncbag, N., Keskin, O. & Gursoy, A. HotSprint: Database of computational hot spots in protein inter-

faces. Nucleic Acids Res. 36, (2008).

63. Tuncbag, N., Gursoy, A. & Keskin, O. Identification of computational hot spots in protein interfaces: Combining solvent accessibility and inter-residue potentials improves the accuracy. Bioinformatics 25, 1513–1520 (2009).

64. Halgren, T. A. Identifying and characterizing binding sites and assessing druggability. J. Chem. Inf. Model. 49, 377–389 (2009).

65. Cheng, L. S. et al. Ensemble-based virtual screening reveals potential novel antiviral compounds for avian influenza neuraminidase. J. Med. Chem. 51, 3878–3894 (2008).

Chapter 2

Nanotechnology and Nanomedicine: Going Small Means Aiming Big

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Abstract

Nanotechnology is an emerging multidisciplinary field of science and technology that works at the molecular and atomic level. It has a major contribution in the development of key novel products in different regimes of science. In field of medicine, development of novel imaging agents, pharmaceutical nano-formulations are being developed for the betterment of the human health. Drug delivery using nanoparticles is an attractive advancement in medicine that enables targeted drug-delivery to attain attractive therapeutic efficacy. Recently used drug delivery system includes, polymeric micelles, liposomes, dendrimers and many others reveal a wide variety of useful properties. Conventional drug delivery methods represent several disadvantages because of off-target action and shows severe toxic health effects. Nanomedicine is an approach of nanotechnology, being applied for diagnosis and treatment of highly infectious diseases like cardiovascular diseases, Alzheimer's disease, Parkinson's disease, using nanoscale particles and nanorobots which is a major risk to mankind. Here, we provide a broad overview of nanoparticles, its novel applications, formulations and commercialization in the field of medicine using synthetic and natural nanopolymers.

Keywords: nanotechnology; nanomedicine; nanoparticles; drug delivery; cancer therapy.

1. Introduction

Nanotechnology is an area of science that involves working with materials and devices on a nanoscale level. This molecular level investigation is at a range usually below 100 nm. On scalable terms, a nanometer is approximately 1/80,000 of the diameter of a human hair,

or 10 times the diameter of a hydrogen atom (**Figure 1**). Advancements in the emergence of biological probes, nanomaterials and analytical tools of nanoscale range, referred to as "nano-technology" are currently being applied in diagnosis and treatment of human health disorders. Its functions are spread across all areas of sciences including physics, chemistry and biology.

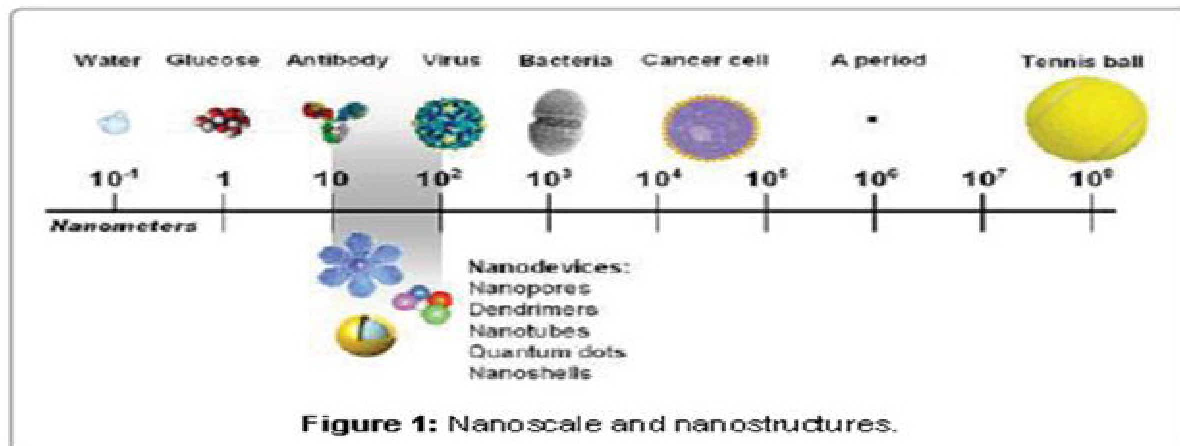


Figure 1: Nanoscale and nanostructures [1]

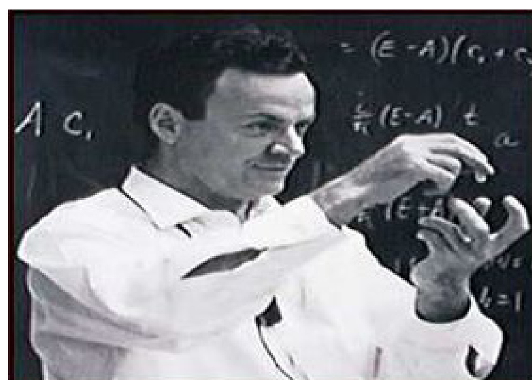
Nanotechnology has grown in leaps and bounds over the last few years. Although, the initial properties of nanomaterials studied were for its physical, mechanical, electrical, magnetic, chemical and biological applications, recently, attention has been geared towards its pharmaceutical application, especially in the area of drug delivery. Nanotechnology will offer the tools to explore the frontiers of medical science at a cellular level. Nanostructures display unique mechanical, electrical, chemical and optical properties. Understanding and controlling such properties is challenging, but harnessing them will provide exciting new opportunities for research, diagnosis and therapy of heart, lung, and blood and sleep disorders. Applications of nanotechnology for the diagnosis, treatment, monitoring and control of biological systems have recently been called as "Nanomedicine" by the National Institutes of Health (NIH).

Recent researches in nanodrug delivery have been designed to overcome the challenges through the development and fabrication of nanostructures. The technology helps in delivery of drugs that are poorly water soluble and can provide means of bypassing the liver, thereby preventing the first pass metabolism. Nanotechnology improves performance and acceptability of dosage forms by increasing their effectiveness, safety, patient adherence, as well as ultimately reducing health care costs. It may also enhance the performance of drugs that are unable to pass clinical trial phases. It definitely promises to serve as drug delivery carrier of choice for the more challenging conventional drugs used for the treatment and management of chronic diseases such as cancer, asthma, hypertension, HIV and diabetes.

2. A Brief History of Nanotechnology

'Nano' comes from the Greek word 'dwarf'. Nanotechnology is defined as the research and development of materials, devices, and systems exhibiting physical, chemical and biological properties that are different from those found on a larger scale (matter smaller than scale of

things like molecules and viruses). The vision of nanotechnology introduced in 1959 by late Nobel Physicist Richard P Feynman in dinner talk said, “There is plenty of room at the bottom,” [2].



R. P. Feynman- Father of Nanotechnology

He suggested nanomachines, nanorobots and nanodevices ultimately could be used to develop a wide range of automatically precise microscopic instrumentation and manufacturing tools, could be applied to produce a vast quantity of ultra small computers and various nanoscale microscale robots.

3. Potential of Nanotechnology

Nanotechnology has received a lot of consideration because of its future prospective that can factually reform each field in which it is being applied. The current drug delivery systems are remnants of conventional drug delivery mechanisms that occur in the nanometer array like dendrimers, liposomes, nanocrystals and polymeric micelles, referred to as “nanovehicles”. Colloidal gold nanoparticles were first prepared by Michael Faraday more than 150 years ago, but were never related with nanotechnology till now. For targeting the staining techniques, the particles were conjugated with antibodies termed as ‘immunogold staining’ and it may be considered as a precursor of recent explosive applications of gold particles in nanotechnology. The importance of nanotechnology in drug delivery is its ability to manipulate the molecules and supramolecular structures to generate the devices with programmed utility.

4. Clinical Efficacy of Nanotechnology in Drug Delivery

Clinically useful drug delivery systems need to deliver a certain amount of a drug that can be therapeutically effective over an extensive period of time. Such prerequisites can be met by the nano scale drug delivery system produced by nanotechnology. The existing techniques of constructing nanoparticles are chiefly based on double emulsion methods or solvent exchange technique. The main drawbacks with the existing methods are the low drug loading capability, low loading effectiveness and reduced ability to control the size distribution. Utilizing nanotechniques such as nanopatterning may allow construction of nanoparticles with high loading efficiency and highly homogeneous particle sizes [3].

5. Nanoparticles as Drug Delivery Carrier

Nanoparticles have been documented to in use since the use 9th century in Mesopotamia for ancient pottery. Nanoparticles represent a promising drug delivery system of controlled and targeted release. A schematic comparison of untargeted and targeted drug delivery systems is shown in **Figure 2**. Nanoparticles cover mostly all types of sciences and manufacturing technologies. The properties of this particle are flying over today scientific barriers and have passed the limitations of conventional sciences. This is the reason why nanoparticles have been evaluated for the use in many fields.

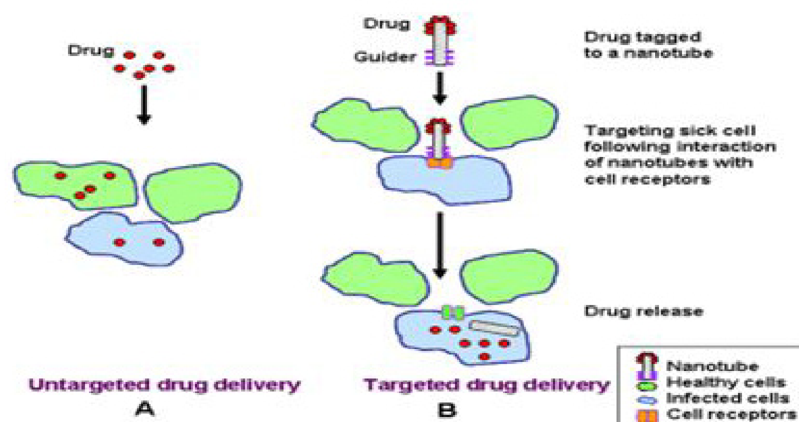


Figure 2: Untargeted and targeted drug delivery systems [4]

5.1. Assets of nanoparticles

Several properties of nanoparticles that are important for application in drug delivery mechanism comprise simple, inexpensive manufacturing method that is easy to scale up. The manufacturing process eliminates potentially toxic ingredients or organic solvents. All the components of the formulation should be commercially accessible, affordable, non-hazardous, safe and eco-friendly. The nanoparticles should be stable with respect to size, size distribution, surface morphology and other significant physical and chemical properties.

6. Formulations of Nanoparticles used in Drug Delivery Mechanism

Nanoparticles applied in drug delivery mechanisms are submicronsized particles (3-200 nm), or devices that uses a wide variety of materials which includes viruses (viral nanoparticles), polymers (polymeric nanoparticles, micelles, or dendrimers), lipids (liposomes) and organometallic compound (**Table 1**).

6.1. Polymer-drug conjugates

Polymer–drug conjugates come under the class of polymer therapeutics which consists of water-soluble polymers which are chemically conjugated to a drug through a biodegradable linker **(Figure 3B)**. The idea came into subsistence in 1975 when Ringsdorf proposed the use of polymer–drug conjugates to transport hydrophobic small molecules [6] because small drug molecules, particularly hydrophobic compounds have a low aqueous solubility and an

extensive tissue distribution profile such that administration of the free drug may cause severe side effects. Therefore, conjugation of these compounds to the biocompatible, hydrophilic polymers would considerably increase their aqueous solubility, modify their tissue distribution profile and boost the half-life in plasma circulation. The colloidal character or size of these vehicles can assist their retention within the circulation for extended periods as compared with small low molecular weight molecules.

Polymer-conjugate technology has proved to be a feasible formulation approach. A number of reports are available [7,8] on bioconjugation of protein and peptide to PEG that significantly improve the usefulness of these polymer drugs by decreasing their immunogenicity and increasing their stability in the presence of proteases. In 1994, in therapeutics, these were first used in anti-cancer therapy approved by FDA through introduction of PEG-L-asparagines (Oncaspar1). The conjugate consists of polymer of PEG having a molecular weight of 5Kd which is attached to the L-asparagines enzyme for the treatment of acute lymphoblastic leukemia [9].

Table 1: Types of nanocarriers for drug delivery [5]

System	Structure	Characteristics	Examples of compounds
Polymeric nanoparticles (polymer-drug conjugates)	Drugs are conjugated to theside chain of a linear polymer with a linker (deavable bond)	(a)Water-soluble, nontoxic, biodegradable (b)Surface modification (pegylation) (c)Selective accumulation and retention in tumor tissue (EPR effect) (d)Specific targeting of cancer cells while sparing normal cells—receptor-mediated targeting with a ligand	Albumin-Taxol (Abraxane) PGA-Taxol (Xyotax) PGA-Camptotheon (CT-2106) HPMA-DOX (PK1) HPMA-DOX- galactosamine (PK2)
Polymeric micelles	Amphiphilic block copolymers assemble and form a micelle With a hydrophobic core and hydrophilic shell	(a)Suitable carrier for water-Insoluble drug (b)Biocompatible, self-assembling, biodegradable (c)Ease of functional modification (d)Targeting potential	PEG-pluronic-DOX PEG-PAA-DOX (NK911) PEG-PLA-TaXol (GeneXol-PM)
Dendrimers	Radially emerging hyperbranched synthetic hyperbranched synthetic and repeated units	(a)Biodistribution and PK can be tuned (b)High structural and chemical homogeneity (c)Ease of functionalizati on, high ligand density (d)Controlled degradation (e)Multifunctionality	PAMAM-MTX PAMAM-plati nate
Liposomes	Self-assembling closed colloidal structures composed of lipid bilayers	(a)Amphiphilic, blocompatible (b)Ease of modification (c)Targeting potential	Pegylated liposomal DOX (Doxil) Non- pegylated liposomal DOX (Myocet) Liposomal daunorubicin

Viral nanoparticles	Protein cages, which are multivalent, self-assembled structures	(a)Surface modification by mutagenesis or bioconjugation—multivalenCY (b)Specific tumor targeting, multifunctional-ity (c)Defined geometry and remarkable unifor-mity (d)Biological compatibility and inert nature	HSP-DOX CPMV-DOX
Carbon nanotubes	Carbon cylinders composed of benzene ring	(a) Water-soluble and biocompatible through chemical modification (organic functlonaliza-tion) (b) Multifunctionality	CNT-MTX CNT-amphoteri O n B

Abbreviations: PGA: poly-(L-glutamate); HPMA: N-(2- hydroxypropyI)-methacrylamide copolymer; PEG: polyethylene glycol; PAA: poly-(--aspartate); PLA: poly-(.-lactide); PAMAM: poly(amidoamine); DOX: doxorubicin; MTX: methotrexate; PK: pharmacokinetics; EPR: enhanced permeability and retention; CNT: carbon nanotube; HSP: heat shock protein; CPMV: cowpea mosaic virus.

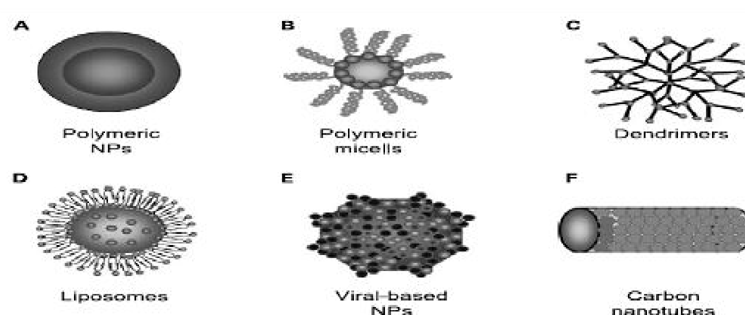


Figure 3: Types of nanocarriers for drug delivery. A, polymeric nanoparticles. B, polymeric micelles. C, dendrimers. D, liposomes. E, viral-based nanoparticles. F, carbon nanotubes [5].

6.2. Polymeric micelles

The efficient properties of micelles are based on amphiphilic block copolymers, which bring together to form a nano sized shell structure in aqueous media (**Figure 3B**). The reservoir for hydrophobic drugs are hydrophobic core region while the hydrophilic shell region stabilizes the hydrophobic core and turn into water-soluble polymers, making it an apposite candidate for *in vitro* administration [10]. The drug can be laden into a polymeric micelle in two ways: physical encapsulation [11] or chemical covalent binding [12]. Paclitaxel, Genexol-PM (PEG-poly (D, L-lactide-paclitaxel) is the first formulation of polymeric micelle which is a cremophor- free polymeric micelle-formulated paclitaxel. Multifunctional polymeric micelles with targeting ligands and imaging and therapeutic agents are currently being actively developed [13] which will become the conventional between numerous models of micelle formulation in future.

6.3. Dendrimers

A dendrimer is a synthetic polymeric macromolecule (nm), consists of several extremely pronged monomers that appear radially from the central core (**Figure 3C**). Properties associated with these dendrimers such as their monodisperse size, modifiable surface functionality, multivalency, water solubility, and available internal cavity make them attractive for drug

delivery [14]. Polyamidoamine dendrimer, the dendrimer most widely used as a scaffold, was conjugated with cisplatin [15]. The easily modifiable surface characteristic of dendrimers enables them to be simultaneously conjugated with several molecules such as imaging contrast agents, targeting ligands, or therapeutic drugs, yielding a dendrimer-based multifunctional drug delivery system [14].

6.4. Lipid coated nanoparticles (liposomes)

Liposomes are self-assembled closed colloidal structures consists of lipid bilayers with spherical shape in which a central aqueous space is present surrounded by an outer lipid bilayer (**Figure 3D & 4**). At present, different types of anticancer drugs have been applied to the lipid-based system by using an array of preparation techniques. Amongst all, lipid-based formulations of the anthracyclines doxorubicin (Doxil, Myocet) and daunorubicin (DaunoXome) are approved for the treatment of AIDS-related Kaposi's sarcoma and metastatic breast cancer [16]. Furthermore, many liposomal chemotherapeutics approved agents are recently being assessed in clinical trials [17]. The next generation liposomal drugs may be immunoliposomes, which selectively release the drug to the preferred site of action [18].

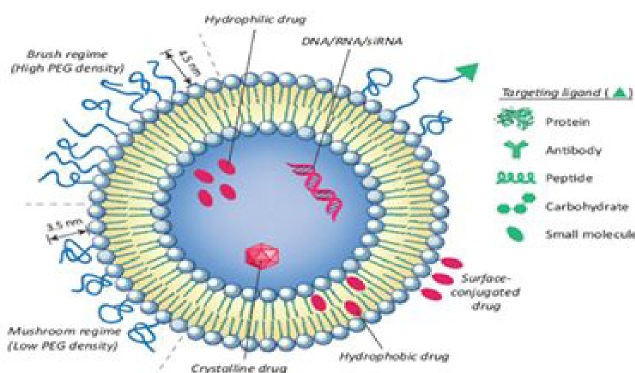


Figure 4: A Liposome used as nanoparticle for drug delivery

6.5. Viral nanoparticles

A diversity of viruses like, canine parvovirus, cowpea chlorotic mottle virus, cowpea mosaic virus and bacteriophages have been constructed for biomedical applications which consist of tissue targeting and drug delivery (**Figure 3E**). A number of peptides and targeting molecules can be exhibited in a biologically active form on the surface their capsid by using genetic or chemical processes. Therefore, a large number of ligands or antibodies plus together with folic acid, transferrin and single-chain antibodies have been conjugated in viruses for *in vivo* tumor specific targeting [19]. Through targeting of heat shock protein, specific targeting can be achieved by a dual-function protein cage that leads to the encapsulation of doxorubicin [20].

6.6. Carbon nanotubes

Carbon nanotubes are carbon cylinders consisting of benzene rings (**Figure 3F**) cur-

rently being applied as sensors in biology for detecting protein and DNA, carriers to transport protein or vaccine or diagnostic tools for the discrimination of variety of proteins from serum samples [21]. These are completely insoluble in almost all types of solvents, causing health related and toxicity problems. On the other hand, introduction of chemical conversion to carbon nanotubes can turn them into functionalized and water-soluble so that they can be associated to a broad range of active molecules, for example, as nucleic acids, therapeutic agents, proteins and peptides. Anticancer drugs (methotrexate) or antifungal agents (amphotericin B) have been covalently attached to carbon nanotubes with a fluorescent agent (FITC).

In vitro studies indicates that the drugs coupled to carbon nanotubes revealed to be more efficiently internalized into cells as compared with free drug alone and proved to have effective antifungal activity [22]. The multiple covalent functionalizations on the tips of carbon nanotubes permit them to transport a large number of molecules instantly and this approach offers a fundamental advantage in anticancer therapy.

6.7. Gold nanoparticles (GNPs)

Gold nanoparticles (GNPs) have several implementations in various fields, for example, diagnosis and cancer therapy, protein and DNA determination, drug and gene delivery, etc. Because of their exclusive properties such as, large surface area to volume ratio, small size, constancy over high temperatures, high reactivity to the living cells and translocation into the cells [23]. GNPs are appropriate for the transport of drugs to cellular targets due to their simplicity of synthesis, biocompatibility and functionalization (**Figure 5**). GNPs can successfully destroy cancer cells or bacteria functionalized with targeted specific biomolecules [24]. The efficiency of conjugation of GNPs with different kinds of antibiotics had been extensively studied [25], [26]. They observed that conjugates of GNPs showed more efficacy in inhibiting the growth of Gram-positive and Gram-negative bacteria as compared with the same dosage of antibiotics consumed alone. Their results suggested that GNPs can serve to be an efficient drug carrier in a drug delivery system [25], [26]. Some durable gold nanoparticles enclosed with vancomycin showed significant improvement of antibacterial activity compared with free antibiotic [27].

7. Smart Drug Delivery Systems

Preferably, nanoparticles drug delivery system should accumulate selectively in the target organ or tissue and simultaneously, enter target cells to transport the bioactive agent. It has been recommend [28] accumulation in the organ or tissue could be attained by the passive or antibody-mediated active targeting [29] whereas certain ligands or cell-penetrating peptides could mediate the intracellular delivery. Therefore, when necessary, a drug delivery system should be multifunctional and should acquire the ability to switch on and switch off certain functions. Another important prerequisite of multifunctional drug delivery system is

that different properties coordinated in an optimal fashion. Accordingly, if a system is generated which can provide combination of both the requirements i.e. target accumulation and specific cell surface binding; the half life of carrier in circulation should be long enough and subsequently, accumulation of drug delivery system in the target cells should proceed fast so as to prevent carrier degradation and loss of drug in interstitial space. The key problem in drug delivery system is the intracellular transport of bioactive agents. Drug delivery system nanoparticulate, such as micelles, gold nanoparticles and liposomes (**Figure 5**). are repeatedly used to increase the effectiveness of drug and DNA transport and targeting [30].

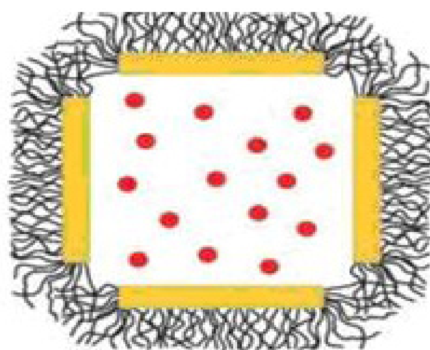


Figure 5: Smart drug delivery system - Gold nanocage covered with polymer

In drug delivery field, the most outstanding achievements were the development of smart drug delivery systems also called as stimuli-sensitive delivery systems. The idea is based on fast transitions of a physicochemical property of polymer systems when got a stimulus. This stimulus comprises physical (light, mechanical stress, electricity, temperature, ultrasound), chemical (ionic strength, pH), or biological (biomolecules, enzymes) signals and these stimuli can either be external stimuli which is artificially induced to trigger the desired events or if internal, it results into change in physicochemical changes in a living thing. Smart drug delivery systems provide a predictable and programmable drug delivery profile in response to different sources of stimulation. Smart drug delivery system has numerous advantages in comparison to conventional drug delivery systems. At the time of application, the conventional controlled transport systems are based on the programmed drug release rate in spite of the external environmental conditions, whereas, smart drug delivery system is based on release-on-demand strategy, which results into the liberation of therapeutic drug by drug carrier only at the time of receiving a specific stimulus. The best example of smart drug delivery system has been shown in self-regulated insulin transport systems which can act in response to changes in the level of glucose environment [31], [32].

7.1. Multifunctional drug carriers

A multifunctional drug delivery system refers to drug delivery carrier which shows several properties of active or passive internalization at specific disease site, capability to transport drug into intracellular target organelles or imaging ability, prolonged blood circulation, stimuli-sensitivity, etc. [33]. Practically, it shows two or more functions, in actual fact, polymer-drug conjugates and smart drug delivery system as discussed above can be regarded

as multifunctional drug delivery system. Multifunctional drug delivery system shows secondary functions as they also have internal hydrolysis inside cells or specific stimuli responsiveness in addition to delivery of drugs.

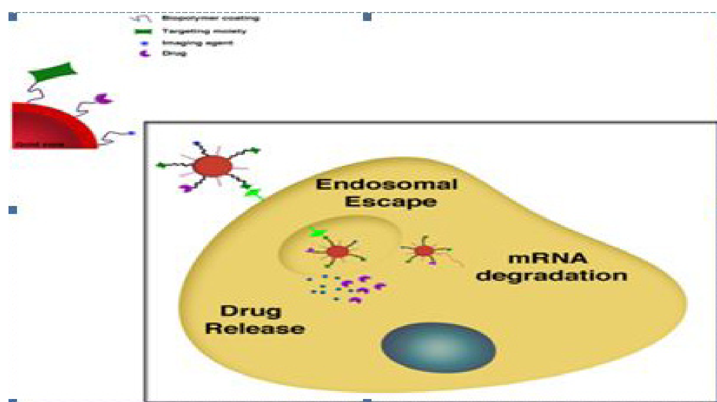


Figure 5: Multifunctional nanoparticle. The following are illustrated: the ability to carry one or more therapeutic agents; biomolecular targeting through one or more conjugated antibodies or other recognition agents; imaging signal amplification, by way of co-encapsulated contrast agents [5].

8. Role of Nanotechnology in Therapeutics

Nanoparticulates and devices can be constructed to interact with cells and tissues at a sub-cellular (i.e. molecular) level, with a high level of functional specificity, for implications in medicine and physiology, therefore, permitting a degree of combination between biological systems and technology not previously achieve. It should be esteemed that nanotechnology is not a distinct emerging scientific subject, but a multidisciplinary of conventional sciences, such as, physics, chemistry, materials science and biology, to assemble the mandatory group expertise required to exploit these novel technologies [34]. The promise that nanotechnology brings is versatile which shows improvements not only in current practices, but may also offering entirely new tools and techniques with full potential. At nanometer scale, manipulation of drugs and other materials may modify the fundamental properties and bioactivity of the materials [35]. These implements can give authorization to control over the several characteristics of drugs or agents such as restricted delivery over short or long periods of time, environmentally initiated restricted discharge or highly specific site-targeted delivery and change in solubility and blood pool retention time.

8.1. Nanomedicine

"Nanomedicine" is the discipline of science and technology dealing with diagnosis, treatment and prevention of diseases, alleviation of pain to recover the human health in short period of time through administration of micro/nanoscale particles, genetic engineering and biotechnology and ultimately multifaceted machinery systems and nonorobots [36]. It was alleged as implementating the five major subdisciplines which may overlap each other through universal technical concerns.

8.1.1. nanaomedicine in anticancer therapy

Cancer is one of the primary causes of death worldwide, inhabiting the second position in developing countries with fast growing occurrence over the time. Existing anti-cancer therapy approaches are based on chemotherapy, radiotherapy and surgery, in which chemotherapy is the one which shows the greater efficacy for cancer remedy, mainly in higher stages [37]. Implementation of new bioactive agents in anticancer therapy has greatly enhanced patient survival rate yet there are different biological hurdles that provoke drug delivery to the targeted cells and tissues. These are mainly critical blood half-life and physiological nature with excessive off-target effects and successful clearance from the human being [38,39].

The advancement and optimization of drug delivery strategies based nanoparticles concerns the early detection of cancer cells and/or specific tumor biomarkers, and the enhancement of the efficacy of the treatments applied [40]. The most important biomedical applications of nanoscale materials can be organized as shown in **Figure 6**.

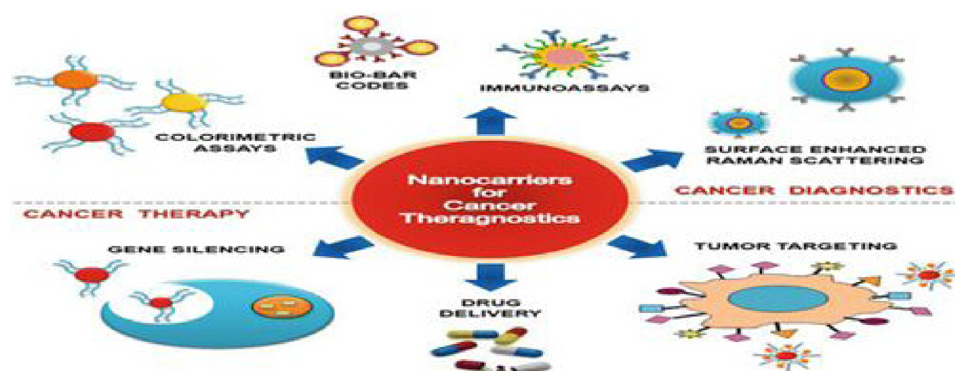


Figure 6: Biomedical applications of nanoscale materials in cancer therapy.

8.1.1.1. tumor cell targeting

Cancer cells display diverse targets on their surface, highly specific in each type of cancer. In cancer treatment, active targeting through nanoparticles is generally associated with specific type of cancer coupled with specific target. Chemotherapy is the leading approach of care for patients with targeted agents in an effort to improve the result. In various types of signaling pathways, these targeted agents are the key components in these pathways. The potential of targeted treatments has activated the study of targeted nanoparticulates that can permit synergistically act by binding and inhibiting cancer pathways while delivering therapeutic payloads. Tumor cell targeting involves many targets associated with the uncontrolled cell proliferation and the angiogenesis and others specifics for the different types of cancer.

9. Nanoparticles in Pulmonary Infections

Micronization of drugs plays a crucial role in enhancing the drug dosage form and therapeutic efficacy today. If a drug is micronized into microspheres with appropriate particle size, it can be delivered directly to the lung by the mechanical interception of capillary bed. If a drug is constructed as microspheres in the range of 7–25 μm, the microspheres can be localized in lung through i.v. administration (**Figure 7**). This approach can improve pulmonary drug con-

centration to maximize its effectiveness against some pulmonary infections such as mycoplasmal pneumonia and reduce the harmful side effects. The final nanoparticulate formulation may be administered either as a dry powder inhalers or nebulizer (metered dose inhalers). Being at nanoscale, nanoparticles are highly suitable for pulmonary transport because they can easily be air borne and delivered to the alveolus. The components of the nanoparticle formulation are biodegradable to evade deposition in the lungs, which prevents irritation of the air ways and lung tissue.

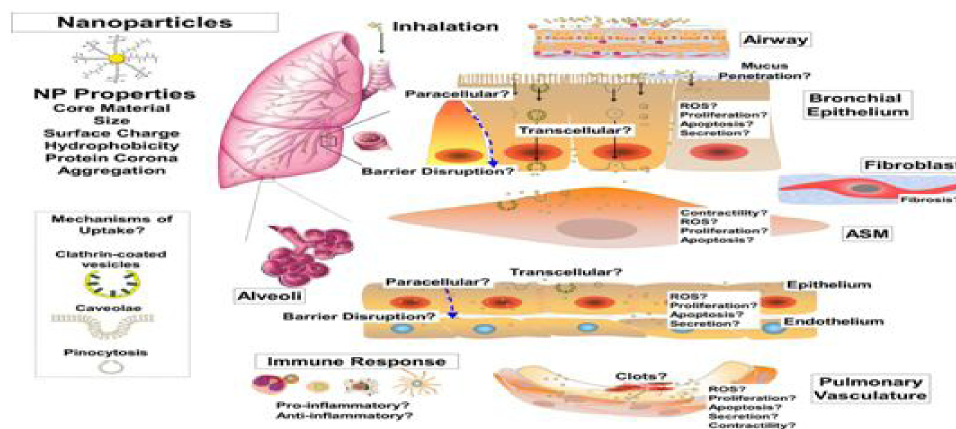


Figure 7: Pulmonary Administration of nanoparticles

10. Nanorobots in Cardiovascular Dieses

The techniques offered by nanotechnology in medical and cardiac sciences are in the fields of, imaging, diagnosis and tissue engineering. Implementation of nanotechnology approaches has offered insight into the potential benefits of nanotechnology in cardiovascular sciences. However, the benefits of nanotechnology exceed all features of medicine which is one of the important applications of nanomedicine in the area of cardiovascular sciences. Conventional surgical practices includes opening of the chest through the sternum and connects the patient to a cardiopulmonary bypass machine and arrested the heart. Different types of surgical techniques are executed on the arrested heart. Although, these practices can lead to additional morbidity such that they trigger central nervous system disorders and also the gastrointestinal complications.

Nanoscience offers approaches to design and build up innovative cardiac equipments, which are smaller in size and also more efficient. Surgical systems using robotics are being developed to give extraordinary control over equipments to offer accuracy to surgeons. This is predominantly useful to minimize invasive cardiac surgery. Instead of manipulating surgical instruments, surgeons use their fingers to move joystick handles on a control console to maneuver robot arms containing miniature instruments that are inserted into ports in the patient. The key novel implementation of nanotechnology in diagnostics and medical research are nanorobots. In the human body, nanorobots could monitor the degree of different compounds and document the information required in their internal memory. They can be quickly used in the inspection of a given tissue, examining its biomechanical and histometrical appearance

in larger aspect. As biotechnology expand the range and efficiency of treatment alternatives available from nanoparticles, the beginning of molecular nanotechnology will again extremely enlarge the comfort, effectiveness and speed of future medical therapies and significantly elevating their cost, risk and invasiveness [41].

11. Alzheimer's Disese with Nanotherapeutics

Alzheimer's disease is the major brain syndrome adversely affecting the elderly population worldwide. It is estimated to become a major health concern with severe socio-economic consequence in the coming decades. The total number of people affected by Alzheimer's disease worldwide today is about 15 million people which are expected to grow by four times by 2050. One of the FDA-approved commercially available drugs used for reducing the symptom in dementia is Rivastigmine. Due to its inability to cross the blood brain barrier and its consequences on peripheral organs, unable to attain its complete therapeutic potential.

In drug delivery mechanism of brain, biodegradable polymeric nanoparticles may be considered as relevant source [42]. Administration of rivastigmine injected intravenously rivastigmine was coupled to Poly (n-butylcyanoacrylate) (PnBCA) nanoparticles coated with the chemical polysorbate 80, which can be efficiently taken up as compared with free drug (an enhancement of up to 3.82 fold was found). This improved delivery is explained in terms of a mechanism which involves the binding of lipoproteins present in the blood to the nanoparticle surface.

11.1. Nanaogels

Ikeda and his coworkers elucidated an example for the Aβ anti-assembly strategy [43]. They designed an amphipathic nanogel that incorporates proteins and controls their folding and aggregation, similar to natural chaperones (proteins assisting the non-covalent folding and/or unfolding). In the case of Aβ, these nanogels would inhibit the amyloidogenesis process effectively through this mechanism (**Figure 8**).

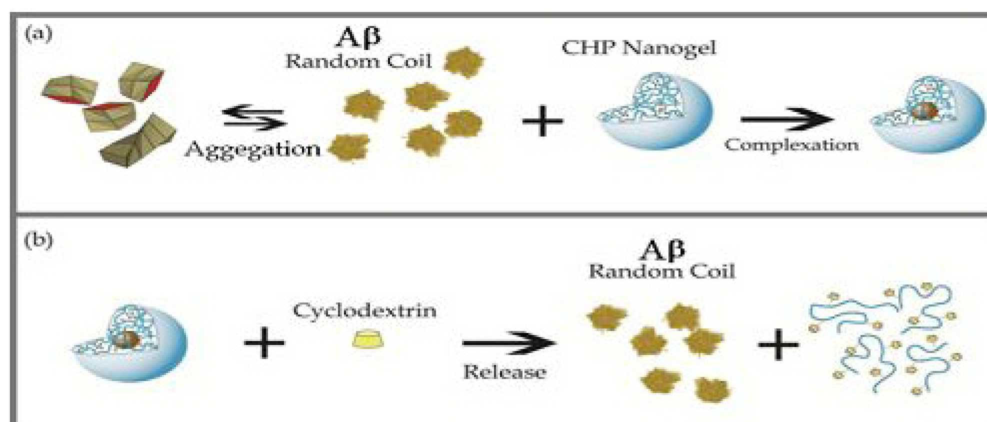


Figure 8: Schematic representation of the interactions between artificial nanoscale chaperone system and misfolded Aβ. (b). Refolded Aβ monomers are released after addition of cyclodextrin.

12. Parkinson's Disease

This can enhance current therapy of Parkinson's disease. Parkinson's disease is the second major neurodegenerative disorder after Alzheimer's disease. It influences one in every 100 persons with age above 65 years. Parkinson's disease is a disorder of the central nervous system. In this disorder, neuro-inflammatory reactions are implicated which leads to serious difficulties in body motions. Recent therapies goal to develop the functional capacity of the patient for long period of time if possible but they cannot modify the succession of the neurodegenerative process.

13. Conclusion

Nanoparticle mediated drug delivery is going to have a great potential impact on the society. It will drastically improve patient's quality of life associated with healthcare, early detection of pathologic conditions, reduce the severity of disease and result in improved clinical outcome for the patient. Together with the progression of nanoscale drug delivery systems, advances in nanoscale imaging suggest the potential for the development of multifunctional ‘‘smart’’ nanoparticles that may facilitate the realization of individualized cancer therapy and early diagnosis and treatment of highly infectious diseases like AD. Almost all types of nanoparticles including polymeric nanoparticles, nanocrystals, polymeric micelles, dendrimers and carbon nanotubes have been evaluated for their suitability as multifunctional nanoparticles that can be applied for simultaneous *in vivo* imaging and treatment of cancers. But realizing such a potential requires harmonized efforts among scientists in different disciplines and continued support by funding agencies.

14. References

1. Nikalje, A.P. (2015). Nanotechnology and its Applications in Medicine. Med Chem. 5: 81-89.

2. Feynman, R.P. (1960). There's plenty of room at the bottom. Eng Sci. 23: 22-36.

3. Rita, M., Fiore, P.N, Sonia, T., Roberta, C., Francesca, I. and Nevio, Pi. (2007). A new crown ether as vesicular carrier for 5-fluoruracil: Synthesis, characterization and drug delivery evaluation. Colloids and Surfaces B: Biointerfaces. 58(2):197-202.

4. Suri, S.S., Fenniri, H. and Singh, B. (2007). "Nanotechnology-based drug delivery systems." J. Occupational Medicine Toxicology. 2(1): 16.

5. Cho, Y.J., Kim, C.J., Kim, N., Kim, C.T. and Park, B. (2008). Some cases in applications of nanotechnology to food and agricultural systems. Biochip J. 2(3): 183-185.

6. Ringsdorf, H. (1975). Structure and properties of pharmacologically active polymers. J Polym Sci Polym Sympo. 51135153.

7. Duncan, R., Ringsdorf, H. and Satchi-Fainaro, R. (2006). Polymer therapeutics: Polymers as drugs, drug and protein conjugates and gene delivery systems: Past, present and future opportunities. Advances in Polymer Sci. 192(1): 18.

8. Harris, J.M. and R.B. (2003). Chess of. Effect on pegylation, pharmaceuticals, Nature Reviews Drug Discovery.

21421.

9. Veronese, F.M., Pasut, G. and P.E. (2005). Gylation, approach. Successful drug to delivery. Nature Reviews Drug Discovery. 14518

10. Adams, M.L., Lavasanifar, A, and Kwon, G.S. (2003). Amphiphilic block copolymers for drug delivery. J Pharm Sci. 92: 1343-1355.

11. Batrakova EV, Dorodnych TY, Klinskii EY, et al. (1996). Anthracycline antibiotics non-covalently incorporated into the block copolymer micelles: *in vivo* evaluation of anti-cancer activity. Br J Cancer.74: 1545-1552.

12. Nakanishi, T,, Fukushima, S., Okamoto, K,, et al. (2001). Development of the polymer micelle carrier system for doxorubicin. J Control Release. 74: 295-302.

13. Nasongkla, N., Bey, E., Ren, J., Ai, H., Khemtong, C. and Guthi, J.S. (2006). Multifunctional polymeric micelles as cancer-targeted, MRI-ultrasensitive drug delivery systems. Nano Lett. 6: 2427-2430.

14. Svenson S, Tomalia DA. (2005). Dendrimers in biomedical applications reflections on the field. Adv Drug Deliv Rev. 57: 2106-2129.

15. Malik, N., Evagorou, E.G. and Duncan, R. (1999). Dendrimerplatinate: a novel approach to cancer chemotherapy. Anticancer Drugs. 10: 767-776.

16. Markman, M. (2006). Pegylated liposomal doxorubicin in the treatment of cancers of the breast and ovary. Expert Opin Pharmacother. 7: 1469-1474.

17. Hofheinz, R.D., Gnad-Vogt, S.U., Beyer, U. and Hochhaus. (2005). A Liposomal encapsulated anti-cancer drugs. Anticancer Drugs. 16: 691-707.

18. Wu, J., Liu, Q. and Lee, R,J. (2006). A folate receptor-targeted liposomal formulation for paclitaxel. Int J Pharm. 316: 148-153.

19. Manchester M, Singh P. (2006). Virus-based nanoparticles (Vnanoparticles): platform technologies for diagnostic imaging. Adv Drug Deliv Rev. 58: 1505-1522.

20. Flenniken, M.L., Willits, D.A., Harmsen, A.L., et al. (2006). Melanoma and lymphocyte cell-specific targeting incorporated into a heat shock protein cage architecture. Chem Biol. 13: 161-170.

21. Bianco, A., Kostarelos, K., Partidos, C.D. and Prato, M. (2005). Biomedical applications of functionalised carbon nanotubes. Chem Commun. 5: 571.

22. Pastorin, G., Wu, W., Wieckowski, S., et al. (2006). Double functionalisation of carbon nanotubes for multimodal drug delivery. Chem Commun. 11: 1182-1184.

23. Sheikhloo, Z., Salouti, M. and Katiraee, F. (2011). Biological synthesis of gold nanoparticles by fungus Epicoccum nigrum. J Clust Sci. 22: 661-665.

24. Tiwari, P.M., Vig, K., Dennis, V.A. and Singh, S.R. (2011). Functionalized Gold Nanoparticles and Their Biomedical Applications. Nanomaterials. 1: 31-63.

25. Grace, N.A. and Pandian. K. (2007). Antibacterial efficacy of aminoglycosidic antibiotics protected gold nanoparticles-A brief study. Colloids Surf A Physicochem Eng Asp. 297: 63-70.

26. Saha, B., Bhattacharya, J., Mukherjee, A., et al. (2007) *In vitro* structural and functional evaluation of gold nanoparticles conjugated antibiotics. Nanoscale Res Lett 2: 614-22.

27. Gu, H. et al. (2003). Presenting vancomycin on nanoparticles to enhance antimicrobial activities. Nano Lett. 3: 1261-1263.

28. Maeda, H., Wu, J., Sawa, T., Matsumura, Y. and Hori, K. (2000).Tumor vascular permeability and the EPR effect in macromolecular therapeutics: A review J. Controlled Release. 6527184

29. Jaracz, S., Chen, J., Kuznetsova, L.V. and Ojima, I. (2005). Recent advances in tumor-targeting anticancer drug conjugates. Bioorg Med Chem. 13504354.

30. Torchilin, V.P. (2005). Recent advances with liposomes as pharmaceutical carriers. Nat Rev Drug Discovery. 414560.

31. Chu, L.Y., Liang, Y.J., Chen, W.M., Ju, X.J. and Wang, H.D. (2004). Preparation of glucose-sensitive microcapsules with a porous membrane and functional gates. Colloids Surf. B: Biointerfaces. 37914.

32. Kim, J.J. and Park, K. (2001). Modulated insulin delivery from glucose-sensitive hydrogel dosage forms. J Control Release. 773947.

33. Lee, E.S., Na, K., and Bae, Y. H. (2005). Super p^H-sensitive multifunctional polymeric micelle. Nano. Lett. 53259.

34. Silva, G.A. (2004). Introduction to nanotechnology and its applications to medicine. Surg Neurol. 61: 216-20.

35. Caruthers, S.D., Wickline, S.A. and Lanza, G.M. (2007). Nanotechnological applications in medicine. Curr Opin Biotechnol. 18: 26–30.

36. Patil, M., Mehta, S.D. and Guvva, S. (2008). Future impact of nanotechnology on medicine and dentistry. J Indian Soc Periodontol. 12(2): 34-40.

37. Blanco, E., Hsiao, A., Mann, A.P., Landry, M.G., Meric-Bernstam, F, and Ferrari, M. (2011). Nanomedicine in cancer therapy: Innovative trends and prospects. Cancer Sci. 102(7): 1247-1252.

38. Conde, J., Doria, G. 0 and Baptista, P. (2012). Noble Metal Nanoparticles Applications in Cancer. J Drug Delivery. 2012(2012): 1-12.

39. Sanna, A., Pala, N., Sechi, M. (2014). Targeted therapy using nanotechnology: focus on cancer. Int J Nanomedicine. 9: 467-483.

40. Baptista, P.V. (2012). Could gold nanoprobes be an important tool in cancer diagnostics? Expert Reviews of Molecular Diagnostics. 12(6): 541-543.

41. Ernest, H. and Rahul, S. (2005). Impact of nanotechnology on biomedical sciences. Review of current concepts on convergence of nanotechnology with biology. Online Journal of Nanotechnology.

42. Wilson, B., Samanta, M.K., Santhi, K., Kumar, K.P.S., Paramakrishnan, N. and Suresh, B. (2008). "Poly(n-butylcyanoacrylate) Nanoparticles Coated with Polysorbate 80 for the Targeted Delivery of Rivastigmine into the Brain to Treat Alzheimer's Disease". Brain Res. 1200: 159-168.

43. Ikeda, K., Okada, T., Sawada, S., Akiyoshi, K. and Matsuzaki. K. (2008). Inhibition of the formation of amyloid beta-protein fibrils using biocompatible nanogels as artificial chaperones. FEBS Lett. 580(28-29): 6587-6595.

Chapter 3

Anti-Gastric Ulcer Activity of Aqueous Extract of *Terminalia Arjuna* Against *Helicobacter Pylori* Lipopolysaccharide

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Abstract

Gastric ulcer is a common disease in humans. Every human might undergo the episode of gastric ulcer at least once in their life time. Among the various causes of gastric ulcer, *Helicobacter pylori* are one among them. The lipopolysaccharide is one of the virulence factors of *H. pylori*. The natural/holistic approach for the treatment/prevention is needed for all diseases including gastric ulcer due to its lack of adverse effects. *Terminalia arjuna* bark is one of the natural drug, which underwent clinical trials for cardio vascular diseases was chosen to study the antiulcer effect of *Terminalia arjuna* aqueous extract in *H. pylori* LPS induced gastric ulcer in rats. Gastric ulcer was induced in Sprague dawley rats with *H. pylori* LPS and *Terminalia arjuna* aqueous extract was administered to study the anti-ulcer activity. The assessment of anti-gastric ulcer was performed by testing the acid secretory and mucosal defensive factors. As a result of this study we could conclude that the aqueous extract of *Terminalia arjuna* offered anti-gastric ulcer effect.

1. Introduction

Gastric ulcer is a very frequent disease in the clinical practice and a challenge in the gastroenterology research [1]. *Helicobacter pylori* after being first isolated in human biopsies by Warren and Marshall in 1983 are now considered to be the major cause of gastric ulcers, duodenal ulcers and gastritis. *H. pylori* infection is also reported to be one of the important causes for relapse of ulcers [2]. Approximately 40 and 80% of individuals in developed and developing countries are infected respectively, making *Helicobacter pylori* as one of the most common bacterial infections in humans [3].

The factors implicated in the virulent action of *H. pylori* towards mucosal integrity include CagA and VacA cytotoxins capable of inducing the release of pro-inflammatory cytokines, excessive production of ammonia known for its strong toxic effect on the gastric epithelium, and the impairment of feedback inhibition of gastrin release by somatostatin [4]. Another product of significance to the virulent action of *H. pylori* is its cell wall lipopolysaccharide [5].

H. pylori lipopolysaccharide elicited within 2 days the pattern of acute mucosal inflammatory responses accompanied by a massive epithelial cell apoptosis, increase in mucosal expression of endothelin- 1, enhancement in TNF-alpha, increase in NOS-2, decrease on cNOS activity [5], excessive nitric oxide generation, apoptotic caspase activation and a marked enhancement in gastric epithelial cell apoptosis [6]. Other pathogenic effects of *H. pylori* LPS involve progression of the mucosal inflammatory process, stimulation of NFκB nuclear translocation, disturbances in mitogen activated protein kinase (MAPK) cascades and a marked up-regulation in gastric mucosal level of endothelin-1 [7].

Eradication of *Helicobacter pylori* seems to be curative of both infection and ulcer disease. Hence successful treatment leads to the resolution of gastritis and diminished ulcer recurrence [8]. The combined treatment of proton pump inhibitors (i.e. omeprazole) with antibiotics (i.e ampicillin, amoxicillin, ofloxacin or tetracycline) have shown to be successful in some of the patients suffering from this complaint, with cure rates up to 90% [9]. Currently, the antiulcer treatment can be performed with antacid drugs, such as proton pump inhibitors (PPIs) or antagonists of the type 2 histamine receptors. However, this therapy produces serious adverse effects, including osteoporotic fracture; renal damage; infection (pneumonia and Clostridium difficile infection); rhabdomyolysis; deficiencies of vitamin B_{12}, magnesium, iron; anemia; thrombocytopenia [10], and is being associated with poor ulcer healing quality and in turn ulcer recurrence [11].

Several plants are used for the treatment of gastric ailments, including stomach ache and ulcers [12]. *Terminalia arjuna* Wight and Arnot known locally as Kumbuk, have a long history of medicinal uses in India [13], including cancer treatment [14]. Prior attempts to

isolate medicinal agents from this tree yielded a variety of relatively simple compounds [15] such as flavonoids [16].

The bark is astringent, sweet, acrid, cooling, aphrodisiac, demulcent, cardiotonic, styptic, antidysenteric, urinary astringent, expectorant, alexiteric, lithontriptic and tonic. It is useful in fractures, ulcers, urethrorrhea, leucorrhea, diabetes, vitiated conditions of pitta, anemia, cardiopathy, hyperhydrosis, fatigue, asthma, bronchitis, tumours, otalgia, dysentery, inflammations, internal and external haemorrhages, cirrhosis of the liver and hypertension [17]. It is reputed as cardiotonic and also possesses hypotensive and hypolipidemic activity [18]. Its use in wound healing has been mentioned by Sushruta in Sushruta Samhita [19]. The bark powder is reported to exert hypocholesterolaemic and antioxidant effect in humans [20]. The chemical constituents isolated from the plant are mainly tannins and various oleanane triterpenoids. Tannins of the leaves had been reported to have anticancer activity [21] and that from the bark possessed antimutagenic effect [22].

Terminalia arjuna bark constitutes high amounts of fibre, sugar, tannin, beta-sitosterol, carbonate of calcium, sodium, aglycones-arjunine, arjunolic acid, arjunoids I, II, III and IV [23]; and also having antioxidant polyphenolics, flavonoids-quercetin, kaempferol, pelargonidin and luteolin [24]. *Terminalia arjuna* exhibited antibacterial activity against *Escherichia coli*, *Klebsiella aerogenes*, *Proteus vulgaris*, and *Pseudomonas aerogenes* (gram-negative bacteria) [25]. An active principle from *Terminalia arjuna* bark, Arjunaphthanoloside-a glycoside, showed potent antioxidant activity and inhibited nitric oxide (NO) production in lipopolysaccharide (LPS)-stimulated rat peritoneal macrophages [26]. Terminoside A, a new oleanane type triterpene, potently inhibited nitric oxide (NO) production and decreased inducible nitric oxide synthase (iNOS) levels in lipopolysaccharide-stimulated macrophages [27]. Hence, the present study was aimed to assess the gastroprotective effect of water extract of *Terminalia arjuna* against *Helicobacter pylori* lipopolysaccharide induced gastric ulcer.

2. Materials and Methods

2.1. Gastric ulcer induced by *Helicobacter pylori* lipopolysaccharide (Hp-LPS)

2.1.1. Preparation of Hp-LPS 26695

Helicobacter pylori lipopolysaccharide was prepared from 26695 strain of *Helicobacter pylori* by the conventional method used for the preparation of lipopolysaccharide from gram-negatives. *Helicobacter pylori* 26695 grown in Brucella broth with 5% FCS was pelleted by centrifugation, washed twice with 0.9% NaCl, heat inactivated for 2 h in steam, washed twice with 0.9% NaCl. Finally the pellet was suspended in 1 mL of 0.9% NaCl and stored at 4˚C. Then it was lyophilized and used.

2.1.2. Procurement and maintenance of animals

Male Sprague-Dawley rats weighing 150-200g were obtained from the National Centre for Laboratory Animal Sciences, National Institute of Nutrition, Hyderabad, India, was used for the study and given standard pellet diet and water *ad libitum* and deprived of food 24 h before sacrifice. All experiments were performed with 6 animals in each group. This study was conducted according to the ethical norms approved by Animal Ethics Committee of our institution (IAEC No. 01/038/07).

2.1.3. Experimental set up for effective dose fixation of TA against Hp-LPS induced gastric ulcers

Group 1: Control rats

Group 2: Ulcerated rats - Ulcer was induced by *Helicobacter pylori* lipopolysaccharide (Hp-LPS) (50µg/animal, orally) for 3 days

Group 3-6: Treatment group - Pretreatment with different doses of *Terminalia arjuna for 7 days* (100, 200, 300 and 400mg/kg bw) and subjected to *Helicobacter pylori* lipopolysaccharide induction for 3 days and then maintained on *Terminalia arjuna* extract for 4 days

Group 7: Reference group - Pretreatment with Sucralfate for 7 days (100mg/kg bw, orally, [28]) and ulcer was induced by *Helicobacter pylori* lipopolysaccharide for 3 days and then maintained on sucralfate for 4 days.

Animals were sacrificed after 24h fasting by cervical decapitation following anesthetization. For studies requiring gastric juice, pyloric ligation was done 4 hours prior to sacrifice.

Gastric juice was collected by a 4 h pyloric ligation [29]. After 4h, the animals were sacrificed. Stomach was dissected out after tying the oesophageal end. The stomach was cut open along the greater curvature and the contents were collected into tubes, centrifuged at 1000 rpm for 10 minutes and were used for the estimation of various biochemical parameters such as volume of gastric juice, acid output, free acidity, total acidity, mucin content etc., Pyloric ligation was done only for the collection of gastric juice. For the collection of gastric mucosal tissue, no pyloric ligation was done; the rats were killed by cervical dislocation under ether anesthesia. After sacrificing the rats, the stomach was excised and cut along the greater curvature, washed carefully with 0.9% NaCl. The mucosa of the stomach was scrapped and used for gastric mucosal parameters. Blood was collected from the jugular vein and plasma was used for estimations.

Ulcer index was calculated according to the method of Okabe et al., [30]. After the collection of gastric juice, the volume was noted and p^H was measured. Free acidity and total

acidity [31] were determined by titrating with 0.01 M NaOH using Toepfer's reagent and phenolphthalein as indicator. The basal and maximum acid output [32] was determined to assess the anti-secretory effect of TA.

2.1.4. Experimental setup for gastro protective evaluation of TA's efficacy against Hp-LPS induced gastric ulcers

Group 1: Normal rats - serve as control (Control)

Group 2: Ulcerated rats - induced for gastric ulcers with *Helicobacter pylori* lipopolysaccharide-26695 (50 µg/animal, orally) for 3 days (Hp-LPS)

Group 3: Treated rats - pretreatment with water extract of *Terminalia arjuna* for 7 days (300 mg/kg bw, orally) and subjected to *Helicobacter pylori* lipopolysaccharide induction for 3 days and then maintained on water extract of *Terminalia arjuna* extract for 4 days (Hp-LPS+TA)

Group 4: Reference group - Pretreatment with Sucralfate for 7 days (100mg/kg, orally, [28]) and ulcer was induced by *Helicobacter pylori* lipopolysaccharide for 3 days and then maintained on sucralfate for 4 days (Hp-LPS+SFT)

After the experimental period, the rats were killed under anaesthesia. Gastric juice, blood and gastric mucosal tissues were collected as that of the above said methods and used for the biochemical analysis.

- Gastric mucosal barrier [33], protein [34], hexose [35], hexoseamine [36], sialic acid [37], fucose [38] were estimated in gastric mucosa and gastric juice to assess the mucoprotective efficacy of TA.
- Gastrin hormone (Gastrin hormone was estimated using RIA kit (Diagnostic products corporation, Los Angeles, USA), pepsin and pepsinogen [39] assays were done.
- Cyclooxygenase 2 expression - Stomach was cut and tissue was utilized for COX-2 analysis so as to assess the prostaglandin levels and the cytoprotective nature of *Terminalia arjuna*. Immunohistochemical analysis of COX-2 was done using Primary Antibody: Goat Anti-COX-2; Secondary Antibody: Rabbit Anti-Goat IgG HRP conjugate. Photomicrographs were obtained using a LABOMED CX RIII microscope (20X /0.454; 10X/0.254; 40X/0.654) connected to a SANYO digital color CCD camera.
- Nitric oxide levels [40] were estimated since it changes during *Helicobacter pylori* lipopolysaccharide pathogenesis.
- Serum tumor necrosis factor-alpha level was determined as it is a marker of inflammation. The level of TNF-α in plasma was measured using ELISA– kit provided by PAN-Biotech,

GmBH. The protocol was a modified version of Sharma and Singh [41]. Primary antibody - Rabbit monoclonal anti TNF-α(1: 5000 dilution), Secondary antibody - Goat antirabbit antibody IgG - HRP conjugated.

➢ The level of IL- β was measured by using Enzyme Linked Immunosorbent assay (ELISA). The protocol was a modified version of Sharma and Singh [41]. Primary antibodies - Rabbit M-Ab Anti - IL- β (1: 5000 dilution), Secondary antibody - Goat antirabbit antibody IgG-HRP conjugated

➢ Proliferating cell nuclear antigen expression was examined for cell proliferation by immunohistochemistry. The gastric tissues were immune stained for PCNA using Monoclonal Mouse Anti-PCNA antibody and detected with Mouse anti- IgG HRP conjugate.

2.2. Statistical Analysis

Values are represented in mean ± SD for six rats in each group and the differences between mean values was determined by one-way analysis of variance (ANOVA) followed by the Dunnett's T3 multiple comparison test by utilizing the SPSS (Statistical Package for Social Science) 10.0 software package. The values are considered significant when $p < 0.001$,$p < 0.01$ and $p < 0.05$.

3. Results and Discussion

3.1.Effective dose fixation for Hp-LPS-26695 for the induction of gastric ulcers

Figure 1a depicts the results of dose fixation for Hp-LPS-26695 for the induction of gastric ulcers. The effective dose of *Helicobacter pylori* lipopolysaccharide (Hp-LPS) - 26695 in inducing gastric ulceration was assessed by orally administering Hp-LPS at the doses of 10, 30, 50 and 70 µg/animal per day for 3 consecutive days by dissolving it in saline. At a dose of 10 and 30 µg/animal for 3 days, mild lesions were observed. Severe gastric lesions were produced at the doses of 50 and 70 µg/animal for 3 days in experimental rats. From these, 50 µg/animal for 3 days was fixed for inducing gastric lesions using Hp-LPS-26695, as it elicits damage at a minimum dose.

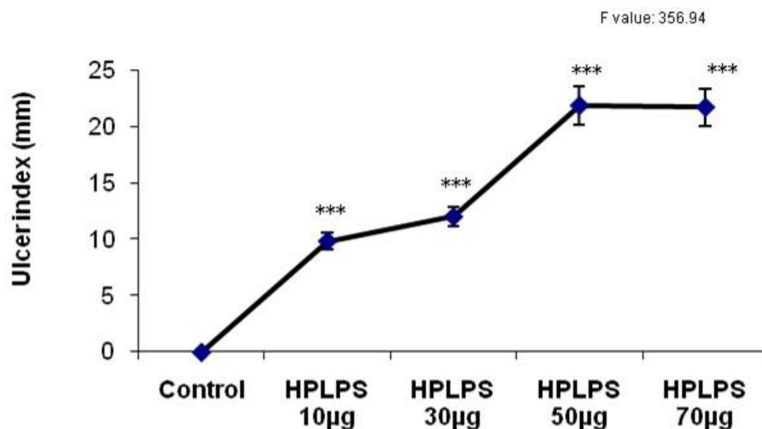

Figure 1a: Dose fixation for HP-LPS for induction of gastric lesions in rats

Values are expressed as mean ± S.D for 6 animals in each group. Groups are compared as follows: Control vs. All groups Significance represented as ***– $p<0.001$

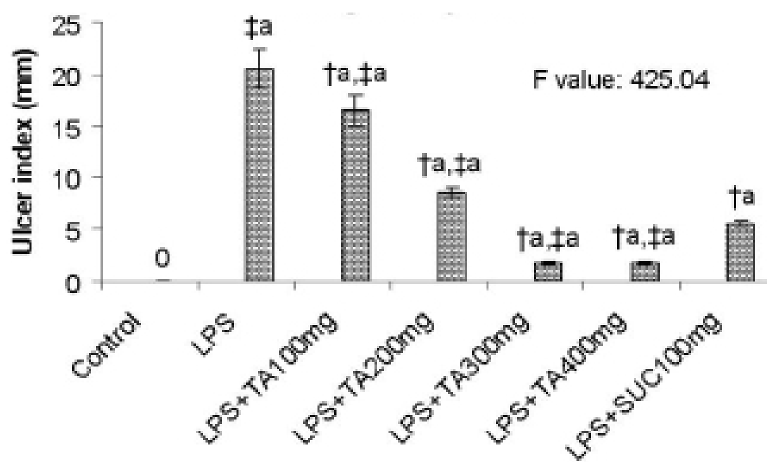

Figure 2a: Effect of TA at various concentration to fix the dose againest Hp-Lps

Values are expresseed as mean ± S. D for 6 animals in each group.
Groups are compared as : + - Hp-Lps vs. All groups; ‡ - Hp-Lps+ SFT vs. All groups. Significance represented as a- $p<0.001$ using one way ANOVA. Dunnett's T3 multiple comparisont est.

3.2. Anti-secretory effect of TA against Hp-LPS induced gastric ulcers

Table 1a: Effect of TA on gastric secretory parameters in control and Hp-LPS induced experimental animal Values are expressed as Mean ± SD for 6 animals in each group

Parameters	Control	Hp-LPS	Hp- LPS +TA 100mg	Hp-LPS + TA 200mg	Hp-LPS+ TA 300mg	Hp-LPS+ TA 400mg	Hp-LPS+ SFT 100mg	F Value
Volume of gastric juice	1.88± 0.15 ‡NS	3.15± 0.24[a,‡a]	3.10± 0.17 †[NS,‡a]	2.5± 0.21†[b,‡a]	1.90± 0.17†[a,‡NS]	1.95± 0.14†[a,‡c]	1.58± 0.15†[a]	75.02
pH	4.2± 0.14 ‡NS	1.87± 0.15[a,‡a]	3.47± 0.18†[a,‡c]	3.97± 0.08 †[a,‡NS]	4.18± 0.15†[a,‡NS]	4.28± 0.15†[a,‡NS]	3.95± 0.19†[a]	193.33
Free acidity	34.67± 2.8[‡NS]	53.67± 2.73 [a,‡a]	52.33± 2.42†[NS,‡a]	42.33± 3.01 †[a,‡b]	33.0± 1.79†[a,‡NS]	35.17± 1.47 †[a,‡NS]	32.5± 3.02†[a]	78.16
Total acidity	64.83± 2.48 ‡NS	88.5± 3.78 [a,‡a]	81.33± 4.55†[NS,‡a]	75.67± 2.8[†b,‡a]	65.83± 4.71 †[a,‡NS]	66.67± 3.01 †[a,‡c]	59.83± 2.93†[a]	50.33
Basal Acid output	38.75± 4.41 ‡a	90.63± 5.5[a,‡a]	23.37± 1.03[†a,‡a]	17.32± 0.81 †[a,‡a]	12.18± 0.86†[a,‡NS]	11.65± 0.87 †[a,‡NS]	29.42± 1.01†[a]	641.27
Maximum Acid Output	37.88± 112.0 ‡a	112.0± 7.64 [a,‡a]	30.68± 1.29[†a,‡a]	18.72± 0.35 †a,‡c	17.48± 0.92†[a,‡NS]	16.17± 1.47 †[a,‡NS]	15.83± 1.47†[a]	726.17

Units: Volume of juice (ml/100g/4h), pH, Free Acidity (mEq/L/100g), Total Acidity(mEq/L/100g), Basal acid output (mEq/100g/4h); Maximum acid output (mEq/100g/4h). Groups are compared as follows: Control vs. Hp-LPS, † - Hp-LPS vs. All groups, ‡ - Hp-LPS+SFT vs. All groups.

Significance represented as [a]– p<0.001, [b]– p<0.01, [c]– p<0.05, [NS]– Non-significant using One way ANOVA- Dunnett's T3 multiple comparison test

Figure 2a shows the effect of TA at various concentrations to fix the dose against Hp-LPS. Hp-LPS 26695 induced a significant increase (p<0.001) in the ulcer index compared to control rats. The ulcer index was significantly decreased (p<0.001) on treatment with TA at a dose of 100 mg/kg body weight compared to Hp-LPS induced rats, whereas a significant decrease (p<0.001) was noted with TA at doses of 200, 300 and 400 mg/kg bw compared to Hp-LPS ulcerated rats.

Table 1a indicates the effect of TA on gastric secretory parameters in control and Hp-LPS induced experimental animals. Hp- LPS 26695 induced a significant increase (p<0.001) in the gastric volume, free acidity, total acidity, maximal and basal acid output and pepsin concentration compared to control rats. In rats treated with different doses of TA a significant decrease was evident in all the secretory parameters in a dose dependent manner. Since reduction in ulcer index along with decrease in secretory parameters were noted at 300 mg/kg body weight, further studies on the assessment of gastroprotective effect of TA against Hp-LPS induced ulceration were performed at this dose.

LPS is a family of glycolipids found in the cell envelope of gram-negative bacteria, including *H. pylori* [42]. LPS from *H. pylori* can stimulate acid secretion, which possibly might contribute to mucosal damage of the stomach. The second possible mechanism by which *H. pylori* LPS can stimulate acid secretion at the gland level is by enhancing histamine release from rat ECL cells [43]. The LPS purified from the known gastric pathogen *H. pylori* has this secretory property greatly impaired and, depending on the strain of the bacterium is able to stimulate directly both pepsinogen [44] and acid secretion, potentially contributing to gastric ulcer.

Terminalia arjuna was reported to have antibacterial effect [45]. The reduction in the ulcer index clearly point towards the antibacterial effect of TA against the toxic effects elicited by Hp-LPS.

Maximal acid output has been indicated in the pathogenesis of mucosal ulceration where low gastric pH resulted in enhancement of *H. pylori*-induced NF-κB nuclear binding [46]. *H. pylori* increases basal gastrin levels, basal acid output, meal-stimulated maximal acid output and 24-h intragastric acidity. The effects on gastric acid production depend on the distribution of gastritis in the stomach [47]. *H. pylori* LPS can stimulate acid secretion at the gland level by increasing histamine release from rat ECL cells [43]. Sucralfate markedly suppresses *H. pylori* infection and the accompanying hypersecretion of acid. These effects are likely to be important mechanisms by which the drug promotes ulcer healing [48].

The Sydney strain of *H. pylori* in mouse model stimulated acid secretion [49] and LPS from *H. pylori* SS1 strain stimulates acid secretion, whereas other LPS preparations did not increase acid secretion. This is probably related to the differences in the molecular structure of the tested LPS preparations [50]. However, in this study the LPS of 26695 strain stimulated acid secretion.

Table 2a: Effect of TA on pepsin, pepsinogen and gastrin in control and Hp-LPS induced experimental animal Values are expressed as mean ± S.D for 6 animals in each group

Parameters	Control	Hp-LPS	Hp-LPS+TA	Hp-LPS+SFT	F value
Pepsin (Gastric Juice-micromole tyrosine liberated/ml)	170.83±14.29	243.83±19.33^{a}	155.33±11.47†^{a,‡NS}	160.0±13.04†^{a}	46.78
Pepsinogen (Gastric Mucosa-micromoles of tyrosine liberated/min/mg protein)	802.12±43.58	1044.17±55.35^{a}	836.67±48.44†^{a,‡NS}	851.67±40.21†^{a}	31.94
Gastrin (plasma-pmol/L)	74.17±7.36	135.5±7.18^{a}	75.5±6.16†^{a,‡NS}	84.17±6.4†^{a}	110.27

Groups are compared as follows: Control vs. Hp-LPS, †- Hp-LPS vs. Hp-LPS+TA and Hp-LPS+SFT, ‡- Hp-LPS+SFT vs. Hp-LPS+TA
Significance represented as ^{a}– p<0.001, ^{NS}- Non-significant using One way ANOVA- Dunnett's T3 multiple comparison test.

Table 2a shows the effect of TA on pepsin, pepsinogen and gastrin in control and Hp-LPS induced experimental animals. Pepsin concentration and Pepsinogen activity was significantly increased ($p<0.001$) in Hp-LPS induced ulcer rats compared to control rats while Hp-LPS+TA and Hp-LPS+SFT rats showed a significant decrease ($p<0.001$) in Pepsin concentration and pepsinogen activity compared to Hp-LPS induced rats. There was no significant alteration in Hp-LPS+TA treated animals compared with Hp-LPS+SFT rats.

Pepsin appears to play a crucial role in ulceration of the stomach and in the absence of pepsin; gastric acid does not cause ulceration. Hence, major benefits of antacid therapy in the treatment of ulcer disease may be inhibition of the conversion of pepsinogen to pepsin and the maintenance of a gastric luminal pH greater than the optimum for the enzyme. Pepsin, a protease present in the gastric lumen, is secreted by the chief cells of the gastric mucosa as an inactive precursor, pepsinogen; pepsinogen is activated by acid present in the gastric lumen, which initiates digestion of protein [51].

Lipopolysaccharide of *H. pylori* (Hp-LPS) affects pepsinogen release by a nontoxic mechanism. This effect was characteristic of the organism and related to the clinical status of the strain. Physical and chemical disruption of LPS suggested that both the structure and the carbohydrate composition of LPS may play a critical role in pepsinogen release. Pepsinogen release is an innate property of all cagA+ *H. pylori* LPS. The structure of the molecule and composition of side-chains are important in this response which appears to be partially lipid A driven [52]. Stimulation of pepsinogen secretion is the important mechanism of Hp-LPS induced mucosal damage [42]. Luminal addition of *H. pylori* lipopolysaccharide resulted in a fifty-fold stimulation of pepsinogen [53].

An increase in pepsinogen activity by Hp-LPS 26695 strongly indicates the ulcerogenic potency of this LPS. Further, a decrease in the pepsinogen activity on TA administration is probably due to the efficacy of TA in inhibiting the hypersecretion of pepsinogen and protecting the mucosa from mucosal damage.

In Hp-LPS induced ulcer rats, the level of plasma gastrin was significantly increased ($p<0.001$) compared to control rats. Hp-LPS+TA and Hp-LPS+SFT rats showed a significant decrease ($p<0.001$) in the level of gastrin compared to Hp-LPS ulcer rats. No significant changes were noted between Hp-LPS+TA and Hp-LPS+SFT rats.

Gastrin is a peptide hormone that stimulates gastric acid secretion and the growth of fundic mucosa in the stomach [54]. *H. pylori* infection is associated with hypergastrinemia [55]. Proinflammatory cytokines including IL-1β, TNF-α, and IL-8 are able to stimulate gastrin release from G cells [56]. In addition, IL-1β, which can also act as a potent inhibitor of acid production, may cause hypochlorhydria resulting in hypergastrinemia [57]. The presence of *H. pylori* colonization was shown in several studies and associated with hypergastrinaemia and

hyperpepsinogenaemia [58].

In the present study, Hp-LPS 26695 also elicited the plasma gastrin levels suggesting onset of inflammatory processes following Hp-LPS administration inducing the release of gastrin from G cells by proinflammatory cytokines like TNF-α and IL-1β. However in TA treated animals reduction in the inflammatory events could have resulted in the maintenance of plasma gastrin levels.

Gastric mucosal protection

Table 3a: Effect of TA on the levels of protein and protein bound carbohydrate complexes in gastric juice of control and Hp –LPS induced experimental animals Values are expressed as mean ± S.D for 6 animals in each group.

Parameters (µg/ ml)	Control	Hp-LPS	Hp-LPS+ TA	Hp-LPS+ SFT	F value
Hexose	405.14±25.64	277.29±28.23[a]	400.49±21.85 †a,‡NS	395.68±23.26†a	36.95
Hexoseamine	174.58±15.58	118.03±11.85[a]	182.11±17.42 † a,‡NS	164.5±14.03 †a	22.48
Sialic acid	40.38±3.17	27.94±1.98[a]	41.67±4.85 † b,‡NS	36.38±3.72 †b	17.95
Fucose	45.02±2.22	31.34±2.33[a]	42.93±3.6 †a,‡NS	42.64±2.23 †a	32.45
Total carbohydrate (TC)	665.12±16.01	454.59±29.95[a]	667.19±29.56 † a,‡NS	639.21±27.61†a	89.67
Protein (P)	272.46±12.65	392.11±25.25[a]	277.36±25.25 †a,‡NS	273.86±22.57 †a	42.68
TC: P ratio	2.45±0.12	1.16±0.13[a]	2.43±0.27† a,‡NS	2.26±0.1 †a	78.18

Groups are compared as follows: Control vs. Hp-LPS, †- Hp-LPS vs. Hp-LPS+TA and Hp-LPS+SFT, ‡- Hp-LPS+SFT vs. Hp-LPS+TA

Significance represented as a– p<0.001, b– p<0.01, NS- Non-significant using One way ANOVA- Dunnett's T3 multiple comparison test.

Table 3a shows the effect of TA on the levels of protein and protein bound carbohydrate complexes in gastric juice of control and Hp-LPS induced experimental animals. A significant decrease (p<0.001) in hexose, hexosamine, sialic acid, fucose, total carbohydrate and Total carbohydrate: protein ratio (TC: P) resulting from a significant increase in protein levels were noted in Hp-LPS ulcerated rats compared to control rats. On the contrary, Hp-LPS+TA treated rats registered a significant increase in hexose (p<0.001), hexoseamine (p<0.001), sialic acid (p<0.01), fucose (p<0.001), total carbohydrate and total carbohydrate: protein ratio (TC: P) (p<0.001) with a concomitant decrease in protein (p<0.001) levels compared to Hp-LPS ulcer rats. Hp-LPS+SFT rats registered a significant increase in hexose (p<0.001), hexoseamine (p<0.001), sialic acid (p<0.01), fucose (p<0.001), total carbohydrate and Total carbohydrate: protein ratio (TC: P) (p<0.001) with a concomitant decrease in protein levels (p<0.001)

compared to Hp-LPS rats. There was no significant alteration in all these parameters in Hp-LPS+TA when compared with Hp-LPS+SFT rats.

Exposure of gastric mucosal cells to the LPS led to a dose-dependent decrease in mucin synthesis, accompanied by a marked increase in caspase-3 activity and apoptosis. A decrease in mucin synthesis following induction with LPS, accompanied by cells proceeding to apoptosis has been reported [59]. Also *H. pylori* LPS cause inhibition of mucin binding to the receptor [60].

H. pylori LPS has been shown to exert an inhibitory effect on the synthesis and secretion of gastric mucin, the glycoprotein that maintains the strength and mucus coat integrity [61]. In the present study, the influence of Hp-LPS 26695 on mucin content was evident from decreases in the hexose, hexoseamine, fucose and sialic acid contents. However the mucoprotective role of TA observed against other ulcer models [62] was also evident with Hp- LPS gastric ulcers and comparable to the effect of SFT.

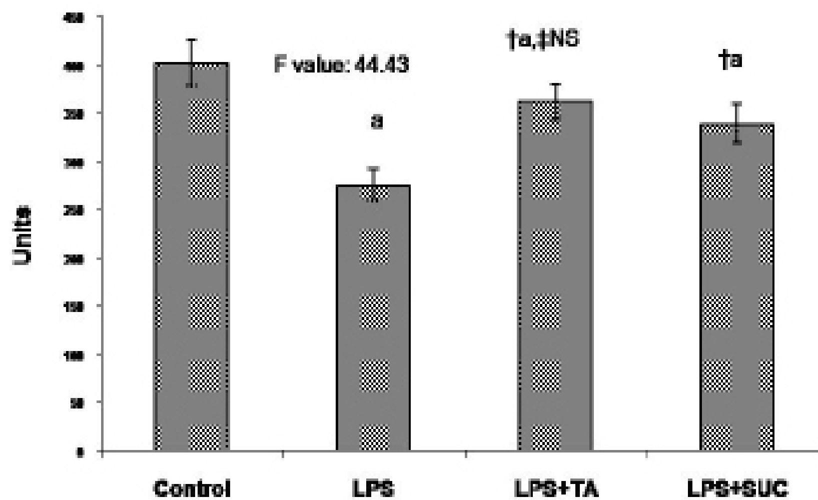

Figure 3a: Effect of TA on the levels of Adherent mucus in control and Hp-LPS induced experimental animals

Units- Micro gram alcian blue/g tissue.
Groups are compared as follows: Control vs. Hp-LPS, †- Hp-LPS vs. Hp-LPS+TA and Hp-LPS+SFT, ‡- Hp-LPS+SFT vs. Hp-LPS+TA Significance represented as a– p<0.001, NS- Non-significant using One way ANOVA- Dunnett's T3 multiple comparison test.

Figure 3a shows the effect of TA on the levels of Adherent mucus in control and Hp-LPS induced experimental animals. The levels of adherent mucus were decreased significantly (p<0.001) in Hp-LPS ulcer rats compared with control rats. In Hp-LPS+TA and Hp-LPS+SFT group, there was a significant increase (p<0.001) in adherent mucus compared to Hp-LPS ulcer rats. No significant difference was observed in Hp-LPS+TA animals compared to Hp-LPS+SFT rats.

Table 4a: Effect of TA on the levels of protein and protein bound carbohydrate complexes in the gastric mucosa of control and Hp-LPS induced experimental animals Values are expressed as mean ± S.D for 6 animals in each group

Parameters (mg/g)	Control	Hp-LPS	Hp-LPS+TA	Hp-LPS+SFT	F value
Hexose	14.35±0.95	7.9±0.47^{a}	15.58±1.23$\dagger^{a,\ddagger c}$	13.05±1.09$\dagger^{a}$	71.18
Hexoseamine	8.78±0.42	4.53±0.22^{a}	9.13±0.46$\dagger^{a,\ddagger NS}$	8.62±0.4$\dagger^{a}$	190.03
Sialic acid	1.78±0.05	0.81±0.07^{a}	1.93±0.29$\dagger^{a,\ddagger NS}$	1.71±0.11$\dagger^{a}$	58.99
Fucose	3.45±0.3	2.22±0.15^{a}	3.53±0.34$\dagger^{a,\ddagger NS}$	3.42±0.33$\dagger^{a}$	27.99
Total carbohydrate (TC)	28.37±1.11	15.46±0.6^{a}	30.18±1.13$\dagger^{a,\ddagger C}$	26.79±1.54$\dagger^{a}$	202.43
Protein (P)	22.37±1.28	17.0±0.96^{a}	24.61±2.04$\dagger^{a,\ddagger NS}$	23.25±1.53$\dagger^{a}$	29.47
TC: P ratio	1.27±0.08	0.91±0.07^{a}	1.23±0.09$^{\dagger a,\ddagger NS}$	1.12±0.11$\dagger^{c}$	19.86

Groups are compared as follows: Control vs. Hp-LPS, †- Hp-LPS vs. Hp-LPS+TA and Hp-LPS+SFT, ‡- Hp-LPS+SFT vs. Hp-LPS+TA. Significance represented as ^{a}– p<0.001, ^{c}– p<0.05, $^{NS-}$ Non-significant using One way ANOVA- Dunnett's T3 multiple comparison test.

Table 4a shows the effect of TA on the levels of protein and protein bound carbohydrate complexes in gastric mucosa of control and Hp-LPS induced experimental animals. The levels of hexose, hexosamine, sialic acid, fucose, total carbohydrate, total carbohydrate: protein ratio (TC: P) and protein were decreased significantly (p<0.001) in Hp-LPS ulcer group compared with control rats whereas, these levels were significantly increased (p<0.001) in Hp-LPS+TA and Hp-LPS+SFT rats compared with Hp-LPS ulcerated rats. A non significant change was observed in Hp-LPS+TA compared with Hp-LPS+SFT rats in all these parameters except a significant increase (p<0.05) in hexose and total carbohydrate levels in Hp-LPS+TA animals.

Although the causative factors for ulcerogenesis may vary, the net imbalances in offensive and defensive factors are involved in ulcerogenesis [63]. The luminal surface of the gastrointestinal tract is covered by a viscoelastic mucous gel layer that acts as a protective barrier against the harsh luminal environment. The structural characteristics of this barrier are primary indicators of its physiological function and changes to its composition have long been identified in gastrointestinal pathologies. The high molecular weight mucins are responsible for the viscoelastic properties of the mucous barrier. Mucins are implicated in the aetiology and may assist in the diagnosis of gastric intestinal metaplasia associated with gastric ulceration, *H. pylori* infection, and the risk of gastric cancer [64].

Inhibition of sulphated mucin synthesis and stimulation of pepsinogen secretion by LPS *in vitro* suggest the mechanisms for *H. pylori*-induced mucosal damage [42]. LPS, primarily through the lipid A component, stimulates the release of cytokines and possesses endotoxic properties including interference with the gastric epithelial cell-laminin interaction, which may lead to loss of mucosal integrity; inhibition of mucin synthesis; and stimulation of pepsinogen secretion [42]. Microvascular dysfunction was provoked by Hp-LPS [65]. Hp-LPS also exhibited alterations in the vascular permeability and the protective effect of TA may

be attributed to the cytoprotective activity by increasing the integrity of mucus status.

Mucus serves as first line of defense against ulcerogens. Mucus is secreted by the mucus neck cells and covers the gastric mucosa thereby preventing physical damage and back diffusion of hydrogen ions [66]. TA significantly increased mucus secretion as observed from the increase in TC: P ratio, which is taken as reliable marker for mucin secretion [67]. This was primarily due to increase in the individual mucopolysaccharides. Further, strengthening of the gastric mucosa is evident from the decrease in the leakage of protein into the gastric juice [68]. Increase in glycoprotein content of gastric mucosa is evidenced from increase in TC: P ratio of the mucosal cells, which is taken as marker for cellular mucus [69]. This increase was due to increase in mucopolysaccharides, the major constituent of mucus and also which are responsible for viscous nature and gel-forming properties of the mucus. The gel is reported to be resistant to a number of ulcerogens including acid, ethanol and NSAIDs, i.e. indomethacin [70]. Hence an increase in the synthesis of mucus may be one of the important contributing factors for ulcer protective role of TA as against other models of gastric ulcers [62,71].

Plate 1a: Effect of TA on gastric mucosal Cyclooxygenase 2 expression

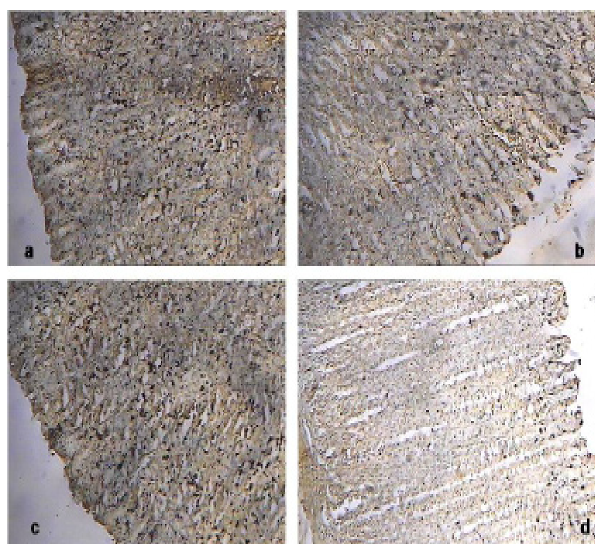

Plate 1a shows the effect of TA on gastric mucosal COX-2 expression in control and Hp-LPS induced experimental animals. Control rats showed normal expression of COX- 2 in gastric mucosa, Hp-LPS induced ulcerated rats showed an abrupt decrease in COX-2 expression in gastric mucosa, Hp-LPS+TA treated rats showed regeneration of mucosal cells and increased expression of COX-2 and Hp-LPS+SFT rats also showed regeneration of mucosal cells with reduced expression of COX-2.

Prostaglandins are known to protect the gastric mucosa against a wide variety of insults [72] and contribute to the maintenance of gastric mucosal integrity by influencing gastric mucus, bicarbonate and acid secretion as well as mucosal blood flow and epithelial cell proliferation rate [73]. *H. pylori* infection failed to induce COX-2 in gastric mucosa [74].

Likewise Hp-LPS 26695 inhibited the expression of COX-2 that could have reduced the

levels of prostaglandins and resulted in the mucosal damage. The diterpene derivative ecabet sodium improves the wound repair in intestinal epithelial cells elicited by hydrogen peroxide by inducing the expression of COX-2 [75]. Terpenes present in TA might have induced COX-2 expression in Hp-LPS+TA rats.

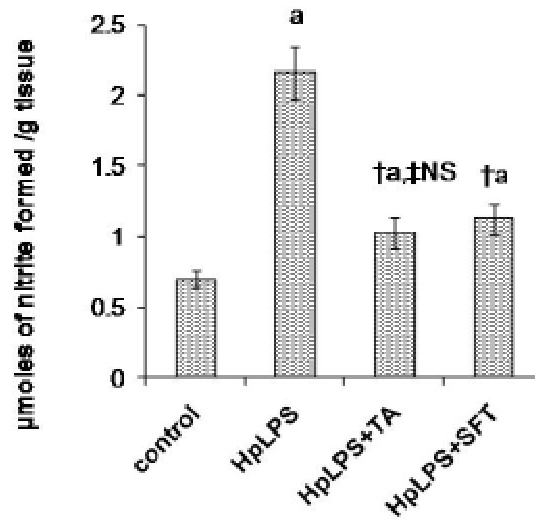

Figure 4a: Effect of TA on nitric oxide levels

Groups are compared as follows: Control vs. Hp-LPS, †- Hp-LPS vs. Hp-LPS+TA and Hp-LPS+SFT, ‡- Hp-LPS+SFT vs. Hp-LPS+TA

Significance represented as a– $p<0.001$, NS- Non-significant using One way ANOVA- Dunnett's T3 multiple comparison test.

Figure 4a illustrates the effect of TA on the levels of nitric oxide in gastric mucosa of control and Hp-LPS induced experimental animals. There was a significant increase ($p<0.001$) in nitric oxide level in Hp-LPS induced animals compared to control rats. Hp-LPS+TA and Hp-LPS+SFT rats showed a significant decrease ($p<0.001$) in nitric oxide level compared to Hp-LPS ulcer group. No significant differences were observed between Hp-LPS+TA and Hp-LPS+SFT rats.

Nitric oxide (NO) is also recognized as an important mediator of gastrointestinal mucosal defense, exerting many of the same actions as prostaglandins (PGs) in these tissues [76]. Both the mediators (NO and PG) are capable of modulating mucosal blood flow, mucus and bicarbonate secretions as well as the repair of gastric injury [77]. Three isoforms of NO synthase (NOS) exist: endothelial NOS, neuronal NOS, and inducible NOS (iNOS) [78].

NO plays a biphasic role in the ulcerogenic response in the gastrointestinal mucosa, as a protective effect of cNOS/NO and a proulcerogenic effect of iNOS/NO have been reported [78]. *H. pylori* LPS can initiate the expression of iNOS in the stomach following a systemic challenge, which can evoke microvascular dysfunction [65].

H. pylori LPS-induced gastric mucosal damage is manifested by the increase in pro-inflammatory cytokine production, excessive NO and prostaglandin generation, massive rise in epithelial cell apoptosis, and a marked up-regulation in gastric mucosal ET-1 level [79]. Induction of NOS-2 leads to pro-apoptotic caspase-3 activation and the excessive formation of

NO-related species that evoke transcriptional disturbances, cause alterations in prostaglandin formation, and leads to the up-regulation of pro-inflammatory cytokine production [80].

H. pylori infection may provoke damage in the stomach and duodenum by releasing soluble factors that activate inflammatory cells such as neutrophils, to produce cytotoxic mediators such as superoxide [81] and nitric oxide (NO) [82]. High concentrations of NO are known to be cytotoxic, and in combination with the superoxide radical, leads to the subsequent formation of the moieties, peroxynitrite and hydroxyl radicals, which are highly injurious to cells [83].

However, NO/iNOS also contributed to the gastric mucosal protection as induced by a mild irritant at a later time period or observed in arthritic rats [84]. The dual action of NO is not determined by the source enzyme, cNOS or iNOS, but depends more on the circumstance where NO is acting. On the other hand, Konturek *et al*. [85] reported that the healing of acetic acid-induced gastric ulcers was delayed and promoted by administration of NOS inhibitors and L-arginine, respectively. However, the inhibition of NO production by NG-nitro-L-arginine methyl ester (L-NAME) impaired gastric mucosal blood flow and delayed healing of acute gastric injury [86].

An increase in the levels of NO following Hp-LPS 26695 induction supports the theories of pro-ulcerogenic role of NO in Hp-LPS 26695 induced gastric ulcer. The decrease in NO levels with reduction in the ulcer size on TA administration suggests attenuation of cellular damage, mucosal injury and apoptosis. Hence the phytoconstituents present in TA may offer gastroprotection by decreasing NO production.

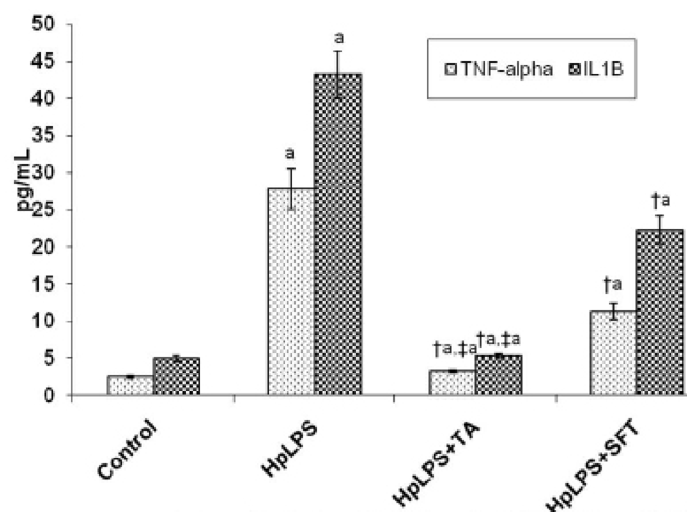

Figure 5a: Effect of TA on the levels of TNF-alpha and IL-1beta

Groups are compared as follows: Control vs. Hp-LPS, †- Hp-LPS vs. Hp-LPS+TA and Hp-LPS+SFT, ‡- Hp-LPS+SFT vs. Hp-LPS+TA

Significance represented as a– $p<0.001$, NS- Non-significant using One way ANOVA- Dunnett's T3 multiple comparison test.

Plate 2a: Effect of TA on PCNA expression

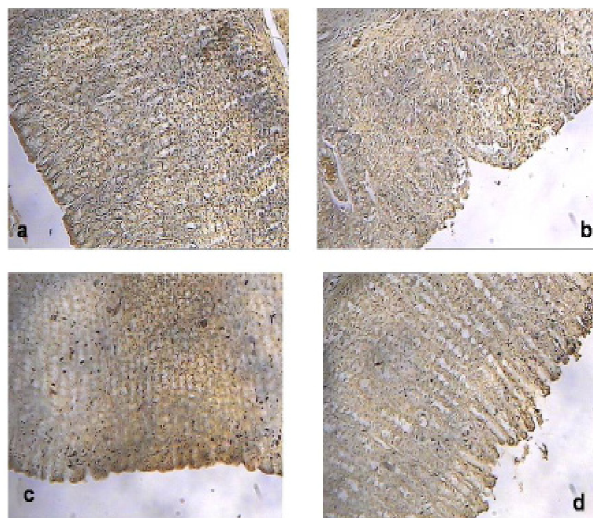

Plate 2a shows the effect of TA on PCNA expression in control and Hp-LPS induced experimental animals. PCNA positive cells were observed in gastric mucosa of control rats, Hp-LPS ulcerated rats showed lesser number of PCNA positive cells, whereas in Hp-LPS+TA rats and Hp-LPS+SFT rats abundant number of PCNA positive cells were observed.

Re-epithelialization is a key process in the ulcer-healing after mucosal injury. To restore the mucosal integrity the filling of the mucosal defect with proliferating and migrating epithelial and connective tissue cells is necessary [87]. The capacity of re-epithelialization is crucial in the recovery of the gastric mucosa after ulceration. Ulcer healing is a complex and tightly regulated process of filling the mucosal defect with proliferating and migrating epithelial and connective tissue cells [87]. Proliferating cell nuclear antigen (PCNA) plays an important role during DNA synthesis and cell proliferation. PCNA increases with *H. pylori* infection [88]. Sun *et al*. [89] found PCNA was increased during healing of gastric mucosal injury. There are several evidences that PCNA assessment is a useful tool to evaluate cell proliferation [88].

Acute gastric mucosal injury is often accompanied with decreased cell proliferation and increased cell apoptosis, while cell apoptosis decreases during the healing of gastric ulcers [90]. There is a balance between cell apoptosis and proliferation in normal gastric mucosa [91]. A few apoptotic cells exist in epithelial cells of normal gastric mucosa, but concentrated necrosis and apoptotic cells are found on the surface of ulcers [92]. In the present study, Hp-LPS induced gastric mucosal damage witnessed a significant decrease in the expression of PCNA. However, regeneration was marked in TA treated rats from a significant increase in the number of PCNA positive cells.

Figure 5a shows the effect of TA on the levels of plasma TNF-α and IL-1β in Hp-LPS induced experimental animals. Hp-LPS induced rats showed a significant increase ($p<0.001$) in TNF-α and IL-1 β levels compared to control rats, whereas Hp-LPS+TA and Hp-LPS+SFT animals showed a significant decrease ($p<0.001$) compared to Hp-LPS induced ulcer rats. A significant decrease ($p<0.001$) was observed in Hp-LPS+TA rats in both TNF α and IL-1 β

levels compared with Hp-LPS+SFT.

Enhancement in gastric mucosal TNF-α production, excessive NO and prostaglandin generation, and alteration in the extent of epithelial cell apoptosis are associated with mucosal inflammatory responses in the animal model of *H. pylori* LPS-induced gastritis [93].

Acute mucosal inflammatory responses are accompanied by a massive epithelial cell apoptosis, and a marked increase in the expression of membrane-bound and soluble forms of TNF-α [94].

IL-1β is a potent inflammatory cytokine that is released as a component of the host response against bacterial infection. It is primarily expressed by activated monocytes/macrophages. IL-1β is produced as a precursor molecule, pro-IL-1β, in the cytosol of macrophages. Pro-IL-1β is a 31–34-kDa inactive form of the cytokine, which is later cleaved by caspase-1 to active 17-kDa IL-1β [95].

Soluble mediators of *H. pylori* are known to induce IL-1β. Of particular significance is the finding that IL-1β gene cluster polymorphisms suspected of enhancing production of IL-1β are associated with an increased risk of gastric cancer [96]. This makes it worthwhile to explore the mechanism of induction of IL-1β by *H. pylori*, and in particular, the role of LPS. The expression of IL-1β is regulated at the level of transcription [97], mRNA stabilization, and post-translational proteolytic processing [98].

Hp-LPS 26695 increased the levels of TNF-α and IL-1β probably in consequence to inflammatory response. As in other models of ulcers, TA also reduced the levels of TNF-α and IL-1β. Mucosal Sucralfate administration produced a reduction in the mucosal expression of TNF-α [5] which is in agreement with the present study.

4. Conclusion

The aqueous extract of *Terminalia arjuna* showed beneficial role in treating gastric ulcer induced by *Helicobacter pylori* lipopolysaccharide (one of the virulence factor for *Helicobacter pylori*). From the results observed in this study, it can be concluded that the evaluated anti-ulcer effect of aqueous extract of *Terminalia arjuna* had significant impact on inhibiting the aggressive factors such as acid and pepsin. The cytoprotective effect was evident from mucosal integrity and ulcer healing effect was mediated by maintenance of proinflammatory processes, gastric mucosal regeneration property.

5. References

1. Yazbek PB, Trindade AB, Chin CM, and Dos Santos JL, "Challenges to the treatment and new perspectives for the eradication of *Helicobacter pylori*," Digestive Diseases and Sciences, 2015; 60: 2901–2912.

2. Parmar NS, Desai JK. *Helicobacter pylori* and gastroduodenal diseases. *Indian Drugs* 1994; 31: 175.

3. Mitchell HM. In: *Helicobacter pylori*: biology and clinical practice. Goodwin CS, Worsley BW (eds), CRC Press, Boca Raton, 1993; pp 95-114.

4. Konturek PC, Pierzchalski P, Konturek SJ, Meixner H, Faller G, Kirchner T, Hahn EG. *Helicobacter pylori* induces apoptosis in gastric mucosa through an upregulation of Bax expression in humans. *Scand J Gastroenterol.,* 1999; 34: 375-383.

5. Slomiany BL, Piotrowski J, Slomiany A. Up-regulation of endothelin-1 in gastric mucosal inflammatory responses to *Helicobacter pylori* Lipopolysaccharide: effect of omeprazole and sucralfate. *J Physiol Pharmacol.,* 2000; 51: 179-192.

6. Slomiany BL, Piotrowski J, Slomiany A. Gastric mucosal inflammatory responses to *Helicobacter pylori* lipopolysaccharide: down-regulation of nitric oxide synthase-2 and caspase-3 by sulglycotide. *Biochem Biophys Res Commun.,* 1999; 261: 15-20.

7. Gupta RA, Polk DB, Krishna U, Israel DA, Yan F, DuBois RN, Peek RM. Activation of peroxisome proliferators-activated receptor γ suppresses nuclear factor κB-mediated apoptosis induced by *Helicobacter pylori* in gastric epithelial cells. *J. Biol. Chem.,* 2001a; 276: 31059–31066.

8. Germano MP, Sanogo R, Guglielmo M, De Pasquale R, Crisafi G, Bisignano G. Effect of Pteleopsis suberosa extracts on experimental gastric ulcers and *Helicobacter pylori* growth. *J. Ethnopharmacol.,* 1998; 59: 167–172.

9. Korman MG, Bolin TD, Engelmann JI, Pianko S. Sucralfate as an alternative to bismuth in quadruple therapy for *Helicobacter pylori* eradication. *Helicobacter* 1997; 2: 140–143.

10. Yu LY, Sun LN,. Zhang XH et al., "A review of the novel application and potential adverse effects of proton pump inhibitors," Advances in Therapy, 2017; 34.

11. Kangwan N, Park JM, Kim EH, Hahm KB, "Quality of healing of gastric ulcers: natural products beyond acid suppression," World Journal of Gastrointestinal Pathophysiology, 2014; 5: 40–47.

12. Sezik E, Yesilada E, Tabata M, Honda G, Takaishi Y, Fujita T, Tanaka T, Takeda Y. Traditional medicine in Turkey VIII. Folk medicine in east Anatolia; Erzurum, Erzincan, Agri, Kars, Igdir provinces. *Economic Botany* 1997; 51: 195–211.

13. Dwivedi S, Udupa N. *Terminalia arjuna*: Pharmacognosy, phytochemistry, pharmacology and clinical use. A review. *Fitoterapia* 1989; 60: 413–420.

14. Hartwell JL. *Plants Used Against Cancer*. Quarterman Publications, Inc., Lawrence, MA. 1982.

15. Prabhakar YS, Kumar DS. The chemistry of *Terminalia arjuna* (Roxb.) Wight and Arnot with reference to its medicinal uses. *Plantes Med. Phytother.* 1988; 22: 30–39

16. Ramanathan R, Tan CH, Das NP. Cytotoxic effect of plant polyphenols and fat-soluble vitamins on malignant human cultured cells. *Cancer Lett.*1992; 62: 217–224.

17. Varier PS. *Indian Medicinal Plants*, vol. 5, first ed., Orient Longman, Arya Vaidya Sala, Kottakal. 1997; pp 253–257.

18. Vaidyaratnam PS. *Varier's Indian Medicinal Plants, A Compendium of 500 Species.* Orient Longman, Arya Vaidya Sala, Kottakal. 1994.

19. Ghanekar BG. *Ayurveda rahasyadipika* (Sushruta Samhita with Hindi commentary),1st edn. Lahore: Meharchand

Lachhmandas. 1936.

20. Gupta R, Singhal S, Goyle A, Sharma VN. Antioxidant and hypocholesterolaemic effects of *Terminalia arjuna* tree-bark powder: a randomised placebo-controlled trial. *J. Assoc. Physicians India* 2001b; 49: 231–235.

21. Kandil FE, Nassar M I. A tannin anti-cancer promoter from *Terminalia arjuna*. *Phytochemistry* 1998; 47: 1567–1568.

22. Kaur S, Grover IS, Kumar S. Antimutagenic potential of extracts isolated from *Terminalia arjuna*. *J Environ Pathol Toxicol Oncol* 2001; 20: 9-14.

23. Vaidya AB. *Terminalia arjuna* in cardiovascular therapy. *J. Assoc. Physicians India* 1994; 42: 281–282.

24. Nair S, Nagar R, Gupta R. Dietary anti-oxidant phenolics and flavonoids in coronary heart disease. *Indian Heart J.,* 1996; 48: 545.

25. Perumal Samy R, Ignacimuthu S, Sen A. Screening of 34 Indian medicinal plants for antibacterial properties. *J. Ethnopharmacol.*1998; 62: 173–182.

26. Ali A, Kaur G, Hayat K, Ali M, Ather M. A novel naphthanol glycoside from *Terminalia arjuna* with antioxidant and nitric oxide inhibitory activities. *Pharmazie* 2003b; 58: 932–934.

27. Ali A, Kaur G, Hamid H, Abdullah T, Ali M, Niwa M, Alam MS. Terminoside A, a new triterpene glycoside from the bark of *Terminalia arjuna* inhibits nitric oxide production in murine macrophages. *J. Asian Nat. Prod. Res.,* 2003a; 5: 137–142.

28. Slomiany BL, Piotrowski J, Slomiany A. Effect of sucralfate on gastric mucosal inflammatory responses induced by helicobacter lipopolysaccharide. Scand J Gastroenterol 1998, 33, 916-922.

29. Shay M, Komarov SA, Fels D, Meranze D, Gruenstein H, Siplet H. A simple method for the uniform production of gastric ulceration in the rat. *Gastroenterology,* 1945; 5: 43-61.

30. Okabe S, Hung CR, Takeuchi K, Takagi K. Effects of L-glutamine of acetylsalicylic acid or taurocholic acid-induced gastric lesions and secretory changes in pylorus-ligated rats under normal or stress conditions. Jpn J Pharmacol 1976 Aug;26(4):455-60.

31. Card WI, Marks IN. 1960. The relationship between the acid output of the stomach following "maximal" histamine stimulation and the parietal cell mass. Clin Sci 19, 147- 163.

32. Kay AW. Effect of large doses of histamine on gastric secretion of hydrochloric acid: an augmented histamine test. *Br. Med. J.,* 1953; 2: 77- 80.

33. Corney SJ, Morrisay SM, Woods RJ. 1974. A method for the quantitative estimation of gastric barrier mucus. Proc Physiol Soc 242, 116-117.

34. Lowry OH, Rosenbrough NJ, Farr AL, Randall RJ. 1951. Protein measurement with folin-phenol reagent. J Biol Chem 193, 265–275.

35. Niebes P. Determination of enzymes and degradation products of glycosaminoglycan metabolism in healthy and various subjects. Clin. Chem. Acta 1972; 42: 399-408.

36. Wagner WD. More sensitive assay for discriminating galactosamine and glycosamine in mixtures. *Anal. Biochem.,* 1979; 94: 394-397.

37. Warren M. The thiobarbituric acid assay of sialic acid. *J. Bio.l Chem.,* 1959; 234, 1971-1975.

38. Dische Z, Shettles SB. 1948. A specific colour reaction of methyl pentoses and a spectrophotometric micro method for the determination. J Biol Chem 175, 595-604.

39. Anson ML. 1938. The estimation of pepsin, trypsin, papain, and cathepsin with haemoglobin. J Gen Physiol 22, 79.

40. Green LC, Ruiz de Luzuriaga K, Wagner DA, Rand W, Istfan N, Young VR, Tannenbaum SR. Nitrate biosynthesis in man. *Proc Natl Acad Sci USA.,* 1981; 78: 7764-7768.

41. Sharma M, Singh O. Immunoassays. In: *Murine and human monoclonal antibody production, Purification and applications. Practical Manual,* National Institute of Immunology, New Delhi. 1989; pp. 27-29.

42. Moran AP. The role of lipopolysaccharide in *Helicobacter pylori* pathogenesis. *Aliment Pharmacol Ther.,* 1996; 10: 39-50.

43. Kidd M, Miu K, Tang LH, Perez-Perez GI, Blazer MJ, Sandor A, Modlin IM. *Helicobacter pylori* lipopolysaccharide stimulates histamine release and DNA synthesis in rat enterochromaffin-like cells. *Gastroenterology,* 1997; 113: 1110-1117.

44. Moran AP, Young GO, Lastovica A. Pepsinogen induction by *Helicobacter pylori* lipopolysaccharides. *Gut* 1998; 43: A15.

45. Ray PG, Majumdar SK. Antimicrobial activity of some Indian plants. *Econ Bot.,* 1976; 30: 317-320.

46. O'Toole D, Abdel-Latif MM, Long A,Windle HJ, Murphy AM, Bowie A, O'Neill LA, Weir DG, Kelleher D. Low pH and *Helicobacter pylori* increase nuclear factor kappa B binding in gastric epithelial cells: a common pathway for epithelial cell injury? *J. Cell Biochem*., 2005; 96: 589-98.

47. Loffeld RJ, van der Hulst RW. *Helicobacter pylori* and gastro-oesophageal reflux disease: association and clinical implications. To treat or not to treat with anti-*H. pylori* therapy? *Scand J Gastroenterol Suppl.,* 2002; 236: 15-18.

48. Banerjee S, El-Omar E, Mowat A, Ardill JE, Park RH,Watson W, Beattie AD, McColl KE. Sucralfate suppresses *Helicobacter pylori* infection and reduces gastric acid secretion by 50% in patients with duodenal ulcer. *Gastroenterology*, 1996; 110: 947-950.

49. Lee A, O'Rourke J, Corazon de Ungria M, Robertson B, Daskalopoulos G, Dixon MF. A standardized mouse model of *Helicobacter pylori* infection: introducing the Sydney strain. *Gastroenterology,* 1997; 112: 1386-1397.

50. Padol IT, Moran AP and Hunt RH. Effect of purified lipopolysaccharides from strains of *Helicobacter pylori* and *Helicobacter felis* on acid secretion in mouse gastric glands *in vitro*. *Infect Immun.*, 2001; 69: 3891-3896.

51. Raufman JP. Pepsin. In: *Encyclopedia of Gastroenterology,* 2004; pp. 147-148.

52. Young GO, Brown S, Stemmet N, Lastovica AJ, Marks IN, Modlin IM, Kidd M. The pepsinogen releasing effect of *Helicobacter pylori* lipopolysaccharide. *Helicobacter* 2002; 7: 30-38.

53. Young GO, Stemmet N, Lastovica A, van der Merwe EL, Louw JA, Modlin IM, Marks IN. *Helicobacter pylori* lipopolysaccharide stimulates gastric mucosal pepsinogen secretion. *Aliment Pharmacol Ther.,* 1992; 6: 169-177.

54. Walsh JH. Gastrin. In: *Gut Peptide Biochemistry and Physiology*, Walsh JH , Dockray GJ (Editors). Raven Press, New York: 1994, pp. 75-122.

55. Mulholland G, Ardill JES, Fillmore D, Chittajallu RS, Fullarton GM, McColl KE. *Helicobacter pylori* related hypergastrinemia is the result of selective increase in gastrin 17. *Gut* 1993; 34: 757-761.

56. Beales ILP, Calam J. *Helicobacter pylori* infection and tumor necrosis factor-a increase gastrin release from human gastric antral fragments. *Eu.r J. Gastroenterol. Hepatol.,* 1997; 9: 773-777.

57. Yasunaga Y, Shinomura Y, Kanayama S, Higashimoto Y, Yabu M, Miyazaki Y, Murayama Y, Nishibayashi H, Kitamura S, Matsuzawa Y. Mucosal interleukin-1b production and acid secretion in enlarged fold gastritis. *Aliment Pharmacol Ther.,* 1997; 11: 801-809.

58. Perez-Paramo M, Albillos A, Calleja JL, Salas C, Marin MC, Marcos ML, Cacho G, Escartin P, Ortiz-Berrocal J. Changes in gastrin and serum pepsinogens in monitoring of *Helicobacter pylori* response to therapy. *Dig. Dis. Sci*., 1997; 42: 1734-1740.

59. Slomiany BL, Slomiany A. Disruption in gastric mucin synthesis by *Helicobacter pylori* lipopolysaccharide involves ERK and p38 mitogen-activated protein kinase participation. *Biochem Biophys Res Commun.,* 2002; 294: 220-224.

60. Piotrowski J, Majka J, Slomiany A, Slomiany BL. *Helicobacter pylori* lipopolysaccharide inhibition of gastric mucosal somatostatin receptor. *Biochem. Mol. Biol. Int.* 1995; 36: 491–498.

61. Liau YH, Lopez RA, Slomiany A, Slomiany BL. *Helicobacter pylori* lipopolysaccharide effect on the synthesis and secretion of sulfated gastric mucin. *Biochem Biophys Res Commun.,* 1992; 113: 455-464.

62. Devi RS, Narayan S, Vani G, Srinivasan P, Mohan KV, Sabitha KE, Devi CS. Ulcer protective effect of *Terminalia arjuna* on gastric mucosal defensive mechanism in experimental rats. *Phytother Res.,* 2007b; 21: 762-767.

63. Goel RK, Bhattacharya SK. Gastroduodenal mucosal defense and mucosal protective agents. *Indian J Exp Biol.,* 1991; 29: 701-714.

64. Corfield AP, Myerscough N, Longman R, Sylvester P, Arul S, Pignatelli M. Mucins and mucosal protection in the gastrointestinal tract: new prospects for mucins in the pathology of gastrointestinal disease. *Gut,* 2000; 47: 589-594.

65. Kiss J, Lamarque D, Moran AP, Pozsar J, Morschl E, Laszlo F, Whittle BJ. *Helicobacter pylori* lipopolysaccharide-provoked injury to rat gastroduodenal microvasculature involves inducible nitric oxide synthase. *Eur J Pharmacol.,* 2001; 420: 175-179.

66. Williams SE, Turnberg LA.Retardation of acid diffusion by pig gastric mucus: a potential role in mucosal protection. *Gastroenterology,* 1980; 79: 299-304.

67. Goel RK, Govinda Das D, Sanyal AK. Effect of vegetable banana power on changes induced by ulcerogenic agents on dissolved mucosubstances in gastric juice. *Indian J Gastroenterol,* 1985; 4: 249-251.

68. Goel RK, Gupta S, Shankar R, Sanyal AK. Antiulcerogenic effect of Banana powder (*Musa sapientum var. paradisiaca*) and its effect on mucosal resistance. *J Ethnopharmacol.,* 1986; 18: 33-44.

69. Goel RK, Maiti RN, Mukhopadhyaya K. Effect of Tamrabhasma, an indigenous preparation of copper, on rat gastric mucosal resistance. *Indian J Exp Biol.,* 1994; 32: 559-561.

70. Bell AE, Sellers LA, Allen A, Cunliffe WJ, Morris ER, Ross-Murphy SB. Properties of gastric and duodenal mucus: effect of proteolysis, disulfide reduction, bile, acid, ethanol, and hypertonicity on mucus gel structure. *Gastroenterology,* 1985; 88: 263-268.

71. Devi RS, Narayan S, Vani G, Shyamala Devi CS. Gastroprotective effect of *Terminalia arjuna* bark on diclofenac sodium induced gastric ulcer. *Chem Biol Interact.,* 2007a; 167: 71-83.

72. Terano A, Ota S, Mach T, Hiraishi H, Stachura J, Parnawski A, Ivey KJ. Prostaglandin protects against Taurocholate induced damage to rat gastric mucosa cell culture. *Gastroenterology,* 1986; 92; 669-677.

73. Lichtenberger LM, Graziani LA, Dial EJ, Butter BD, Hills BA. Role of surface-active phospholipids in gastric cytoprotection. *Science,* 1983; 219: 1327-1329.

74. Shah AA, Byrne MF, Cullen L, Walsh T, Fitzgerald DJ, Murray FE. Effect of *H. pylori* infection on the expression of cyclooxygenase-2 in human gastric mucosa. *Prostaglandins, Leukot Essent Fatty Acids,* 2003; 68: 1-8.

75. Sasaki K, Iizuka M, Konno S, Shindo K, Sato A, Horie Y,Watanabe S. Ecabet sodium prevents the delay of wound repair in intestinal epithelial cells duced by hydrogen peroxide. *J Gastroenterol,* 2005; 40: 474-482.

76. Hirata T, Ukawa H, Yamakuni H, Kato S, Takeuchi K. Cyclooxygenase isozymes in acute mucosal ulcerogenic and functional responses following barrier disruption in rat stomachs. *Br J Pharmacol.,* 1997; 122: 447-454.

77. Takeeda M, Yamato M, Kato S, Takeuchi K. Cyclooxygenase isozymes involved in adaptive functional responses in rat stomachs following barrier disruption. *J Pharmacol Exp Therap.,* 2003; 307: 713-719.

78. Tanaka A, Kunikata T, Konaka A, Kato S, Takeuchi K. Dual action of nitric oxide in pathogenesis of indomethacin-

induced small intestinal ulceration in rats. *J Physiol Pharmacol.,* 1999; 50: 405-417.

79. Fu S, Ramanujam KS, Wong A, Fantry GT, Drachen-Berg CB, James SP, Meltzer SJ, Wilson KT. Increased expression and cellular localization of inducible nitric oxide synthase and cyclooxygenase 2 in *Helicobacter pylori* gastritis. *Gastroenterology,* 1999; 116: 1319-1329.

80. Marnett LJ, Wright TL, Crews BC, Tannenbaum SR, Morrow JD. Regulation of prostaglandin biosynthesis by nitric oxide is revealed by target deletion of inducible nitric-oxide synthase. *J Biol Chem.,* 2000; 275: 13427-13430

81. Mooney C, Keenan J, Munster D, Wilson I, Allardyce R, Bagshaw P, Chapman B, Chadwick V. Neutrophil activation by *Helicobacter pylori*. *Gut,* 1991; 32: 853-857.

82. Mccall TB, Boughton-Smith NG, Palmer RMJ, Whittle BJR, Moncada S. Synthesis of nitric oxide from L-arginine by neutrophils. Release and interaction with super- oxide anion. *Biochem J.,* 1989; 261: 293-296.

83. Ischiropoulos H, Al-Medhi AB, Fisher AB. Reactive species in ischemic rat lung injury: contribution of peroxynitrite. *Am J Physiol.,* 1995; 269: L158-L164.

84. Kato S, Tanaka A, Konaka A, Kunikata T, Takeuchi K. Changes in gastric mucosal ulcerogenic responses in rats with adjuvant arthritis: role of nitric oxide. *Alimen Pharmacol Therap.,* 1999; 13: 833-840.

85. Konturek SJ, Brzozowski T, Majka J, Polmczyk J, Stachula J. Inhibition of nitric oxide synthase delays healing of chronic gastric ulcers. *Eur J Pharmacol.,* 1993; 239: 215-217.

86. Takeuchi K, Kato S, Takehara K, Asada Y, Yasuhiro T. Role of nitric oxide in mucosal blood flow response and healing of HCl-induced lesions in rat stomachs. *Digestion,* 1997a; 58: 19-27.

87. Tarnawski A, Szabo IL, Husain SS, Soreghan B. Regeneration of gastric mucosa during ulcer healing is triggered by growth factors and signal transduction pathways. *J Physiol Paris*, 2001; 95: 337-344.

88. Aydemir S, Ozdemir BH, Gur G, Dogan I, Yilmaz U, Boyacioglu S. Effects of *Helicobacter pylori* infection on gastric epithelial cell kinetics in patients with chronic renal failure. *World J Gastroenterol*., 2005; 11: 7183-7187.

89. Sun WH, Ou XL, Yu Q, Cao DZ, Chen H, Yu T, Shao H, Zhu F, Sun YL. Effects of cyclooxygenase-2 inhibitors on gastric epithelial cell proliferating and gastric healing following hydrochloric acid-induced injury in rats. Zhongguo Bingli Shengli Zazhi. 2003;19:1508.

90. Zhang FC, Liang LX, Zhang G, Cai LY, Liu MC. The relationship between apoptosis, expression of EGFR and gastric ulcer. *Linchuang Neike Zazhi.,* 2003; 20: 382-383.

91. Ma ZF, Ma HS. Correlations of *Helicobacter pylori*-related gastric mucosal inflammation and apoptosis with lactic acid bacteria. *Shijie Huaren Xiaohua Zazhi.,* 2006; 14: 312-317.

92. Tang ZP, Xu XM, Ye LZ, Kuang ZS, Xie YH. Effect of Jianzhongyuyangpian (JZYYP) on acetic acid-induced gastric ulcer margin mucosal cell kinetics in rats. *Zhongguo Zhongxiyi Jiehe Xiaohua Zazhi.,* 2001; 9: 201-203.

93. Poligone B, Baldwin AS. Positive and negative regulation of NF-κB by COX-2. *J Biol Chem.,* 2001; 276: 38658-38664.

94. Slomiany BL, Piotrowski J, Slomiany A. Up-regulation of gastric mucosal inflammatory responses to *Helicobacter pylori* lipopolysaccharide by aspirin but not indomethacin. *J Endotoxin Res.,* 2001; 7: 203-209.

95. Cerretti DP, Kozlosky CJ, Mosley B, Nelson N, Van Ness K, Greenstreet TA, March CJ, Kronheim SR, Druck T, Cannizzaro LA, Huebner K, Black RA. Molecular cloning of the interleukin-1 beta converting enzyme. *Science,* 1992; 256: 97-100.

96. El-Omar EM, Carrington M, Chow WH, McColl KE, Bream JH, Young HA, Herrera J, Lissowska J, Yuan CC, Rothman N, Lanyon G, Martin M, Fraumeni JF Jr., Rabkin CS. Interleukin-1 polymorphisms associated with increased risk of gastric cancer. *Nature*, 2000; 404: 398-402.

97. Cahill CM, Waterman WR, Xie Y, Auron PE, Calderwood SK. *J Biol Chem.,* 1996; 271: 24874-24879.

98. Auron PE, Webb AC. Interleukin-1: a gene expression system regulated at multiple levels. *Eur Cytokine Netw.* 1994; 5: 573-592.

Chapter 4

Recent Updates on Biomarkers of Gastric Cancer

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Abstract

Gastric cancer (GC) or stomach cancer, is one of the most common malignancies worldwide. It ranks second in terms of global cancer-related mortality and fourth in terms of incidence among various ethnic groups. The etiology of GC is multifactorial and includes dietary as well as non-dietary factors. Despite many efforts, GC remains to be the condition without clear symptoms at the onset, poor prognosis and high recurrence. Thus, there is an urgent need to find efficient assays to identify gastric cancer biomarkers for treatment of the disease. Here, we review the most effective biomarkers for gastric cancer with a potential for the early detection and treatment of GC.

Keywords: Cancer; GC biomarkers; Treatment; Biomarker assays

1. Introduction

GC is the fourth most common cancer and the second leading cause of cancer related deaths, with nearly one million newly diagnosed cases every year [1]. According to WHO, around 7.6 million people worldwide die of cancer annually, and it is estimated that by 2030, the deaths from cancer will rise to over 11 million [1,2]. The number of deaths due to GC amount to 3.4 per 100,000 per year [3]. GC is an aggressive malignancy that is difficult to be detected at an early stage. It is a complex, multi step process involving several genetic and epigenetic alterations that lead to aberrant expression of specific genes. GC is defined as the

malignancy of the gastric mucosa epithelium with glandular differentiation. About half of the GCs are located in the lower stomach. GC is caused by altered regulation of oncogenes, tumor suppressor genes, DNA repair genes, cell cycle regulators, and signaling molecules [4]. It occurs when cells in the lining of the stomach grow uncontrollably and form tumors that can invade normal tissues and spread to other parts of the body [6]. Anticancer research has led to the development of many novel target molecules and techniques to diagnose and treat cancers [8-11]. As GC is often without the clear symptoms, early diagnosis of GC requires novel biomarkers and techniques with high sensitivity and specificity.

GC can be classified into intestinal and diffuse types based on epidemiological and histo-pathological features [14,15]. Adenocarcinoma is the major histological type of GC, accounting for upto 95% of all gastric malignancies [16]. Tumor node metastasis (TNM) staging system is used worldwide for prognosis and direct therapeutic decisions for patients with GC [17]. Accumulating evidence has implicated the role of *H. pylori* infection in the pathogenesis of GC by causing a chronic gastritis, the precursor to all the pathophysiologic abnormalities characteristic of gastric carcinogenesis [19,20]. Intestinal type GC is associated with *H. pylori* infection, obesity, and certain dietary factors, such as high intake of salt, smoked meats, and food preserved with nitrites or nitrates, smoking and alcohol [18]. During last few decades the studies of GC showed that it results from the complex gene-environment interactions [21,22]. New high-throughput techniques have revealed its association with alterations of many genes, deregulation of signaling pathways, aberrant DNA methylation patterns, and chromosomal imbalances [23]. Despite advances in diagnosis and treatment, the five year survival rate for GC is poor, with only 20% of the patients surviving [18,24].

2. GC Biomarkers

GC is a biologically complex disease arises evolves due to various genetic and epigenetic alterations. Therefore, it is essential to understand the molecular variables that affect GC in order to develop clinical strategies for its treatment. A great deal of effort has been made in the search of tumor biomarkers, in order to improve the understanding of GC and to identify biomarkers that would improve cure by early detection and diagnosis. The biomarkers are biological variables that correlate with clinico-biological outcomes. The discovery of cancer biomarkers to devise strategies that target expressed proteins are becoming increasingly popular [25]. Overall, the molecular phenotyping of GC is still in its infancy and the search for novel diagnostic and predictive biomarkers continues [26]. Identifying more biomarkers (e.g. COX-2, c-myc, p27 or p53) will have a major impact for diagnosis and making clinical decisions. Analysis of cancer tissues revealed that miRNAs could be important molecular markers useful for cancer classification, prognosis and therapy. miRNAs also emerged as circulating markers, which may become valuable for early diagnosis and follow-up investigations [27]. miRNAs have become important biomarkers, thanks to their stability and the availability of assays to

quantify them [28,29]. Cancer cells employ multiple and diverse survival pathways and there is an increasing need to define a battery of biomarkers [30]. Such signatures might appropriately represent the breadth of molecular diversity inherent in cancers in general, and pave the way to understand the molecular genetics of gastric carcinogenesis. In the present era of personalized medicine, genomic and proteomic profiling attempts for mining novel biomarkers of GC may provide basis for individualized therapy to cancer patients.

Some of the well-known GC causing factors are the consumption of nitrate- or nitrite-rich food (grilled, salted, or pickled foods) [31], *H. pylori* infection [32], old age (>60 years), and a history of stomach disorders [33]. Proteomics based biomarkers can help in the accurate diagnosis and treatment of GC patients [34,35]. To identify potential target proteins that can serve as novel GC biomarkers, a combination of various proteomics techniques have been used, such as 2-DE, iTRAQ, ICAT, ProteinChip array, hydrophilic interaction liquid chromatography (HILIC), and 2-D LC [36]. The identity of the fractionated proteins has been revealed by MS [37]. Other validating techniques such as western blotting and immunohistochemistry (IHC), can be used to confirm these proteins [38]. Galectins are an important proteomics based GC biomarkers with significant change in expression in GC [39-46].

A number of GC biomarkers have been identified. However, not all have been equally effective. We first describe the two effective assays to treat GC and then list some important GC biomarkers.

2.1.1 Bevacizumab assay

Bevacizumab is a humanized monoclonal antibody against human vascular endothelial cell growth factor (VEGF). Bevacizumab is effective against GCs as VEGF is involved in the development of gastric cancers. There are, however, no established methods to predict the clinical efficacy of using bevacizumab. Although patients treated with bevacizumab in addition to chemo-therapy respond well and seem to benefit from it.

The antitumor activity of bevacizumab has been examined in the MKN-45 human gastric xenograft models and was found to show significant effect against MKN-45 tumor growth. The antitumor activity of bevacizumab against colorectal tumors has also been tested and found to be comparable to that of the gastric cancers. The efficacy of bevacizumab in gastric cancers depends on VEGF. VEGF is significantly expressed in bevacizumab-sensitive tumors compared with bevacizumab-insensitive tumors [47]. Bevacizumab also inhibits angiogenesis in gastric cancers

The microvessel density (MVD) measurements suggest that angiogenic factors other than VEGF are involved in angiogenesis in bevacizumab-insensitive tumors. However, expression levels of different angiogenic factors did not significantly correlate with bevacizumab efficacy

[47]. Human cancer xenograft models have been used to study the effects of bevacizumab in gastric cancers for which assays have been developed by inoculating human gastric cancer cells into T-cell-deficient mice [47]. Bevacizumab showed significant antitumor activity in MKN-45. However, the sensitivity of the gastric cancer models to bevacizumab was found to be unrelated to the histological type. Some studies have suggested that bevacizumab directly inhibits the growth of tumor cells [48]. However, in the gastric cancer cell lines the direct antitumor activity of bevacizumab was not observed [47][1]. Thus the levels of VEGF are closely related with the sensitivity of bevacizumab in the tested cell lines.

2.1.2 HUVEC pVEGFR2 assay:

Angiogenesis, in which new blood vessels are generated, plays a critical role in tumor development. VEGF triggers angiogenesis by dimerization of the receptor tyrosine kinases. In HUVEC pVEGFR2 assay, tumor samples are homogenized in HuMedia-EB2 basal media. The VEGF concentration in the samples can be measured and HUVEC seeded at a density of approx. 3×10^5 in HuMedia-EG2. The medium can then be changed to an assay medium which should have been pre-treated with bevacizumab or human IgG and the cells washed with PBS. The cells are lysed and pVEGFR2 detected by immunoblotting.

2.2.3 Cancerous inhibitor of protein phosphatase 2A

Cancerous inhibitor of protein phosphatase 2A (CIP2A) is an oncogenic factor that can serve as marker for gastric cancer. It stabilizes c-MYC by inhibiting its degradation and drives the neoplastic transformation [49]. Cancers are characterized by unregulated cell proliferation and differentiation [50]**.** CIP2A expression in malignant gastric tissues can be used to determine the role of CIP2A as a diagnostic marker for gastric cancer. Gastric cancer specimens could be analyzed for CIP2A expression and compared with normal WT expression. In one study, the presence of CIP2A transcripts was significantly higher in 90% of cancer samples analysed [51]. However, there does not seem to be a direct link between CIP2A expression and the stage of tumor.

Unlike other cancers, there is a dearth of novel diagnostic markers for gastric cancer. CIP2A, a potent endogenous inhibitor of PP2A is an oncoprotein required for anchorage-independent growth and transformation of human cells. CIP2A is an important oncogenic factor and may thus serve as a diagnostic marker for gastric cancer. However, a potential link between depletion of CIP2A and induction of senescence remains to be defined. It has recently been showed that the deletion of the c-MYC gene causes premature senescence of human fibroblasts [52]. Intriguingly, CIP2A depletion leads to partial differentiation of leukemic HL60 cells due to the reduced expression of c-MYC in HL60 cells [53] Thus overexpression of CIP2A seen in many tumors may interfere with cellular differentiation leading to malignant

transformation. Since CIP2A gene is highly expressed in gastric cancer tissues compared to normal tissues, it can be an important candidate for the diagnosis and treatment of gastric cancer.

2.2.4 *Helicobacter pylori* infection as the indicator of Gastric Cancer

H. pylori causes the development of gastric cancer [54]. However, the degree of infection in gastric cancer patients varies in different studies. These variations may be due to differences in the detection methods of *H. pylori* in the gastric cancer patients. Many diagnostic tests for *H. pylori* yield false results, and therefore multiple tests should be carried to provide accurate diagnosis of *H. pylori* infection [55]. In many countries endoscopy is performed for the early detection of gastric cancers. In one study, the relationship between *H. pylori* infection and the incidence of gastric cancer has been studied by endoscopy and biopsy, followed by histological and serologic testing [56]. It showed that gastric cancer developed in patients having *H. pylori* infection whereas uninfected patients did not develop gastric cancer. Other epidemiologic studies have also showed a close relation between *H. pylori* infection and occurrence of gastric cancer. Serum antibodies have also been successfully used to estimate the H. pylori infection in patients with gastric cancers. However, recent studies have demonstrated that serum antibody assay can yield false negatives, thus underestimating the degree of *H. pylori* infection in gastric cancer patients. To circumvent this problem, tissues must be taken from the greater curvature of the uppergastric body that results in fewer false negatives. Hence, H. pylori infection can act as the marker of gastric cancer and eradication of *H. pylori* could prevent the development of new gastric cancers. It has previously been shown that suppression of *H. pylori* infection inhibits the development of gastric cancer or growth of occult cancer.

2.2.5 Insulin-like growth factor type 1 receptor, epidermal growth factor receptor and HER2

Insulin-like growth factor type 1 receptor (IGF-IR), epidermal growth factor receptor (EGFR) and HER2 have been linked to several tumors and are considered as important biomarkers of gastric cancer. However, the clinical significance of these gastric cancer biomarkers remains to be investigated beyond doubt. Estimation of IGF-IR levels in surgical GC specimens and diffuse type tumors are significant prognosticators of GC. Thus devising strategies to target IGF-IR may prove valuable in treating GC patients [57]. As GC remains an aggressive malignancy, with an average survival of approx. 10 months in patients with metastatic GC, emphasis on the improved techniques for the diagnosis and treatment may facilitate the therapy which would result in better survival. IGF-IR is a membrane resident receptor activated by IGF-I and IGF-II. IGF-IR signalling functions in cell proliferation and malignant transformation [58]. IGF-IR–directed cancer strategies have been developed for breast cancer and other solid tumors [59]. However, the role of IGF-IR expression in GC is

poorly understood. Many studies suggest that IGF-IR can interact with EGFR to augment the malignancy of tumors [60,61]. EGFR and its homologue HER2 (also called erbB-2) encode for receptor tyrosine kinases. These receptors play a crucial role in cancer cell proliferation, differentiation, survival and angiogenesis [62]. Epidermal growth factors stimulate EGFR for the synthesis of DNA and cell growth affecting various cellular processes. The co-receptor HER2 forms dimers with EGFR and acts synergistically to promote malignant transformation [63]. The expression levels of these receptors is related to many cancers and the ensuing survival rate [64] Immunohistochemical assays to assess the prognostic relevance of these receptors have been designed to determine the expression profile of IGF-IR, EGFR, and HER2 in GC [57]. Therapeutic strategies co-targeting IGF-IR, EGFR or HER2 have resulted in enhanced antitumor activity [65] The roles of anti-EGFR and anti-HER2 monoclonal antibodies (cetuximab and trastuzumab respectively), or a dual inhibitor of EGFR and HER2 (lapatinib) have been reported [66-68]. Therefore it is important to understand the clinical significance of IGF-IR and its molecular interactions with EGFR and HER2 to devise strategies for GC. Although there is significant evidence that IGF-IR, EGFR and HER2 are important predictors of GC, their role as indicators of GC however remains contentious.

2.2.6 Circulating micro-RNAs

Plasma microRNAs (miRNAs) can be used to diagnose and monitor GC. Serum tumour markers, such as carcinoembryonic antigen and carbohydrate antigen 19-9 have been used in GC diagnostics [69]. However, these conventional serum markers are non-specific and lack sensitivity to detect early cancer. Recently, several studies have provided evidence that miRNAs involved in tumourigenesis can be detected stably in blood plasma of cancer patients [70-72]. Circulating miRNAs that originate from cancerous tissues and are not lysed by endogenous RNases are stable blood-based biomarkers for the detection of cancer [73]. These serum based biomarkers are interesting molecules for screening of cancers.

A large number of genetic and epigenetic changes are known to be involved in tumour progression and maintenance. Several studies have identified that plasma/serum nucleic acids levels are altered in cancer patients [74-76]. However, recently miRNAs have been identified as novel factors related to oncogenesis [77,78]. The characteristics of miRNAs as tissue-specific molecular signatures and the presence of multiple copies per cell makes miRNAs as potential biomarkers. The diagnostic potential of plasma miRNAs has been validated and the plasma concentrations of *miR-17-5p*, *miR-21*, *miR-106a* and *miR-106b* are significantly higher in GC patients [79].

Plasma miRNA assays can be potentially used to screen high risk patients and monitor disease recurrence in GC. miRNA biomarkers are also powerful tools for evaluating the efficacy of adjuvant therapies. However, further clinical studies using a variety of tumour-

specific plasma miRNAs should be carried out to identify the potential application of these biomarkers in GC diagnosis and treatment.

3. Conclusion

Globally, GC continues to be one of the leading causes of cancer related deaths. Despite the development of several novel and effective classes of anticancer drugs, GC remains an aggressive clinical condition, with a median survival of only a few months in patients with unresectable cancers. GC is incurable and chemotherapy can only be palliative once it metastasizes. Therefore, the identification of specific biomarkers to diagnose gastric cancers is important. Modern technologies such as genome sequencing and expression arrays have helped to identify various novel biomarkers with prognostic value. However, advanced clinical trials for the screening of novel biomarkers need to be initiated for better results. Emphasis on the need for improved techniques for identifying GC biomarkers may facilitate the targeted chemotherapy, resulting in better outcomes.

4. References

1. Ferlay, J., et al., Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. International Journal of Cancer. Journal International Du Cancer, 2010; 127(12): p. 2893-2917.

2. Jemal, A., et al., Global cancer statistics. CA: a cancer journal for clinicians, 2011; 61(2): p. 69-90.

3. Cancer of the Stomach - SEER Stat Fact Sheets. 2015/07/23/11:22:33; A

4. Bai, Y., et al., HOXA11 gene is hypermethylation and aberrant expression in gastric cancer. Cancer Cell International, 2014; 14(1): p. 79.

5. Dicken, B.J., et al., Gastric Adenocarcinoma. Annals of Surgery, 2005; 241(1): p. 27-39.

6. Gastric Cancer: The Basics | Oncolink - Cancer Resources. 2015/07/23/10:24:47.

7. Dinis-Ribeiro, M., et al., Management of precancerous conditions and lesions in the stomach (MAPS): guideline from the European Society of Gastrointestinal Endoscopy (ESGE), European Helicobacter Study Group (EHSG), European Society of Pathology (ESP), and the Sociedade Portuguesa de Endoscopia Digestiva (SPED). Endoscopy, 2012; 44(1): p. 74-94.

8. Tabrez, S., et al., Cancer chemoprevention by polyphenols and their potential application as nanomedicine. Journal of Environmental Science and Health. Part C, Environmental Carcinogenesis & Ecotoxicology Reviews, 2013; 31(1): p. 67-98.

9. Jabir, N.R., et al., Nanotechnology-based approaches in anticancer research. International Journal of Nanomedicine, 2012; 7: p. 4391-4408.

10. Ali, R., et al., New anticancer agents: recent developments in tumor therapy. Anticancer Research, 2012. 32(7): p. 2999-3005.

11. Athanasios Alexiou, C.V. Application of Efficient Nanoparticles for Early Diagnosis and Treatment of Cancer.

12. Saka, M., et al., Present and future status of gastric cancer surgery. Japanese Journal of Clinical Oncology, 2011. 41(3): p. 307-313.

13. Wang, Z., et al., Clinical application value of bone turnover markers in non-small-cell lung cancer patients with bone metastases. The Chinese-German Journal of Clinical Oncology, 2011. 10(2): p. 81-84.

14. Lauren, P., THE TWO HISTOLOGICAL MAIN TYPES OF GASTRIC CARCINOMA: DIFFUSE AND SO-CALLED INTESTINAL-TYPE CARCINOMA. AN ATTEMPT AT A HISTO-CLINICAL CLASSIFICATION. Acta Pathologica Et Microbiologica Scandinavica, 1965. 64: p. 31-49.

15. Hwang, J.J., et al., Characteristics of gastric cancer in peptic ulcer patients with Helicobacter pylori infection. World Journal of Gastroenterology : WJG, 2015. 21(16): p. 4954-4960.

16. Hu, B., et al., Gastric cancer: Classification, histology and application of molecular pathology. Journal of Gastrointestinal Oncology, 2012. 3(3): p. 251-261.

17. Sousa, M.T.D.d., P.R. Savassi-Rocha, and M.M.D.Á. Cabral, Impact of using a lymph node revealing solution in surgical specimens for pathological staging of gastric cancer. Jornal Brasileiro de Patologia e Medicina Laboratorial, 2014. 50(6): p. 445-451.

18. Nagini, S., Carcinoma of the stomach: A review of epidemiology, pathogenesis, molecular genetics and chemoprevention. World Journal of Gastrointestinal Oncology, 2012. 4(7): p. 156-169.

19. Shi, J., Y.-P. Qu, and P. Hou, Pathogenetic mechanisms in gastric cancer. World Journal of Gastroenterology : WJG, 2014. 20(38): p. 13804-13819.

20. Watari, J., et al., Helicobacter pylori associated chronic gastritis, clinical syndromes, precancerous lesions, and pathogenesis of gastric cancer development. World Journal of Gastroenterology : WJG, 2014. 20(18): p. 5461-5473.

21. Panani, A.D., Cytogenetic and molecular aspects of gastric cancer: clinical implications. Cancer Letters, 2008. 266(2): p. 99-115.

22. McLean, M.H. and E.M. El-Omar, Genetics of gastric cancer. Nature Reviews Gastroenterology & Hepatology, 2014. 11(11): p. 664-674.

23. Hudler, P., Genetic Aspects of Gastric Cancer Instability. The Scientific World Journal, 2012. 2012: p. e761909.

24. Karimi, P., et al., Gastric cancer: descriptive epidemiology, risk factors, screening, and prevention. Cancer Epidemiology, Biomarkers & Prevention: A Publication of the American Association for Cancer Research, Cosponsored by the American Society of Preventive Oncology, 2014. 23(5): p. 700-713.

25. Sallam, R.M., Proteomics in Cancer Biomarkers Discovery: Challenges and Applications. Disease Markers, 2015. 2015: p. e321370.

26. Röcken, C. and V. Warneke, [Molecular pathology of gastric cancer]. Der Pathologe, 2012. 33 Suppl 2: p. 235-240.

27. Berger, F. and M.F. Reiser, Micro-RNAs as Potential New Molecular Biomarkers in Oncology: Have They Reached Relevance for the Clinical Imaging Sciences? Theranostics, 2013. 3(12): p. 943-952.

28. Denk, J., et al., MicroRNA Profiling of CSF Reveals Potential Biomarkers to Detect Alzheimer`s Disease. PLoS ONE, 2015. 10(5).

29. Lan, H., et al., MicroRNAs as Potential Biomarkers in Cancer: Opportunities and Challenges. BioMed Research International, 2015. 2015: p. e125094.

30. Hahn, W.C. and R.A. Weinberg, Modelling the molecular circuitry of cancer. Nature Reviews. Cancer, 2002. 2(5): p. 331-341.

31. Mirvish, S.S., Gastric cancer and salivary nitrate and nitrite. Nature, 1985. 315(6019): p. 461-462.

32. Parsonnet, J., et al., Helicobacter pylori infection and the risk of gastric carcinoma. The New England Journal of

Medicine, 1991. 325(16): p. 1127-1131.

33. Lo, S.S., et al., Relationship between age and clinical characteristics of patients with gastric cancer. Journal of Gastroenterology and Hepatology, 1996. 11(6): p. 511-514.

34. Ding, X., et al., The Potential Biomarker Panels for Identification of Major Depressive Disorder (MDD) Patients with and without Early Life Stress (ELS) by Metabonomic Analysis. PLoS ONE, 2014. 9(5): p. e97479.

35. Leal, M.F., et al., Searching for Gastric Cancer Biomarkers Through Proteomic Approaches. Journal of Gastroenterology and Hepatology Research, 2014. 3(3).

36. Cortes, D.F., et al., Recent Advances in the Mass Spectrometric Analysis of Glycoproteins: Capillary and Microfluidic Workflows. Electrophoresis, 2011. 32(1): p. 14-29.

37. Tuli, L. and H.W. Ressom, LC–MS Based Detection of Differential Protein Expression. Journal of proteomics & bioinformatics, 2009. 2: p. 416-438.

38. Howat, W.J., et al., Antibody validation of immunohistochemistry for biomarker discovery: Recommendations of a consortium of academic and pharmaceutical based histopathology researchers. Methods (San Diego, Calif.), 2014. 70(1): p. 34-38.

39. Ashraf, G.M., et al., Purification, characterization, sequencing and biological chemistry of galectin-1 purified from Capra hircus (goat) heart. The Protein Journal, 2011. 30(1): p. 39-51.

40. Ashraf, G.M., et al., Glycosylation of purified buffalo heart galectin-1 plays crucial role in maintaining its structural and functional integrity. Biochemistry. Biokhimii͡a, 2010. 75(12): p. 1450-1457.

41. Ashraf, G.M., et al., Protein misfolding and aggregation in Alzheimer's disease and type 2 diabetes mellitus. CNS & neurological disorders drug targets, 2014. 13(7): p. 1280-1293.

42. Ashraf, G.M., et al., Altered galectin glycosylation: potential factor for the diagnostics and therapeutics of various cardiovascular and neurological disorders. Advances in Experimental Medicine and Biology, 2015. 822: p. 67-84.

43. Hasan, S.S., G.M. Ashraf, and N. Banu, Galectins – Potential targets for cancer therapy. Cancer Letters, 2007. 253(1): p. 25-33.

44. Ashraf, G.M., et al., Studies on the role of goat heart galectin-1 as a tool for detecting post-malignant changes in glycosylation pattern. Saudi Journal of Biological Sciences, 2015. 22(1): p. 85-89.

45. Ashraf, G.M., et al., Studies on the role of goat heart galectin-1 as an erythrocyte membrane perturbing agent. Saudi Journal of Biological Sciences, 2015. 22(1): p. 112-116.

46. Ashraf, G.M., et al., Purification, characterization, structural analysis and protein chemistry of a buffalo heart galectin-1. Amino Acids, 2010. 39(5): p. 1321-1332.

47. Yamashita-Kashima et al., Biomarkers for Antitumor Activity of Bevacizumab in Gastric Cancer Models. BMC Cancer, 2012. 12 (37)

48. Liang Y. and Hyder S.M., Proliferation of Endothelial and Tumor Epithelial Cells by Progestin-Induced Vascular Endothelial Growth Factor from Human Breast Cancer Cells: Paracrine and Autocrine Effects. Endocrinology, 2005. 146 (8): p. 3632-41

49. Junttila et al., CIP2A Inhibits PP2A in Human Malignancies. Cell, 2007. 130 (1): p. 51-62

50. Hanahan D. and Weinberg R.A. , The Hallmarks of Cancer. Cell, 2011. 144 (5): p. 646-74

51. Li W. et al., CIP2A Is Overexpressed in Gastric Cancer and Its Depletion Leads to Impaired Clonogenicity, Senescence,

or Differentiation of Tumor Cells. Clinical Cancer Research. 2008. 14 (12): p. 3722-8

52. Guney I. et al., Reduced c-Myc Signaling Triggers Telomere-Independent Senescence by Regulating Bmi-1 and p16(INK4a). Proceedings of National Academy of Sciences, U S A. 2006. 103 (10): p. 3645-50

53. Eckhardt S.G. et al., Induction of Differentiation in HL60 Cells by the Reduction of Extrachromosomally Amplified c-Myc. Proceedings of National Academy of Sciences, U S A. 1994. 91 (14): p. 6674-8

54. Forman D. et al., Association between Infection with Helicobacter Pylori and Risk of Gastric Cancer: Evidence from a Prospective Investigation. British Medical Journal. 1991. 302 (6788): p.1302–5

55. Tabata H. et al., Helicobacter Pylori and Mucosal Atrophy in Patients with Gastric Cancer: A Special Study Regarding the Methods for Detecting Helicobacter Pylori. Digestive Diseases and Sciences. 1999. 44 (10): p. 2027-34

56. Uemura N.et al., Helicobacter Pylori Infection and the Development of Gastric Cancer. New England Journal Medicine. 2001. 345 (11): p. 784-9

57. Matsubara J. et al., Impact of Insulin-like Growth Factor Type 1 Receptor, Epidermal Growth Factor Receptor, and HER2 Expressions on Outcomes of Patients with Gastric Cancer." Clinical Cancer Research. 2008. 14 (10): p. 3022-9

58. Brodt P. et al., Inhibition of the Type I Insulin-like Growth Factor Receptor Expression and Signaling: Novel Strategies for Antimetastatic Therapy. Biochemical Pharmacology. 2000. 60 (8): p. 1101-7

59. Bonneterre J. et al., Prognostic Significance of Insulin-like Growth Factor 1 Receptors in Human Breast Cancer. Cancer Research. 1990 . 50 (21): p. 6931-5

60. Adams, T.E et al., Signalling by the Type 1 Insulin-like Growth Factor Receptor: Interplay with the Epidermal Growth Factor Receptor. Growth Factors. 2004. 22 (2): p. 89-95.

61. Gilmore A.P. et al., Activation of BAD by Therapeutic Inhibition of Epidermal Growth Factor Receptor and Transactivation by Insulin-like Growth Factor Receptor.s Journal of Biological Chemistry. 2002. 277 (31): p. 27643-50

62. Huang S.M and Harari P.M., Epidermal Growth Factor Receptor Inhibition in Cancer Therapy: Biology, Rationale and Preliminary Clinical Results. Investigation New Drugs. 1999.17 (3): p. 259-69.

63. Graus-Porta D. et al., ErbB-2, the Preferred Heterodimerization Partner of All ErbB Receptors, Is a Mediator of Lateral Signaling. EMBO Journal. 1997. 16 (7):p. 1647-55

64. Nicholson, R.I., EGFR and Cancer Prognosis. European Journal Cancer. 2001, 37 (4): p 9-15.

65. Camirand, A. et al., Co-Targeting HER2/ErbB2 and Insulin-like Growth Factor-1 Receptors Causes Synergistic Inhibition of Growth in HER2-Overexpressing Breast Cancer Cells. Medical Science Monitor. 2002. 8 (12): p. 521-6

66. Pinto C. et al., Phase II Study of Cetuximab in Combination with FOLFIRI in Patients with Untreated Advanced Gastric or Gastroesophageal Junction Adenocarcinoma (FOLCETUX Study). Annals of Oncology. 2007. 18 (3): p. 510-7

67. Grávalos, C. et al., Phase II Study of Trastuzumab and Cisplatin as First-Line Therapy in Patients with HER2-Positive Advanced Gastric or Gastroesophageal Junction Cancer. Clinical and Translational Oncology. 2011. 13 (3): p.179-84

68. Iqbal, S. et al., Southwest Oncology Group Study S0413: A Phase II Trial of Lapatinib (GW572016) as First-Line Therapy in Patients with Advanced or Metastatic Gastric Cancer. Annals of Oncology. 2011. 22 (12): p. 2610-5

69. Ishigami, S. et al., Clinical Importance of Preoperative Carcinoembryonic Antigen and Carbohydrate Antigen 19-9 Levels in Gastric Cancer. Journal of Clinical Gastroenterology. 2001. 32 (1): p. 41-4

70. Calin, G.A and Croce, C.M., MicroRNA Signatures in Human Cancers. Nature Review Cancer. 2006. 6 (11): p. 857-66

71. Chen X. et al., Characterization of microRNAs in Serum: A Novel Class of Biomarkers for Diagnosis of Cancer and Other Diseases. Cell Research. 2008. 18 (10): p. 997-06

72. Filipowicz, W. et al., Mechanisms of Post-Transcriptional Regulation by microRNAs: Are the Answers in Sight. Nature Review Genetics. 2008. 9 (2): p. 102-14

73. Mitchell P.S. et al., Circulating microRNAs as Stable Blood-Based Markers for Cancer Detection. Proceedings of National Academy of Science, U S A. 2008. 105 (30): p. 10513-8

74. Sidransky D., Nucleic Acid-Based Methods for the Detection of Cancer. Science. 1997. 278 (5340): P. 1054-9

75. Anker P. et al., Circulating Nucleic Acids in Plasma or Serum. Clinica Chimica Acta. 2003. 13 (1-2): p. 143-6

76. Chan K.C. and Lo Y.M., Circulating Tumour-Derived Nucleic Acids in Cancer Patients: Potential Applications as Tumour Markers. British Journal of Cancer. 2007. 96 (5):p. 681-5

77. He L. et al., A microRNA Polycistron as a Potential Human Oncogene. Nature. 2005. 435 (7043): p. 828-33.

78. Lu J. et al., MicroRNA Expression Profiles Classify Human Cancers. Nature. 2005. 435 (7043): p. 834-8.

79. Tsujiura M.et al., Circulating microRNAs in Plasma of Patients with Gastric Cancers. British Journal of Cancer. 2010. 102 (7): p. 1174-9

Chapter 5

Application of Mass Spectrometry to Analyse Protein Structure within a Live Cell

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1. Introduction

In depth understanding of molecular mechanisms of cellular processes requires protein structure-function correlation to investigate in its endogenous environment. Due to identical chromophoric groups present across all proteins within a cell, the different spectroscopic tools that are utilized to visualize protein structure cannot be used within a crowded molecular environment *in vivo*. Molecular mass is the molecular signature of a molecule. Thus, mass spectrometry that measures molecular mass, could be used to investigate a single molecular entity in an unpurified sample. In fact, mass spectrometry might be an appropriate biophysical technique that could be used to solve protein structure even in a heterogeneous molecular pool such as inside a live cell. *In vivo* application of mass spectrometry to monitor protein structure and stability is little older than a decade. In this chapter we highlighted following three different types of mass spectrometry based biophysical techniques that had been used to elucidate structure and conformational dynamics of proteins and to map protein-protein interactions within a live cell: Hydrogen deuterium exchange of back bone amide hydrogens of a protein, Free radical mediated fast photochemical oxidation and Cross linking of proteins.

2. Molecular Crowding *in vivo*

Proteins in biological cells are in a highly crowded environment. Molecular crowding

in a cell is essential for the biochemical processes such as protein folding, protein nucleic acid interaction, oligomerization etc [1]. Arthur Konerberg reported that the replication of oriC plasmid in a cell free environment became possible using high concentration of polyethylene glycol (PEG). In fact PEG occupies major portion of aqueous volume which results in restoration of the required crowded environment for the event [2]. Another important example of molecular crowding is polymerization of sickle hemoglobin (HbS) in sickle cell anemia. A very high concentration of hemoglobin in red blood cell results in a crowded environment that is essential for the polymerization of deoxy-HbS [3]. Theoretically, the rate of homogenous nucleation of sickle hemoglobin is expected to decrease by 10^{10} fold on reducing HbS concentration by 20%. In practice, the replacement of HbS with HbF by 20% results in reduced homogenous nucleation rate by a factor of 10^3 fold. Although HbF does not participate in polymerization but it preserves the molecular crowding inside RBCs resulting in a large difference (10^7 fold) in sickle hemoglobin polymerization [4]. A significant increase in the molecular crowding is observed with aging, which could be associated with a reduction in the cellular volume and retardation in the protein degradation rate [5]. One of the hypotheses for the brain cells being susceptible to Parkinson's disease with aging is increase in the rate of aggregation of α-synuclein [6]. Thus to understand a molecular mechanism in a biological system, it is important to explore the molecular interactions inside a living cell.

Function of a protein is completely guided by its structure. Various spectroscopic tools that are used to visualise protein structure namely circular dichroism, fluorescence, infrared, nuclear magnetic resonance utilize intrinsic chromophores of the protein molecule. A major limitation in using these spectroscopic methods in a living cell arises from the difficulty in differentiating the contribution of chromophoric groups that are similar among all proteins. Atomically-resolved structural information is obtained from X-Ray crystallography where the experimental molecule needs to be crystallized. High concentration of complex macromolecules results in a crowded environment in a biological cell [7]. Thus, it is impossible to compartmentalize a protein molecule from its surroundings within a live cell. Therefore, the classical approaches for most of the structural investigations of proteins using above mentioned techniques are restricted to purified molecules *in vitro*. Subsequently, *in vitro* observation obtained by spectroscopic methods is extrapolated *in vivo* for its functional correlation [8]. However, crowding of macromolecules represents a significant functional feature of cellular complexity and subsequently its importance on biochemical processes as mentioned earlier.

3. Mass Spectrometry Based Structural Analysis *in vivo*

To understand the molecular mechanism of a biological process it is crucial to analyze the macromolecular binding stoichiometry and conformational dynamics associated with the event *in vivo*. However, it is impossible to mimic the complex cellular environment *in vitro* [9]. Unlike the other molecular spectroscopic tools, mass spectrometry is specific to molecular

mass, which enables the technique to monitor individual molecular entity even in a crowded impure molecular milieu. The bottom line of mass spectrometry based protein structure analysis is to monitor the changes in molecular mass as the process progresses. To investigate the conformational dynamics of macromolecules, it is essential to label the experimental molecule with a molecular probe. Therefore, to monitor an event *in vivo* using mass spectrometry, the molecular probe must be permeable across the cell membrane and execute the required chemical modification of experimental molecule inside the live cell. A couple of mass spectrometry based methods have been used in last few years to investigate structure and conformational dynamics of proteins inside living cells.

In the conformation analysis using hydrogen deuterium exchange, the polar hydrogens of a protein molecule are replaced with deuterium from solvent D_2O [10]. Narayanan S. *et al.* showed that exploiting permeability of D_2O across cell membrane followed by the isotope exchange of polypeptide backbone amide hydrogens, the structure-function correlation of human hemoglobin can be studied within live red blood cells (RBCs) [11]. Ghaemmaghami S. *et al*. reported that allowing urea and D_2O to penetrate *E.Coli* cells, the stability of truncated N-terminal domain of λ repressor (λ_{6-85}) protein was measured through isotope exchange based mass spectrometry [12]. In free radical mediated oxidative modification, the structural changes of protein were monitored through radical induced oxidation of the constituent amino acid residues [13]. Using laser photolysis of hydrogen peroxide, the hydroxyl radical induced oxidation of cytoplasmic proteins in Vero cells were studied by Espino JA, *et. al* [14]. In cross linking method, a cross linker gets covalently bonded with the reactive functional groups of either two parts of a molecule or between parts of different molecules [15]. Using formaldehyde as a cross linker, protein-protein interactions network was mapped in yeast cells by Cortnie and co-workers [16]. In these methods the observed increase in mass of a protein is translated in terms of its structural changes. In this chapter we described the application of aforementioned techniques in the analysis of protein conformation within live cells (*in vivo*).

4. Hydrogen Deuterium Exchange in Protein

The vibrational degrees of freedom of a protein molecule results in breathing motion of the molecule that manifests as local unfolding thereby causing transient open conformations to exist [17,18]. During this transition, the polar hydrogens that are bonded covalently with oxygen, nitrogen and sulphur, become exposed to the solvents and subsequently undergo exchange with a rate that depends on differential solvent accessibility, inductive effect and field effect of neighbouring groups, pH and temperature [19]. The above exchange can be kinetically monitored using heavy water [D_2O] as solvent [20]. In practice, the isotope exchange of the peptide backbone amide hydrogens of a protein molecule that are involved in the formation of secondary structure of protein, α helices and β sheets, are studied [21]. Eventually, the isotope exchange kinetics of peptide backbone amide hydrogens of a protein can be translated

to its conformational dynamics in the solution phase [22].

5. Kinetics of Peptide Backbone Amide Hydrogen Deuterium Exchange

The local unfolding followed by the transient exposure of protein conformations and subsequent isotope exchange of backbone amide hydrogens from solvent can be explained by Linderstrom Lang's model [23,24]:

where, NH_{closed} and NH_{open} are backbone amide hydrogens in the closed and in the open conformation respectively. ND is the isotopically exchanged amide NH. k_{op}, k_{cl} and k_{ex} are rate constants of opening, closing and intrinsic isotope exchange steps respectively. The experimentally measured hydrogen deuterium exchange rate constant k_{HX}, can be expressed as

$$k_{HX}=\frac{k_{op}\times k_{ex}}{k_{cl}+k_{ex}+k_{op}}$$

Thus, in the presence of large excess of D_2O, the isotope exchange reaction follows pseudo first order kinetics. In general the rate constant of isotope exchange reaction k_{ex} of peptide backbone amide hydrogens reaches minimum at pH 3. Additionally, every 10^0C rise in temperature increases k_{ex} by 2 to 3 folds [22].

Protein in its native state exists mainly in closed conformations and it might be approximated that under physiological condition the open conformation of a protein molecule is unstable and transient [25]. Thus, $k_{cl} >> k_{op}$ and k_{HX} is given by,

$$k_{HX}=\frac{k_{op}\times k_{ex}}{k_{cl}+k_{ex}}$$

The rates of hydrogen deuterium exchange in a protein molecule are primarily determined by either the rate of opening of the closed conformation or by the intrinsic isotope exchange rate. These two conditions are referred as EX_1 mechanism and EX_2 mechanism respectively. Under EX_1 condition, $k_{ex} >> k_{cl}$, implying that the exchange occurs immediately upon opening event [26,27]

$$k_{HX}=k_{op}$$

In general EX_1 mechanism is observed on denaturation of protein molecules. Under EX_2 condition $k_{ex} << k_{cl}$, implying that several opening closing events occur before the exchange event.

$$k_{HX}=\frac{k_{op}\times k_{ex}}{k_{cl}}$$

or, $k_{HX} = K_{op} \times k_{ex}$

where $K_{op} = (k_{op}/k_{cl})$ is the equilibrium constant of opening event. In general, under physiologi-

cal condition protein exhibits the isotope exchange via EX_2 mechanism.

At the experimental temperature and pressure, the free energy change associated with the local fluctuation followed by the opening of native structure is given by [28]:

$$\Delta G_{HX} = -RT\ \log_e K_{op} = -RT\ \log_e\left(\frac{k_{HX}}{k_{ex}}\right) = -RT\ \log_e\left(\frac{1}{P}\right)$$

where P is protection factor providing information on the stability of the native conformer. The backbone amide hydrogens that are in the loop regions are unprotected and remain in the open conformation. Hydrogen deuterium exchange in these regions occurs rapidly with rate constant k_{ex}.

Permeability of D_2O across the cell membrane can be exploited to execute hydrogen/deuterium exchange inside a live cell [29]. The replacement of hydrogen with deuterium does not lead to change in the structure and chemical property of a molecule. The hydrogen deuterium exchange data of backbone amide hydrogens across the peptide backbone of a protein mirrors the conformational dynamics of a particular state [30]. Every isotope exchange reaction has its own kinetics. Thus to monitor the kinetics of a biological event, different intermediate states must be trapped and the hydrogen deuterium exchange must be executed for all those states separately. In practice, the isotope exchange reaction is quenched at different time points by reducing pH to 3 and temperature to 4^0C. Both the cell lysis and proteolytic digestion of isotope exchanged protein are performed under quenched condition to minimize the back exchange of deuterium. The number of deuterium incorporated 'D' in a peptide at a given time 't' can be calculated as follows [31]:

$$D_{(t)} = \left(\frac{M_t - M_0}{M_\infty - M_0}\right) \times N$$

where M_t is the observed isotope averaged centroid mass of deuterated peptide at time t, M_0 and M_∞ are the isotope averaged centroid mass of undeuterated and fully deuterated peptides respectively and N is the total number of backbone amide hydrogens in a peptide excluding amino terminal hydrogens. At a fixed pH and temperature and in presence of large excess of D_2O, the isotope exchange of each backbone amide hydrogens follows pseudo first order kinetics and D(t) can be expressed as

$$D_{(t)} = N - \sum_{i=1}^{N} \exp^{-k_i t}$$

where, k_i is the exchange rate constant of backbone amide hydrogen at i^{th} position.

In practice, backbone amide hydrogens of a peptide can be grouped into following three categories: fast, intermediate and slow exchanging [23]. Thus,

$$D_{(t)} = N - \left[P_A e^{-k_1 \times t} + P_B e^{-k_2 \times t} + P_C e^{-k_3 \times t}\right]$$

where, P_A, P_B, P_C are the population of fast, intermediate and slow exchanging amide hydrogens with average rate constants k_1, k_2, k_3 respectively. These kinetic parameters can be

obtained from D (t) vs. t plot.

6. In vivo Application of Hydrogen Deuterium Exchange

6.1. Conformational dynamics of protein

Structure-function correlation of protein *in vivo* might be explored by studying the change in conformational dynamics of a protein molecule accompanying ligand binding in-cell. Using continuous labeling method in hydrogen deuteriuim exchange Narayanan S. *et.al*., reported the structural transition of human hemoglobin associated with its oxygenation inside live Red Blood Cells [11]. Authors described the different step involved in hydrogen deuterium exchange in live cells followed by mass spectrometric data analysis in detail [11]. The cooperativity and allosteric regulation of oxygenation and the associated change in the conformational flexibility across various regions of globin chains in hemoglobin were monitored through hydrogen deuterium exchange of fully oxygenated and deoxygenated RBCs by incubating it in 300 mOsm D_2O buffer, isosmotic to human blood. To understand the change in conformational dynamics, a comparative analysis of six rate constants and six populations of oxy and deoxy states of hemoglobin are required. The analysis method was simplified by calculating the rate of exchange reaction for each category of amide hydrogens in both oxy and deoxy states of hemoglobin. The rate of pseudo first order hydrogen deuterium exchange reaction was calculated using the method of initial rates where the product of rate constant (k_i) and the respective population (P_i) of each group of backbone amide hydrogens were used as rate of exchange reaction. The algebraic summation of exchange rates across two states of hemoglobin was considered to assess whether the experimental region of the molecule became flexible or rigid in its conformational dynamics on deoxy to oxy transition.

The movement of heme Fe^{2+} towards the plane of porphyrin nucleus on O_2 binding to deoxy hemoglobin is transmitted to the proximal His residue resulting in the expulsion of penultimate Tyr residue from the pocket between F and H helices. Subsequently salt bridges of carboxyl group of the terminal residue and hydrogen bonds of penultimate residue are ruptured [32,33]. Disappearance of above interactions was reflected in the increased flexibility of a peptide β^{86}ATLSELHCDKLHVDPEN^{102} in the *in vivo* hydrogen deuterium exchange analysis [11]. The allosteric regulator 2,3-diphosphoglycerate is bound, one per deoxy hemoglobin tetramer, between two β-subunits through ionic interactions with βVal^{1}, βHis^{2}, βLys^{82} and βHis^{143} residues of both β globin chains [32]. Oxygenation causes contraction in the DPG binding pocket followed by the dissociation of interactions with all above residues and the associated structural transition was reflected in the increased flexibility in the *in vivo* hydrogen deuterium exchange of the peptide β1VHLTPEEKSAVTAL14 [11]. Another important factor in the cooperative oxygen binding to heme unit is Bohr effect, which is contributed by the imidazolium hydrogen of βHis^{146} and αHis^{122} and N-terminal NH_2 of αVal^{1}. Oxygenation results in the dis-

sociation of salt bridges involving those residues which in turn causes decrease in pK_a followed by the release of Bohr protons from above residues. Subsequently the released protons combine with bicarbonate to form carbonic acid [34]. The structural transitions associated with these crucial steps were reflected in the *in vivo* hydrogen deuterium exchange analysis through the change in the conformational dynamics of the peptides β^{130}YQKVVAGVANALHKYH^{146}, α^{110}AAHLPAEFTPAVHASLDKFLASVSTVLTSKYR^{141} and α^{1}VLSPADKTNVKAAWGKV GAHAGEYGAEALERMF^{33} respectively [11].

The structure function relationship of hemoglobin is well established from *in vitro* research over couple of decades. The hemoglobin content of RBCs is 95% of total protein. Thus RBCs might be considered as an *in vivo* system of pure hemoglobin. From the observed correlation between *in vitro* and *in vivo* results of structure-function correlation of human hemoglobin, authors claimed that *in vivo* hydrogen deuterium exchange method might be successful to monitor conformational dynamics of any protein in cell irrespective of its size, location and structural complexity. Mass spectrometry based visualization of proteins in a proteome is limited by the competition in the ionization between various proteins that depends on their ionization probability and abundance, dynamic range. Thus, the described *in vivo* method is limited by the coverage of cellular proteins which is primarily defined by the relative abundance within a live cell. For low abundant proteins in cell, liquid chromatography based prefractionation at low temperature and acidic pH might help in hydrogen deuterium exchange analysis.

6.2. Protein folding

The correlation between in cell stability measurement and degradation rate of full length protein provides important information on biological insights *in vivo*. The thermodynamic stability of a truncated N-terminal domain of λ repressor, $\lambda_{6\text{-}85}$, was measured within the E.Coli cells using SUPREX (Stability of Unpurified Proteins from Rates of hydrogen deuterium exchange) [12]. Both, heavy water (D_2O) and urea, a denaturant, were allowed to permeate across *E.Coli* cell membrane and subsequently the hydrogen deuterium exchange rate of unfolded protein was measured *in vivo*. The thermodynamic stability of the protein was calculated. In this experiment *in vivo* stability measurement of $\lambda_{6\text{-}85}$ was possible till 3M urea. With higher concentration of urea cell viability dropped drastically due to the denaturation of important proteins in cell.

In hydrogen deuterium exchange kinetics the observed rate constant of exchange for each hydrogen is given by,

$$k_{HX} = \frac{k_{op} \times k_{ex}}{k_{cl} + k_{ex} + k_{op}}$$

Under the condition where both k_{close} and k_{open} are much greater than k_{ex},

$$k_{HX}=\frac{K_{op}\times k_{ex}}{K_{op}+1}$$

where, K_{op} = (k_{open}/ k_{close}). For hydrogen exchange following global unfolding mechanism where k_{ex} is similar for entire peptide backbone amide hydrogens, K_{op} = (1/K_{fold}),

$$k_{HX}=\frac{<k_{ex}>}{[1+K_{fold}]}$$

where $<k_{ex}>$ is the average hydrogen deuterium exchange rate of unprotected amide hydrogens. The increase in mass as a function of time can be expressed as [35]

$$\Delta M=\Delta M+(\Delta M_0-\Delta M_\infty)e^{-[<k_{ex}>/(1+K_{fold})]t}$$

where ΔM_0 and ΔM_∞ are the increase in mass before and after complete exchange respectively.

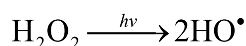
$$K_{fold}=e^{-(\Delta G_f+m[urea])/RT}$$

ΔG_f is the free energy change on folding and m = (d[ΔG_f] /d[urea]). The above equation can be used to fit hydrogen deuterium exchange rates for both *in vivo* and *in vitro* experiments. From the obtained stability parameter of protein it was observed that *in vivo* stability of λ_{6-85} in cell is very similar compared to that *in vitro*.

7. Hydroxyl Radical Labeling in Protein Footprinting

Hydroxyl radical mediated oxidative modification of proteins can be used as a probe for monitoring protein structure and dynamics [36]. Highly reactive hydroxyl radical forms covalent bonds with solvent exposed amino acid side chains. This strategy has been used in protein footprinting, an assay that traces protein conformational changes and ligand binding through the accessibility of backbone and side chain residues of amino acids [37]. In general there is incorporation of oxygen atom on modified amino acid residues. Differences in oxidative modification between multiple states of a protein, e.g., ligand bound versus unbound states provides residue level information on ligand binding sites [38,39]. Hydroxyl radical can be generated by various methods such as decomposition of H_2O_2 by Fenton chemistry, homolytic cleavage of H_2O_2 using UV radiation, radiolysis of water using synchrotron X-ray pulses or by γ-ray [40,41,42]. Optimized exposure of radicals is necessary to avoid any radical induced unfolding of the protein which might lead to the exposure of inaccessible regions of the protein molecule. Therefore, while employing the methods to generate hydroxyl radicals as covalent probes, considerable attention should be taken to ensure that oxidation reaction occurs prior to any changes in the protein conformation. Hambly and Gross demonstrated fast photochemical oxidation of protein (FPOP) where pulsed laser was used to generate hydroxyl radicals from H_2O_2 followed by oxidation of proteins to study the conformational changes and ligand binding sites *in vitro* [43]. Although, the hydroxyl radical induced unfolding of the protein occurs in a timescale of milli second or longer duration, the pulsed laser in FPOP takes an advantage of exposing the protein to the radical in less than a microsecond. Also, the presence of the

radical scavenger in the process helps in shortening the exposure lifetime of the radicals. This allows the labeling to occur in a microsecond time scale, ensuring that labeling occurs faster than the unfolding of most of the proteins [44,45]. Laser photolysis of H_2O_2 using UV-light induces a homolytic cleavage of H_2O_2 to generate highly reactive hydroxyl radical in an aqueous solution [46]. The primary quantum yield of H_2O_2 decomposition was observed to be close to 0.5 [47].



The hydroxyl radicals produced undergoes Haber-Weiss chain reaction which increases the total quantum yield of H_2O_2 decomposition to 1-2 [46],

$HO^{\bullet}$ undergoes diffusion controlled recombination to generate H_2O_2

Hydroxyl radical mediated footprinting of proteins involves the modification of amino acid residues either by an abstraction of hydrogen from saturated carbon atom or by the addition of hydroxyl group to an unsaturated carbon atom. Hydroxyl radical reaction via the hydrogen abstraction from C–H bond forms a carbon centered radical which then interacts with O_2 to form a peroxyl radical [48,49]. This peroxyl radical can get involved in a series of radical reactions resulting in major and minor products with respective mass shifts. The reaction on the amino acid residues with $HO^{\bullet}$, a week electrophile, is also significantly affected by the type of C-H ($3^0>2^0>1^0$), nature of neighbouring functional groups, stability of nascent radical and steric effect at the target site. Hydrogen abstraction is favoured when it is located adjacent to electron delocalising groups such as hydroxyl, carboxyl or amide groups that help in stabilization of the radical formed [50]. Due to steric hindrance, side chains of amino acids are preferentially attacked than α-carbon sites [51]. The peptide backbone can also be cleaved via the attack of hydroxyl radical at β-carbon atom [52]. In addition to the solvent accessibility, the reactivity of individual side chains is also determined by its chemical nature. Hydroxyl radical attack on the side chains takes place at a rate of 10 to 1000 folds faster than the hydrogen abstraction from the α-carbon atom at the peptide backbone [53].

The hydroxyl radical reaction on side chains is well characterized and developed using mass spectrometry based approach in both aerobic and anaerobic environments. Most of the amino acid residues on reaction with hydroxyl radical showed a mass shift of +16 Da on addition of hydroxyl group (A, R, N, D, E, Q, H, I, L, K, M, F, P, W, Y, V, S, T) [46]. Carbonylation (+14 Da) that leads to a mass increment of 14 Da, was observed for the following amino

acid residues: L, I, Q, R, V, P and K [46]. In addition to +16 Da, acidic amino acids, D and E might undergo loss of CO_2 (-44 Da); decarboxylation (-30 Da) and carbonylation (+14 Da). Similarly, a series of additional products with a mass shift of +5 Da, -22 Da, -23 Da, and -10 Da were observed on oxidation of His residue [46]. In addition to +16 Da and +14 Da, Arg showed a reduction in mass -43 Da by deguanidination [46]. Aromatic amino acids, Trp, Tyr, Phe, on oxidation results in multiple numbers of major and minor products with a mass shift of +16 Da, +32 Da, +48 Da etc [46]. Formation of sulfonic acid, sulfinic acid on oxidation of Cys residue results in a mass shift of +48 Da, +32 Da respectively [46]. Met on oxidation generates methionine sulfoxide, methionine sulfonate or a minor product with a mass shift of +16 Da, +32 Da or -32 Da respectively [46]. A summary of mass shift accompanied by a change in the composition for most of the amino acid residues on hydroxyl radical mediated modification is given in the **Table 1**.

The relative reactivity of amino acid side chains under aerobic condition through MS detection was observed to follow an order: Cys > Met > Trp > Tyr > Phe > His > Leu ~ Ile > Arg ~ Lys ~ Val > Ser ~ Thr ~ Pro > Gln ~ Glu > Asp ~ Asn > Ala > Gly [39]. Lower reactivity of the amino acid residues Asp, Asn, Ala and Gly in the side chains makes it less effective as probes in the experiment [39]. Modifications of Ser and Thr were found to be difficult to detect. Therefore among 20 natural amino acids 14 of them can be used as probes. These 14 amino acid residues comprises of ~65% of the total protein molecule which makes the footprinting technique to be reasonably good for probing the conformational studies of a protein molecule [54]. Following the irradiation of protein molecule with H_2O_2, the proteome is subjected to proteolytic digestion for the characterization of protein through peptide fragmentation.

8. *In vivo* Application of $HO^{\bullet}$ Labeling

The permeability of H_2O_2 across cell membranes via diffusion and/or through channel proteins made the *in vivo* application of FPOP feasible. In order to study the protein conformation in a living cell, FPOP footprinting method was used in cell and the oxidative modification of proteins in various subcellular compartments was studied in African green monkey kidney cells (Vero cell) [14]. After optimum incubation of Vero cells in H_2O_2 followed by pulsed laser exposure, the cells were lysed and proteome pool was subjected to proteolytic using trypsin digestion. LC/MS analysis was performed to identify and characterize the site specific modification and quantification of oxidized proteins in cell. To probe solvent accessibility of protein within the crowded environment in cell, oxidative modification of actin was investigated. The obtained profile of oxidation pattern of different residues of actin was compared with previously reported *in vitro* results. To study the actin conformation in cell, the solvent accessible surface area (SASA) of both open and closed conformations of the homologous (99%) bovine actin were compared with the obtained in cell FPOP data. Although actin is likely to present in multiple conformations inside a cell but the in cell FPOP data and its comparison with SASA

of actin suggested that most abundant conformation of actin in cell is open structure rather than the tightly packed one [14].

Hydroxyl radical labeling is fast and irreversible in nature. Thus, the labeling signature is retained in all post-labeling analytical steps. The conformational dynamics of membrane bound proteins which are difficult to isolate even, can be studied successfully using in cell FPOP method. However, the preferential oxidation of specific residues like Cys, Met limits the applicability of in cell FPOP technique to selective protein sequences. Prolonged exposure of H_2O_2 might turn out to be toxic to a biological cell. Therefore optimum time exposure and concentration of H_2O_2 to use in FPOP method in cell must be standardized very carefully.

9. Protein Crosslinking

Crosslinking is a process of chemical modification which involves the formation of covalent bonds between two molecules or two parts of a molecule through a cross-linker [55]. In protein crosslinking, mass spectrometric based identification of crosslinked peptides has become an amenable toolkit to obtain information on protein structure and topology of a protein complex [56,57]. Despite the growing application of protein crosslinking, mass spectrometry based identification of crosslinked sites was majorly restricted to *in vitro* studies [58]. However, a handful of experiments have been developed by employing cell permeable chemical crosslinkers for investigation of protein crosslinking *in vivo* [59]. Majority of the *in vivo* studies utilize formaldehyde as a crosslinker which is permeable to the cell membrane [60]. In addition, membrane permeable crosslinker, azide-tagged, acid-cleavable disuccinimidyl bis-sulfoxide was recently introduced by Robyn et al., to study protein-protein interaction in an endogenous environment [61].

The efficiency of linking two molecules using cross-linker depend on their chemical specificity (amino, sulfhydryl, carboxyl, guanidinyl etc.) and the distance between the two functional groups (spacer arm) [62]. The most widely used crosslinking reagents in protein crosslinking mass spectrometry as reviewed by Andrea Sinz are amine, sulfhydryl and photoreactive crosslinkers [63]. In general chemical crosslinkers can be broadly classified into homobifunctional, heterobifunctional and trifunctional crosslinkers. Homobifunctional crosslinkers contains identical functional groups on either sides of the spacer arm such as bis(sulfosuccinimidyl) suberate. Heterobifunctional crosslinkers comprises of two different reactive sites on either ends of the spacer arm, e.g., N-Sulfosuccinimidyl 4-(N-maleimido-methyl)cyclohexane-1-carboxylate [64,65]. Trifunctional crosslinkers possess three different reactive sites where the third reactive group can link to a protein molecule or can be used for affinity purification of cross linked molecules [66]. Based on the location of the reaction, a crosslink can be formed within a single polypeptide chain, or between different polypeptide chains resulting in intramolecular or intermolecular crosslinking respectively [67,68]. Intra-

molecular cross-linking provides the information of the amino acid residues responsible in preserving the protein folds whereas intermolecular cross-linking between various proteins gains insights on specificity of the surface residues involved in protein-protein interaction [69].

10. Formaldehyde as Cross-linker for *in vivo* application

Formaldehyde is a water soluble, highly reactive polar compound which is used as a bifunctional cross-linker with a short spacer length of 2.3-2.7 Å [60]. Due to its membrane permeability, it finds more application in protein crosslinking *in vivo*. The chemical modification of protein molecules induced by formaldehyde as a cross-linker involves two steps. In a protein molecule, the nucleophilic amino group attacks the carbonyl carbon atom of formaldehyde forming an unstable carbinol intermediate which subsequently forms a methylene product on dehydration (**Scheme 1**). In the next step, nucleophilic attack on methylene carbon by another nucleophilic amino group of a protein results in cross-linking between two protein molecules (**Scheme 1**). The above reaction can be contributed by a series of side chain residues of the following of amino acids: lysine, histidine, asparagine, tryptophan, tyrosine, arginine; and α-amino group at the N-terminus of a protein molecule [60]. In the study of protein–protein and/or protein-DNA interaction using crosslinking strategy with formaldehyde as a cross linker, less than 1% formaldehyde v/v and limited time period of exposure of the experimental molecules to the cross linker are generally used. However in the histological analysis, formaldehyde with a concentration of more than 1% v/v and the duration of the crosslinking reaction from hours to days are followed to restore the localization of proteins in the tissue samples. It has been reported that use of lower concentration of formaldehyde and shorter time period, the crosslinking reaction is largely limited to the side chains of lysine and trptophan residues and the amino termini of protein molecule.

The methylene bridge that is formed between the two amino acid residues that are cross-linked, results in a mass increment by 12Da. The shorter spacer arm of formaldehyde allows cross linking between two amino acid residues of protein molecules that are located in a very close proximity [70]. In the subsequent analysis step the crosslinked proteins or peptides are purified using affinity chromatography and are subjected for proteolytic digestion for mass spectrometric analysis. Although lysine residues of proteins get crosslinked via ε-amino side chain group, it has been reported that crosslinked proteins are still left with sufficient number of unreacted lysine and arginine residues of proteins to provide global enzymatic digestion using trypsin as a proteolytic enzyme followed by fragmentation of proteolytic peptides for protein identification. In addition, most of the proteomic analysis search engine tools allow one missed cleavage in the proteolytic peptides to process the proteomics data for protein identification against the respective proteome database. Besides trypsin, other proteolytic enzymes such as GluC, chymotrypsin can also be used in protein identification [60].

Cortnie *et al*., used formaldehyde as an *in vivo* cross linker to map the proteins involved in both stable and transient interactions in 26 S proteasome network in yeast cells [16]. In this study formaldehyde was incubated with yeast cells and the cross linking reaction was quenched using glycine as quencher. The cross-linked proteins were isolated using affinity-based purification and interacting partners were characterised by mass spectrometry. Authors were successful to identify 64 proteasome-interacting proteins in yeast 26 S proteasome complex where 42 interactions were found to be novel. Azide-A-DSBSO (Azide-tagged disuccinimidyl bis-sulfoxide), a membrane permeable cross linker was synthesized by Robyn and coworkers for mapping protein-protein interactions in mammalian cells (HEK 293 cells). The synthesized chemical cross linker was incubated with HEK 293 cells and the cross linking reaction was quenched with glycine. The cross linked proteins were purified and identified through affinity purification and tandem mass spectrometry respectively. In this study, 54 crosslinked proteins were identified *in vivo* including both inter and intra-subunit novel crosslinks [61].

In vivo crosslinking coupled to mass spectrometry is a powerful technique to map protein-protein interactions in a crowded cellular environment. In formaldehyde based *in vivo* crosslinking the potential participating amino acid residue is lysine. Thus in mapping of protein-protein interaction depends on physical location of lysine also. Therefore mass spectrometry based *in vivo* cross linking is limited by the event that failure to observe successful cross linking between two proteins in the experimental results does not always mean that there is no interaction between them *in vivo*.

11. Conclusion

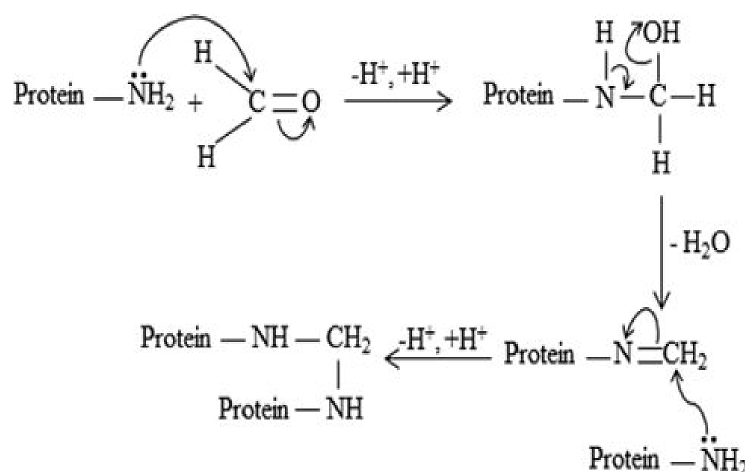
Mass spectrometry based hydrogen deuterium exchange, fast photochemical oxidation and crosslinking of different amino acid residues in a protein molecule enable to explore molecular mechanisms associated with complex biological events *in vivo*. Permeability of molecular probes, D_2O, H_2O_2 and H_2CO across the cell membrane is the crucial step behind above mentioned approaches to be successful. Molecular specificity of mass spectrometry made it feasible to investigate the structural stability, conformational dynamics of a protein and interaction network of a protein in a crowded cellular environment. However, reversible nature of hydrogen deuterium exchange, probability of oxidation induced protein unfolding and surface localization of reactive functional groups that gets crosslinked are the limitations of three aforementioned techniques respectively. Liquid chromatography based pre-fractionation at low temperature and acidic pH might help in visualizing low abundant proteins in cell. Reduced incubation period of a live cell with hydrogen peroxide might help to understand the structure of the protein in its native form. Optimal concentration of crosslinkers as well as surface exposed reactive functional groups of proteins is crucial while exploring the protein interaction network *in vivo*. Rapid advancement of mass spectrometry might provide answer in understanding the mechanisms of many complex cellular and subcellular biological processes

near future.

Table 1: A list of probable chemical modifications and the corresponding mass shifts of various amino acid residues in FPOP

Amino acid residues	Modification of side chains	Respective Mass shifts (Da)
Cysteine	sulfonic acid, sulfinic acid, hydroxy	+48, +32, -16
Methionine	sulfoxide, sulfone, aldehyde	+16, +32, -32
Phenylalanine	hydroxy	+16, +32, +48, etc.
Trptophan	hydroxy, pyrrol ring open	(+16, +32, +48, etc.), (+32, etc.)
Tyrosine	hydroxy	+16, +32, +48, etc.
Histidine	oxo, ring opening	(+16), (-22, -23, +5, -10)
Arginine	deguanidination, hydroxy, carbonyl	-43, +16, +14
Leucine	hydroxy, carbonyl	+16, +14
Isoleucine	hydroxy, carbonyl	+16, +14
Valine	hydroxy, carbonyl	+16, +14
Proline	hydroxy, carbonyl	+16, +14
Lysine	hydroxy, carbonyl	+16, +14
Glutamine	hydroxy, carbonyl	+16, +14
Serine	hydroxy, carbonyl	+16, -2
Threonine	hydroxy, carbonyl	+16, -2
Glutamic acid	decarboxylation, hydroxy, carbonyl	-30, +16, +14
Aspartic acid	decarboxylation, hydroxy	-30, +16
Asparagine	Hydroxy	+16
Alanine	Hydroxy	+16

Scheme 1: Formaldehyde mediated crosslinking through primary amino groups



12. References

1. Zhou H-X. Influence of crowded cellular environments on protein folding, binding, and oligomerization: biological consequences and potentials of atomistic modeling. FEBS Lett [Internet]. 2013; 587(8): 1053–1061.

2. Kornberg A. Ten commandments: lessons from the enzymology of DNA replication. J Bacteriol [Internet]. 2000; 182(13): 3613–3618.

3. Batra J, Xu K, Qin S, Zhou H-X. Effect of Macromolecular Crowding on Protein Binding Stability: Modest Stabilization and Significant Biological Consequences. Biophys J [Internet]. 2009; 97(3): 906–911.

4. Rotter M, Aprelev A, Adachi K, Ferrone FA. Molecular crowding limits the role of fetal hemoglobin in therapy for sickle cell disease. J Mol Biol [Internet]. 2005; 347(5): 1015–1023.

5. Nagy IZ, Nagy K, Lustyik G. Protein and water contents of aging brain. Exp brain Res [Internet]. 1982; Suppl 5: 118–122.

6. Shtilerman MD, Ding TT, Lansbury PT. Molecular crowding accelerates fibrillization of alpha-synuclein: could an increase in the cytoplasmic protein concentration induce Parkinson's disease? Biochemistry [Internet]. 2002; 41(12): 3855–3860.

7. Medalia O, Weber I, Frangakis AS, Nicastro D, Gerisch G, Baumeister W. Macromolecular architecture in eukaryotic cells visualized by cryoelectron tomography. Science [Internet]. 2002; 298(5596): 1209–1213.

8. Ning M, Lopez M, Cao J, Buonanno FS, Lo EH. Application of proteomics to cerebrovascular disease. Electrophoresis [Internet]. 2012; 33(24): 3582–3597.

9. Chebotareva NA, Kurganov BI, Livanova NB. Biochemical effects of molecular crowding. Biochemistry (Mosc) [Internet]. 2004; 69(11): 1239–1251.

10. Morgan CR, Engen JR. Investigating solution-phase protein structure and dynamics by hydrogen exchange mass spectrometry. Curr Protoc protein Sci [Internet]. 2009; Chapter 17: Unit 17.6.1-17.

11. Narayanan S, Mitra G, Muralidharan M, Mathew B, Mandal AK. Protein Structure–Function Correlation in Living Human Red Blood Cells Probed by Isotope Exchange-based Mass Spectrometry. Anal Chem [Internet]. 2015; 87(23): 11812–11818.

12. Ghaemmaghami S, Oas TG. Quantitative protein stability measurement in vivo. Nat Struct Biol [Internet]. 2001; 8(10): 879–882.

13. Calabrese AN, Ault JR, Radford SE, Ashcroft AE. Using hydroxyl radical footprinting to explore the free energy landscape of protein folding. Methods [Internet]. 2015; 89: 38–44.

14. Espino JA, Mali VS, Jones LM. In Cell Footprinting Coupled with Mass Spectrometry for the Structural Analysis of Proteins in Live Cells. Anal Chem [Internet]. 2015; 87(15): 7971–7978.

15. Zybailov BL, Glazko G V, Jaiswal M, Raney KD. Large Scale Chemical Cross-linking Mass Spectrometry Perspectives. J Proteomics Bioinform [Internet]. 2013; 6(Suppl 2):1.

16. Guerrero C, Tagwerker C, Kaiser P, Huang L. An integrated mass spectrometry-based proteomic approach: quantitative analysis of tandem affinity-purified in vivo cross-linked protein complexes (QTAX) to decipher the 26 S proteasome-interacting network. Mol Cell Proteomics [Internet]. 2006; 5(2): 366–78.

17. Karplus M, McCammon JA. Dynamics of proteins: elements and function. Annu Rev Biochem [Internet]. 1983; 52(1): 263–300.

18. Wood E. Proteins: Structures and Molecular Properties. Biochem Educ [Internet]. 1985; 13(2): 88.

19. Bai Y, Milne JS, Mayne L, Englander SW. Primary structure effects on peptide group hydrogen exchange. Proteins [Internet]. 1993; 17(1): 75–86.

20. Englander SW, Sosnick TR, Englander JJ, Mayne L. Mechanisms and uses of hydrogen exchange. Curr Opin Struct Biol [Internet]. 1996; 6(1): 18–23.

21. Hamuro Y, Coales SJ, Southern MR, Nemeth-Cawley JF, Stranz DD, Griffin PR. Rapid analysis of protein structure

and dynamics by hydrogen/deuterium exchange mass spectrometry. J Biomol Tech [Internet]. 2003;14(3):171–182.

22. Maier CS, Deinzer ML. Protein Conformations, Interactions, and H/D Exchange. In 2005. p. 312–60.

23. Weis DD. Hydrogen exchange mass spectrometry of proteins : fundamentals, methods, and applications. United Kingdom: John Wiley & Sons; 2016.

24. Englander SW, Mayne L, Bai Y, Sosnick TR. Hydrogen exchange: the modern legacy of Linderstrøm-Lang. Protein Sci [Internet]. 1997; 6(5): 1101–1109.

25. Murphy KP. Protein Structure, Stability, and Folding [Internet]. Vol. 168. New Jersey: Humana Press; 2001.

26. Miranker A, Robinson C V, Radford SE, Dobson CM. Investigation of protein folding by mass spectrometry. FASEB J [Internet]. 1996; 10(1): 93–101.

27. Rodriguez HM, Robertson AD, Gregoret LM. Native state EX2 and EX1 hydrogen exchange of Escherichia coli CspA, a small beta-sheet protein. Biochemistry [Internet]. 2002; 41(7): 2140–2148.

28. Krishna MMG, Hoang L, Lin Y, Englander SW. Hydrogen exchange methods to study protein folding. Methods [Internet]. 2004; 34(1): 51–64.

29. Karan DM, Macey RI. The permeability of the human red cell to deuterium oxide (heavy water). J Cell Physiol [Internet]. 1980; 104(2): 209–214.

30. Mitra G, Muralidharan M, Narayanan S, Pinto J, Srinivasan K, Mandal AK. Glutathionylation Induced Structural Changes in Oxy Human Hemoglobin Analyzed by Backbone Amide Hydrogen/Deuterium Exchange and MALDI-Mass Spectrometry. Bioconjug Chem [Internet]. 2012; 23(12): 2344–2353.

31. Zhang Z, Smith DL. Determination of amide hydrogen exchange by mass spectrometry: a new tool for protein structure elucidation. Protein Sci [Internet]. 1993; 2(4): 522–531.

32. Perutz MF. Stereochemistry of cooperative effects in haemoglobin. Nature [Internet]. 1970; 228(5273): 726–739.

33. Paoli M, Liddington R, Tame J, Wilkinson A, Dodson G. Crystal Structure of T State Haemoglobin with Oxygen Bound At All Four Haems. J Mol Biol [Internet]. 1996; 256(4): 775–92.

34. Shaanan B. Structure of human oxyhaemoglobin at 2.1 A resolution. J Mol Biol [Internet]. 1983 Nov 25; 171(1): 31–59.

35. Ghaemmaghami S, Fitzgerald MC, Oas TG. A quantitative, high-throughput screen for protein stability. Proc Natl Acad Sci U S A [Internet]. 2000; 97(15): 8296–8301.

36. Maleknia SD, Downard KM. Radical approaches to probe protein structure, folding, and interactions by mass spectrometry. Mass Spectrom Rev [Internet]. 2001; 20(6): 388–401.

37. Sharp JS, Becker JM, Hettich RL. Analysis of Protein Solvent Accessible Surfaces by Photochemical Oxidation and Mass Spectrometry. Anal Chem [Internet]. 2004; 76(3): 672–83.

38. Baichoo N, Heyduk T. Mapping Conformational Changes in a Protein: Application of a Protein Footprinting Technique to cAMP-Induced Conformational Changes in cAMP Receptor Protein †. Biochemistry [Internet]. 1997; 36(36): 10830–10836.

39. Wang L, Chance MR. Structural mass spectrometry of proteins using hydroxyl radical based protein footprinting. Anal Chem [Internet]. 2011; 83(19): 7234–7241.

40. Haber F, Weiss J. The Catalytic Decomposition of Hydrogen Peroxide by Iron Salts. Proc R Soc A Math Phys Eng Sci [Internet]. 1934; 147(861): 332–351.

41. Volman DH, Chen JC. The Photochemical Decomposition of Hydrogen Peroxide in Aqueous Solutions of Allyl Alcohol at 2537 Å. 1. J Am Chem Soc [Internet]. 1959; 81(16): 4141–4144.

42. Xu G, Takamoto K, Chance MR. Radiolytic Modification of Basic Amino Acid Residues in Peptides: Probes for Examining Protein−Protein Interactions. Anal Chem [Internet]. 2003; 75(24): 6995–7007.

43. Hambly DM, Gross ML. Laser flash photolysis of hydrogen peroxide to oxidize protein solvent-accessible residues on the microsecond timescale. J Am Soc Mass Spectrom [Internet]. 2005; 16(12): 2057–2063.

44. Aye TT, Low TY, Sze SK. Nanosecond Laser-Induced Photochemical Oxidation Method for Protein Surface Mapping with Mass Spectrometry. Anal Chem [Internet]. 2005; 77(18): 5814–5822.

45. Gau BC, Sharp JS, Rempel DL, Gross ML. Fast Photochemical Oxidation of Protein Footprints Faster than Protein Unfolding. Anal Chem [Internet]. 2009; 81(16): 6563–6571.

46. Xu G, Chance MR. Hydroxyl radical-mediated modification of proteins as probes for structural proteomics [Internet]. Vol. 107, Chemical Reviews. American Chemical Society; 2007. p. 3514–43.

47. Weeks JL, Matheson MS. The Primary Quantum Yield of Hydrogen Peroxide Decomposition 1. J Am Chem Soc [Internet]. 1956; 78(7): 1273–1278.

48. Garrison WM, Kland-English M, Sokol HA, Jayko ME. Radiolytic degradation of the peptide main chain in dilute aqueous solution containing oxygen. J Phys Chem [Internet]. 1970; 74(26): 4506–4509.

49. Garrison WM. Reaction mechanisms in the radiolysis of peptides, polypeptides, and proteins. Chem Rev [Internet]. 1987; 87(2): 381–398.

50. Taniguchi H, Fukui K, Ohnishi S, Hatano H, Hasegawa H, Maruyama T. Free-radical intermediates in the reaction of the hydroxyl radical with amino acids. J Phys Chem [Internet]. 1968; 72(6): 1926–1931.

51. Hawkins CL, Davies MJ. EPR studies on the selectivity of hydroxyl radical attack on amino acids and peptides. J Chem Soc Perkin Trans 2 [Internet]. 1998 Jan 1 [cited 2017 Oct 29];0(12):2617–22.

52. Headlam HA, Mortimer A, Easton CJ, Davies MJ. beta-Scission of C-3 (beta-carbon) alkoxyl radicals on peptides and proteins: a novel pathway which results in the formation of alpha-carbon radicals and the loss of amino acid side chains. Chem Res Toxicol [Internet]. 2000; 13(11): 1087–1095.

53. Maleknia SD, Brenowitz M, Chance MR. Millisecond radiolytic modification of peptides by synchrotron X-rays identified by mass spectrometry. Anal Chem [Internet]. 1999; 71(18): 3965–3973.

54. Takamoto K, Chance MR. RADIOLYTIC PROTEIN FOOTPRINTING WITH MASS SPECTROMETRY TO PROBE THE STRUCTURE OF MACROMOLECULAR COMPLEXES. Annu Rev Biophys Biomol Struct [Internet]. 2006; 35(1): 251–276.

55. Back JW, de Jong L, Muijsers AO, de Koster CG. Chemical cross-linking and mass spectrometry for protein structural modeling. J Mol Biol [Internet]. 2003; 331(2): 303–313.

56. Back JW, Hartog AF, Dekker HL, Muijsers AO, Koning LJ, Jong L. A new crosslinker for mass spectrometric analysis of the quaternary structure of protein complexes. J Am Soc Mass Spectrom [Internet]. 2001; 12(2): 222–227.

57. Trakselis MA, Stephen C. Alley, Ishmael§ FT. Identification and Mapping of Protein−Protein Interactions by a Combination of Cross-Linking, Cleavage, and Proteomics. 2005; 16(4): 741–50.

58. Schmidt C, Robinson C V. A comparative cross-linking strategy to probe conformational changes in protein complexes. Nat Protoc [Internet]. 2014; 9(9): 2224–2236.

59. Vasilescu J, Guo X, Kast J. Identification of protein-protein interactions usingin vivo cross-linking and mass spectrometry. Proteomics [Internet]. 2004; 4(12): 3845–3854.

60. Sutherland BW, Toews J, Kast J. Utility of formaldehyde cross-linking and mass spectrometry in the study of protein–protein interactions. J Mass Spectrom [Internet]. 2008; 43(6):699–715.

61. Kaake RM, Wang X, Burke A, Yu C, Kandur W, Yang Y, et al. A New in Vivo Cross-linking Mass Spectrometry Platform to Define Protein–Protein Interactions in Living Cells. Mol Cell Proteomics [Internet]. 2014; 13(12): 3533–3543.

62. Jin Lee Y. Mass spectrometric analysis of cross-linking sites for the structure of proteins and protein complexes. Mol Biosyst [Internet]. 2008; 4(8): 816.

63. Sinz A. Chemical cross-linking and mass spectrometry for mapping three-dimensional structures of proteins and protein complexes. J Mass Spectrom [Internet]. 2003; 38(12): 1225–37.

64. Leitner A, Walzthoeni T, Kahraman A, Herzog F, Rinner O, Beck M, *et al*. Probing Native Protein Structures by Chemical Cross-linking, Mass Spectrometry, and Bioinformatics. Mol Cell Proteomics [Internet]. 2010; 9(8): 1634–1649.

65. Wong SS. Chemistry of protein conjugation and cross-linking [Internet]. CRC Press; 1991 [cited 2017 Oct 29]. 340 p.

66. Sinz A. Chemical cross-linking and mass spectrometry to map three-dimensional protein structures and protein–protein interactions. Mass Spectrom Rev [Internet]. 2006; 25(4): 663–682.

67. Young MM, Tang N, Hempel JC, Oshiro CM, Taylor EW, Kuntz ID, et al. High throughput protein fold identification by using experimental constraints derived from intramolecular cross-links and mass spectrometry. Proc Natl Acad Sci U S A [Internet]. 2000; 97(11): 5802–5806.

68. Back JW, Sanz MA, De Jong L, De Koning LJ, Nijtmans LGJ, De Koster CG, et al. A structure for the yeast prohibitin complex: Structure prediction and evidence from chemical crosslinking and mass spectrometry. Protein Sci [Internet]. 2002; 11(10): 2471–2478.

69. Holding AN. XL-MS: Protein cross-linking coupled with mass spectrometry. Methods [Internet]. 2015; 89: 54–63.

70. Toews J, Rogalski JC, Clark TJ, Kast J. Mass spectrometric identification of formaldehyde-induced peptide modifications under *in vivo* protein cross-linking conditions. Anal Chim Acta [Internet]. 2008 Jun 23; 618(2): 168–183.

Chapter 6

Natural Polyphenol-Dendrimers Nano-Formulations for Therapeutic Applications in Medicine

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Abstract

Curcuminoids, alkaloids and flavonoids (or bioflavonoids) constitute the broadest and well-studied classes of natural polyphenols and they belong to plant-derived secondary metabolites. Recently, polyphenols have attracted significant interest in the scientific community due to their antioxidant potential to confront and scavenge the free radicals generated during the various pathological processes including neurodegenerative disorders, cancer, and cardiovascular diseases. Dendrimers are three-dimensional hyperbranched polymeric macromolecular structures consisting of symmetrically repetitive branched moieties around a central core, thus adopting a globular structure. Dendrimers, due to their multi-valency and modifiable surface functionality, can be conjugated with several ligands, thus developing targeted effective dendritic multifunctional drug delivery systems. Nanomedicine, a subcategory of nanotechnology, is a multidisciplinary combination of science and technology based on studies at the molecular and atomic level, and contributes to the development of innovative pharmaceutical nano-formulations in the field of medicine. Dendrimers constitute an advanced targeted drug delivery system, which due to its diversified properties, is a challenging advancement in nanomedicine and ensures the desired therapeutic efficacy. Herein, we present a

concise overview of natural polyphenol-dendrimers nano-formulations along with their novel therapeutic applications in the field of medicine.

Keywords: Dendrimers; Flavonoids; Nanocarriers; Therapeutic applications; Drug delivery; Natural polyphenols

1. Introduction

Plants are the main sources of biologically active substances that participate in their defensive mechanisms against herbivores, insects, and microorganisms. Curcuminoids are a class of linear diarylheptanoids, such as curcumin and its derivatives, bearing variable chemical groups that enhance their aqueous solubility, bioavailability and suitability as drug formulations. They are natural polyphenols with excellent antioxidant, antitumor, anti-inflammatory, radioprotective, anti-acidogenic and neuroprotective properties [1,2]. Encapsulation of curcuminoids into nanoparticulate formulations has been proved to provide stability against hydrolytic degradations, improved bioavailability and pharmacokinetics [3]. Alkaloids are a class of natural polyphenolic compounds with numerous structural diversities and exceptional pharmacological activities such as antimalarial, anticancer, vasolidatory, analgesic, and antibacterial activities [4]. However, their poor aqueous solubility and bioavailability limit their therapeutic potency [5]. Flavonoids constitute a large category of polyphenolic compounds that derive from herbal and plant-based beverages and foods [6]. Flavonoids exhibit various antioxidant, cell-signal modulating, anti-inflammatory, and cardioprotective properties. Furthermore, they can inhibit neurodegeneration and the growth of several viruses and microorganisms [7,8,9]. Despite their health benefits, their therapeutic efficacy depends on the improvement of the pharmacokinetic profile after their oral administration. Flavonoids possess limited water solubility, bioavailability, and permeability, and are considered unstable and sensitive to exogenous factors such as light, pH, and temperature [10,11,12]. As a result, encapsulation in nanocarriers for drug delivery applications can be a viable way to improve bio-efficacy and bioavailability of flavonoids by increasing the solubilization potential, altering the absorption pathways, and preventing the metabolic degradation.

Drug delivery constitutes a feasible way to improve patient compliance and therapeutic index via the controlled and targeted administration of a drug [13]. An efficient drug delivery nano-formulation is sufficiently protecting the drug from degradation processes and enables secure crossing through the biological barriers ensuring its safe and targeted transfer to the specific site of action [14]. Since the 1990s, apart from linear polymers, the polymer science has included a novel class of structurally diversified compounds, the branched polymers, such as cross-linked polymers, dendri-grafts, macromolecules, and most recently, dendrimers [15].

Dendrimers are hyper branched polymeric three-dimensional macromolecular structures **(Figure. 1)** with significantly beneficial, over ordinary linear polymers, utilities such as controlled and globular structure and various functionalities [16]. Dendrimers attracted researchers' scientific attention due to their unique structural characteristics and potential

utilization in drug delivery applications. Since the 1980s, various dendritic formulations have been synthesized with specific architectures according to the divergent or convergent synthetic strategy [17,18], starting from a multifunctional core and subsequently adding repeated branching cycles classified as generations of the dendrimer [19]. Several multifunctional dendritic nano-formulations have been utilized as optimal drug delivery molecules in vaccination and immunology, photodynamic therapy, and cancer treatment for the successful administration of proteins, genes, DNA, solubilizing and diagnostic agents [20], and as nano-particulate multifunctional frameworks for targeted treatments, diagnostics, and imaging applications [21]. Poly(propylene imine) (PPI) and Poly(amidoamine) (PAMAM) dendrimers are the most explored types of dendritic drug delivery vehicles as anticancer agents and nanocarriers for RNAi therapeutics [22,23].

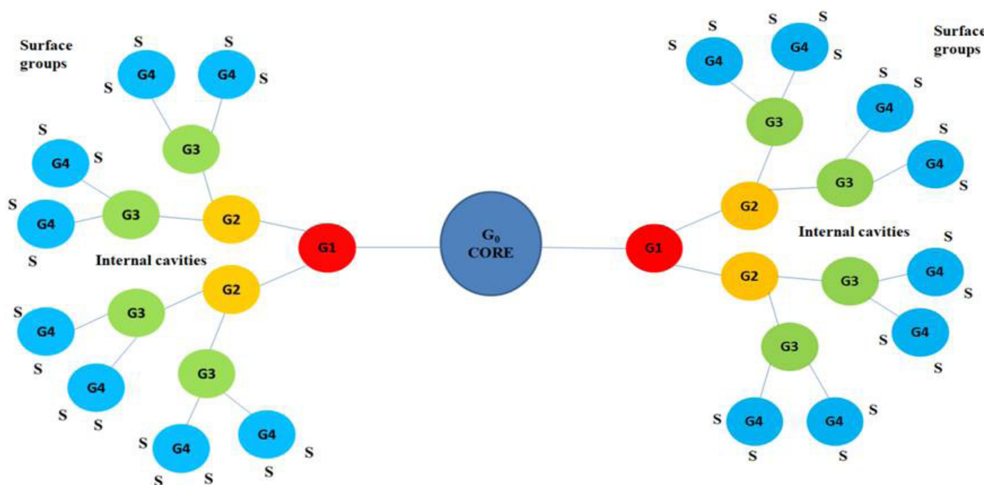
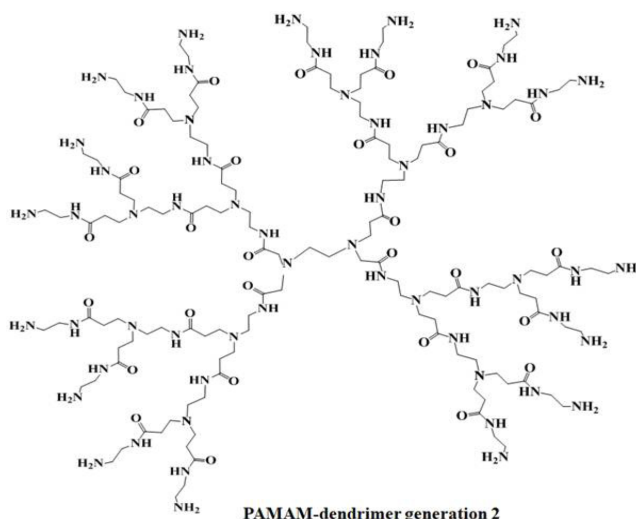


Figure 1: Indicative illustration of a dendritic structure with internal cavities and surface groups.

2. PAMAM dendrimers

PAMAM dendrimers **(Scheme 1)** are well-defined, nano-sized, hyper-branched macromolecules with an ethylene diamine central core and various reactive functional surface groups. PAMAM dendrimers are well-known for their effective enhancement of aqueous solubility and bioavailability of several hydrophobic compounds through encapsulation into their hydrophobic interior [24,25,26]. Furthermore, PAMAM dendrimers possess both active and passive targeting utilities due to their nano-size dimensions and the ability of the reactive peripheral amine groups to conjugate with bio-recognition molecules. However, one major drawback of PAMAM dendrimers is the reported cytotoxic profile of high-generation units. Dendrimers are used in order to enhance the aqueous solubility of water-insoluble drugs [27,28,29]. Interestingly, many researchers have demonstrated that the conjugation process of a drug to PAMAM dendrimers significantly reduces the dendrimer toxicity [30,31]. Folic acid (FA) constitutes the most commonly utilized targeting ligand, especially for its high affinity towards the folate receptors found on the outer membranes of several tumor cells such as colon, ovarian, breast, cervical, and lung cancers [32,33,34]. This specific binding of FA to the folate receptors can sufficiently increase the cellular uptake via clathrin-dependent endocytosis. It

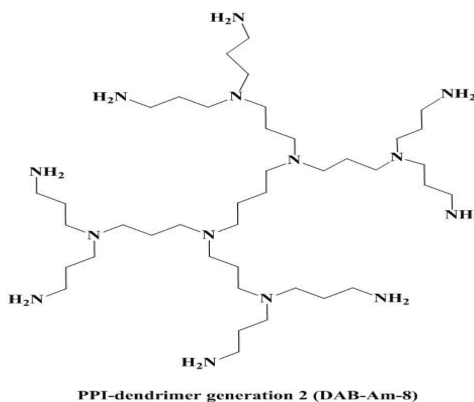
has been proved that FA-conjugated PAMAM dendrimers (FA-PAMAM) present enhanced cellular uptake by targeted cancer cells, improved drug accumulation, and minimum toxicity to normal healthy cells [35,36].



Scheme1: Indicative illustration of a PAMAM-dendrimer generation 2.

3. PPI dendrimers

In general, the main structural components of a dendritic molecule are classified into three distinct parts: a core, an interior, and an exterior. PPI dendrimers **(Scheme 2)** possess a core which is composed of diaminobutane moieties, an exterior that contains the outermost propylene imine chain, and an interior which is characterized as the intermediate component part between the exterior and the core [14]. PPI dendrimers constitute the most substantially explored category of dendrimers, adequate for drug delivery applications. The generations of PPI dendrimers are directly proportional to the surface cationic groups [37,38]. The augmentation of dendritic generations and their surface groups increasingly affects the toxicity of PPI dendrimers, as it has been well documented for PPI-G4 dendrimers which are less toxic compared to PPI-G5 dendrimers [39]. Several parameters such as hemolytic toxicity, drug loading and release behavior can affect the number and generation of PPI dendrimers [40,41], whereas the suitability of each generation depends on the purpose of the corresponding drug delivery procedure [40,42].

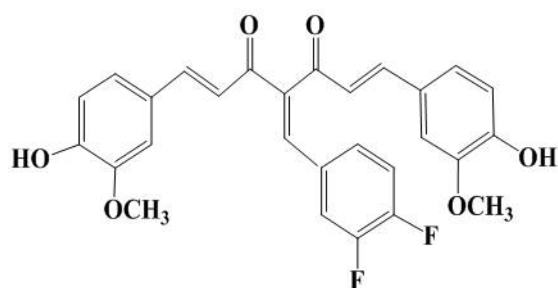


Scheme 2: Indicative illustration of a PPI-dendrimer generation 2 (DAB-Am-8).

4. Natural Polyphenol-Dendrimers Nano-Formulations

4.1. 3,4-Difluorobenzylidene Diferuloylmethane (DFC)

3, 4-difluorobenzylidene diferuloylmethane (DFC), (1E,6E)-4-(3,4-Difluorobenzy-lidene)-1,7-bis(4-hydroxy-3-methoxyphenyl)hepta-1,6-diene-3,5-dione **(Scheme 3)** is a synthetic derivative of diferuloylmethane (curcumin), and an efficient anticancer polyphenol which has exhibited a 16-fold enhanced half-life combined with increased anticancer efficiency compared to its natural precursor, during tests on pancreatic tumors [43,44,45]. These observations are attributed to the improved bioavailability and stability of DFC compared to diferuloylmethane. Guided by these findings, researchers developed DFC-loaded dendritic nano-formulations conjugated to FA for the specific delivery of the drug to cervical cancer cells capable of over-expressing the folate receptors, thus increasing the anticancer activity of the compound. The engineered dendritic delivery nano-system possesses high DFC entrapment efficiency and loading capacity with outstanding bioavailability and aqueous solubility. The positive charge due to the conjugated FA ensures shielding and biological safety, improved anticancer potency *in vitro* and enhanced internalization compared to non-specifically targeted nano-formulations in SKOV3 and HeLa cells. Additionally, the dendritic nano-carriers augmented the apoptotic onset of the tumor cells, down-regulated NFκB and up-regulated the expression of PTEN, thus contributing in the debilitation of tumor recurrence rate and drug resistance after the initial drug treatment [46].



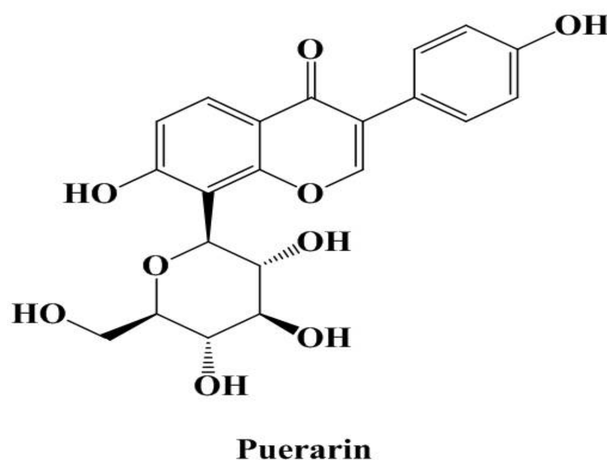
3,4-difluorobenzylidene diferuloylmethane (DFC)

Scheme 3: Formula of 3,4-difluorobenzylidene diferuloylmethane (DFC).

4.2. Puerarin

Puerarin,7-Hydroxy-3-(4-hydroxyphenyl)-8-[(3R,4R,5S,6R)-3,4,5-trihydroxy-6-hydroxyl methyl)oxan-2-yl]chromen-4-one **(Scheme 4)** constitutes a well-known isoflavone with excellent potential against cardiovascular disorders and anxiogenicity [47,48]. Studies on the effect of PAMAM dendrimers (G3, G4, G5, G3.5, G4.5) on the trans-epithelial transport and immortalized human corneal epithelium (HCE) cellular uptake of puerarin proved that PAMAM dendrimers (G3,G4,G5) with a cationic charge could significantly enhance the transport and uptake of the corresponding isoflavone. Confocal laser scanning microscopy

(CLSM) and flow-cytometry (FCM) examinations revealed a significantly strong internalization of PAMAM-G4 in the HCE cellular milieu, presumably due to the charge interactions and the easily manipulated design, size, surface functionality and charge of PAMAM dendrimers in their utilization as ocular drug nano-carriers [49,50]. It is suggested that both the type and generation of PAMAM dendrimers can affect the corneal permeation of puerarin mainly due to the provoked enhancement in its solubility induced by the encapsulation procedure and the ability of PAMAM dendrimers to successfully integrate with the lipid bilayer in the plasma membrane of corneal epithelial cells and loosen the corneal epithelial cell junctions [51,52]. Additional experimental and comparative results on the effects of dendrimer-puerarin complexes and eye drops containing free puerarin showed: a) the ability of these dendritic nanostructures to present long residence times inside the eyes, b) the slow in vitro puerarin release rates in biological buffers (PBS), and c) the absence of significantly different drug corneal permeation levels between the dendrimer-puerarin complexes and the free isoflavone [53]. Moreover, *in vivo* studies on the behavior of amine terminated (full generation) or carboxylate terminated (half generation) PAMAM dendrimers as solubility and bioavailability enhancers of puerarin indicated the ability of full generation PAMAM dendrimers to act as better solubilizing agents mainly due to the electrostatic intermolecular interactions between the phenolic hydroxyl groups of the isoflavone and the surface amine groups of the dendritic molecules. Furthermore, no hemolysis was observed after the administration of puerarin-dendrimer complexes to erythrocytes [54].

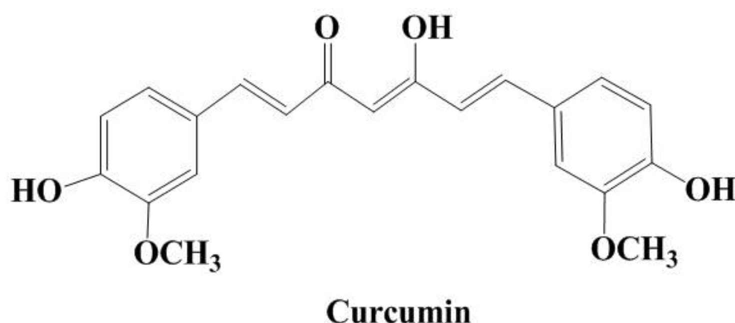


Scheme 4: Formula of Puerarin.

4.3. Curcumin

Curcumin,(1*E*,6*E*)-1,7-Bis(4-hydroxy-3-methoxyphenyl)hepta-1,6-diene-3,5-dione **(Scheme 5)** constitutes one of the three curcuminoid components present in turmeric, with bis-desmethoxycurcumin and desmethoxycurcumin being the other two. It is a natural phenol mainly used as a yellow food additive and colorant and a tautomeric compound present as keto and a more stable enolic form [55,56]. Curcumin has been the subject of much research

due to its various medicinal properties. Research studies have demonstrated its potent anti-inflammatory activity and anticancer role. Moreover, curcumin reduces the proliferation and transformation of tumors by regulating transcription and growth factors, protein kinases, inflammatory cytokines or other enzymes. In animal experimental studies, curcumin has been shown to possess a protective role against blood, mouth, pancreas, lung, skin, and intestinal tract cancer cells [57,58]. Research results on the antiproliferative activity of PAMAM encapsulated and free curcumin on T47D breast cancer cells via TRAP assay and a 24 h study of telomerase activity, indicated that the curcumin-loaded dendritic nano-carriers presented increased inhibitory effect, negligible cytotoxicity and enhanced antiproliferative potency [59]. Further *in vivo* studies on the treatment of various cancer cell lines with curcumin conjugated oligo(ethylene glycol) chains (curc-OEG) displayed high inhibition levels due to intense apoptotic activity. Furthermore, the intravenous injection of curc-OEG at high doses in SKOV-3 and MDA-MB-468 tumors provoked the decrease of tumor numbers and weights, with no observed subchronic and acute toxicities in the peripheral mouse visceral organs of the mice. Curc-OEG nano-formulations can also constitute effective carriers of camptothecin and doxorubicin anticancer drugs for the successful enhancement of cytotoxicity in drug-resistant cancer cells [60,61].

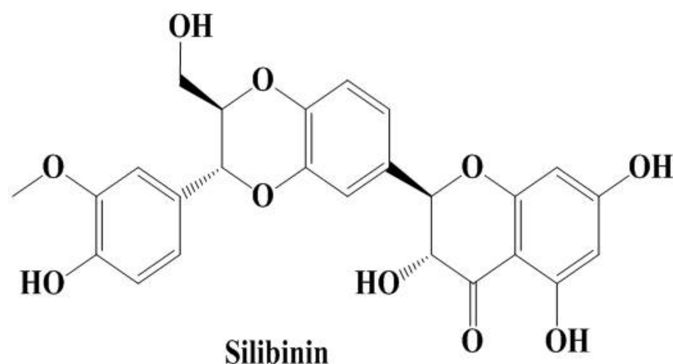


Scheme 5: Formula of Curcumin.

4.4. Silibinin

Silibinin,(2R,3R)-3,5,7-trihydroxy-2-[(2R,3R)-3-(4-hydroxy-3-methoxyphenyl)-2-(hydroxyl-methyl)-2,3-dihydrobenzo[b][1,4]dioxin-6-yl]chroman-4-one**(Scheme6)** constitutes a flavonolignan with low bioavailability and water solubility, and nontoxic behavior even at concentrations of up to 13 g/day [62]. The anticancer potential of silibinin is currently under investigation [63]. However, it possesses anti-inflammatory, chemo-protective, anti-photocarcinogenic, anti-hepatitis, anti-cirrhosis and DNA repair properties [64,65,66]. Comparative studies between the amine-terminated PAMAM–G2 and G3 dendrimers and ester-terminated PAMAM–G1.5 and G2.5 dendrimers on their solubility enhancing properties as nano-carriers of silibinin indicated the improved solubilizing activity of the amine-terminated dendritic molecules mainly due to electrostatic interactions provoked by the formation of a complex between the corresponding dendritic nano-molecules and the phenolic hydroxyl moieties of the flavonolignan [67]. This complexation further leads to improved in vitro drug

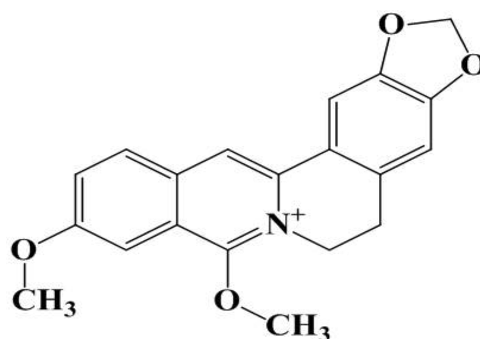
release rates and in vivo bioavailability profiles of the encapsulated compound. Synthetic efforts on the development of surface-modified magnetic Fe_3O_4 nanotubes (Fe_3O_4NT) grafted with PAMAM-G3 dendrimers as nano-carriers of hydrophobic silibinin revealed the potential effect of PAMAM incorporation in the enhancement of silibinin loading capacity and sustainability of drug release rate, yet maintaining the magnetic properties of the nano-formulation compared to the unmodified Fe_3O_4 nanotubes and other similar nano-systems. As a result, the Fe_3O_4NT-PAMAM system could be utilized as a promising nano-carrier for drug-delivery applications [68].



Scheme 6: Formula of Silibinin.

4.5. Berberine

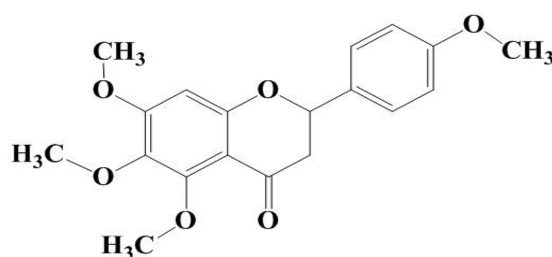
Berberine,5,6-Dihydro-9,10-dimethoxybenzo[*g*]-1,3-benzodioxolo[5,6-a]quinolizinium **(Scheme 7)** constitutes a naturally produced nitrogenous cyclic benzylisoquinoline alkaloid with proved anticancer, apoptotic and antiproliferative activity [69]. However, the low bioavailability and poor pharmacokinetics have limited its utilization as an anticancer compound [70]. Recent studies on the synthesis of berberine-conjugated or encapsulated PAMAM-G4 dendrimers indicated the development of significantly nano-sized formulations (100-200 nm) with similar *z*-potential to that of an empty or unmodified PAMAM-G4 dendrimer. Furthermore, the conjugation percentage of berberine exceeded the encapsulation percentage of the corresponding compound, whereas both types of nano-formulations presented a sustained release rate of berberine in biological buffers and aqueous media. *Ex-vivo* and *in vivo* hemolytic studies on toxicity showed that both dendritic nanostructures are considered significantly biocompatible and relatively nontoxic. Moreover, the MTT results proved that the berberine-conjugated dendritic molecules showed excellent anticancer activity against MDA-MB-468 and MCF-7 breast cancer cells. Additionally, the *in vivo* pharmacokinetic parameters such as AUC (area under the curve) and half-life ($t_{1/2}$) of the novel dendritic formulations and the conjugated or encapsulated berberine were significantly improved. The overall conclusions imply that the conjugation of natural compounds to PAMAM dendrimers leads to improved delivery of these molecules and the development of more efficiently therapeutic dendritic nano-formulations [71].



Scheme 7: Formula of Berberine.

4.6. Tetramethylscutellarein

Tetramethylscutellarein, [5,6,7-trimethoxy-2-(4-methoxyphenyl)-4H-chromen-4-one] **(Scheme 8)** is a flavonoid with well-established anti-inflammatory potential [72], moderate cytotoxic activity against MCF-7 breast cancer cells and anti-tuberculosis properties [73]. Experimental studies on the synthesis of tetramethylscutellarein-loaded PAMAM-G4 dendrimers with amines as terminal moieties confirmed the solubility enhancement provoked by a complex formation between the surface functional amine moieties of the dendrimer and the flavonoid mainly due to electrostatic interactions and hydrogen bonding. Furthermore, the results at hand indicated the influence of pH on the solubility of the dendritic formulations and the *in vitro* release rates of the encapsulated flavonoid [72].

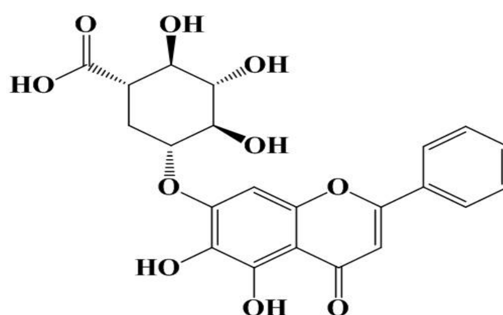


Scheme 8: Formula of Tetramethylscutellarein.

4.7. Baicalin

Baicalin, (2*S*,3*S*,4*S*,5*R*,6*S*)-6-(5,6-dihydroxy-4-oxo-2-phenyl-chromen-7-yl) oxy-3,4,5-trihydroxy-tetrahydropyran-2-carboxylic acid **(Scheme 9)**, the glucunoride derivative of baicalein, constitutes a flavonoid with excellent potential for the prevention and treatment of allergy, hypertension, cardiovascular disorders, inflammation, and bacterial infections [74,75]. Moreover, baicalin presents cytostatic and cytotoxic potency *in vitro*, antitumor growth effect *in vivo* [76], and sufficient anticancer activity on lung, bladder, prostate, breast, liver, ovarian, and colorectal cancer cells [77]. However, the limited bioavailability and water solubility

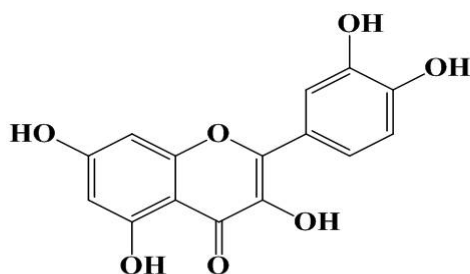
in combination with the non-specific targeting of tumors limits the clinical application of the corresponding flavonoid [78]. Recent studies on the development of baicalin-loaded FA-conjugated PAMAM dendrimers indicated the beneficial effect of dendrimers and the corresponding generation of the PAMAM formulations on the aqueous dispersion, biological and physicochemical profile of baicalin [79]. More specifically, FA-modified PAMAM-G3 and PAMAM-G6 dendrimers demonstrated excellent entrapment efficiency and sustained release rates of baicalin in acidic PBS (pH 5.4). Furthermore, the corresponding nano-formulations provided targeted delivery of the encapsulated flavonoid and enhanced toxicity and flavonoid uptake in the folate receptor (FR) of HeLa cells as proved by MTT assays. All the experimental results were significantly related to the generation of the utilized PAMAM dendrimers. Further cell apoptosis and cell cycle analyses indicated the tumor-specific efficacy of the novel dendritic nano-formulations for the targeted delivery of baicalin to tumor cells [80].



Scheme 9: Formula of Baicalin.

4.8. Quercetin

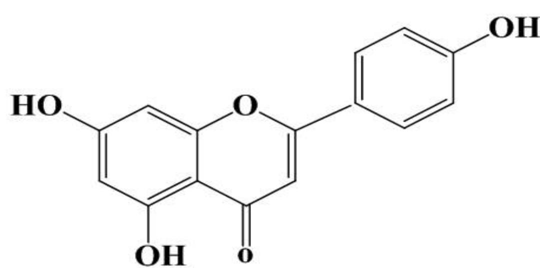
Quercetin, 2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-4*H*-chromen-4-one **(Scheme 10)** constitutes a well-known flavonol with excellent antioxidant [81], cardio-protective [82], anti-ulcer [83], gene regulatory [84], anti-inflammatory [85], anticancer, and neuroprotective properties [86]. However, the poor solubility and bioavailability of quercetin limit its wide therapeutic applicability. Recent research works have established PAMAM dendrimers as effective quercetin delivery systems. More specifically, spherical nano-sized formulations (34.4-100.3 nm) of PAMAM dendrimers G0–G3 were proved to enhance the aqueous solubility of quercetin. Moreover, the novel dendritic quercetin-loaded nanostructures showed sustained *in vitro* release rates and prolonged stability at 4±2 ^{o}C. Furthermore, the results at hand indicated that orally administrated quercetin-loaded dendritic nano-formulations showed improved anti-inflammatory activity in rats compared to free quercetin suggesting that the incorporation of the corresponding flavonoid in PAMAM dendrimers can result in the development of an effective tool for the oral quercetin targeted delivery [87].



Scheme 10: Formula of Quercetin.

4.9. Apigenin

Apigenin, 5,7-Dihydroxy-2-(4-hydroxyphenyl)-4*H*-1-benzopyran-4-one **(Scheme 11)** belongs to the flavone family. It induces autophagy in leukemia cells supporting a chemo-preventive role [88], whereas it possesses a specific function in the prevention of renal damage [89]. Additionally, it exerts sedative and anxiolytic effects [90]. However, *in vitro* studies have shown that apigenin presents toxicity in red blood cells [91]. In recent studies, flavonoid apigenin was utilized as the fluorophore core of benzylic dendrimers. In general, fluorescent dendrimers are used as analytical tools and organic light emitting devices (OLEDs). Apigenin possesses three phenol moieties and weak blue light emission. These properties render apigenin an ideal precursor for the synthesis of Freshet-type dendrimeric structures [92]. Simulations of molecular dynamics were used for the estimation of branching and side effects. The overall data suggested that the 3rd and 4th dendrimer generations possess larger asphericities. Additionally, the fluorescence spectra implied the existence of aggregation phenomena for non-spheric dendritic structures [93].



Scheme 11: Formula of Apigenin.

4.10. Daidzein

Daidzein, 7-Hydroxy-3-(4-hydroxyphenyl)chromen-4-one **(Scheme 12)** is a natural compound which belongs in the class of isoflavones. Recent research results have proved the viability of daidzein treatment as an effective remedy for osteoporosis, blood cholesterol, menopausal relief, heart disease, and hormone-related cancers [94,95]. However, the low bioavailability

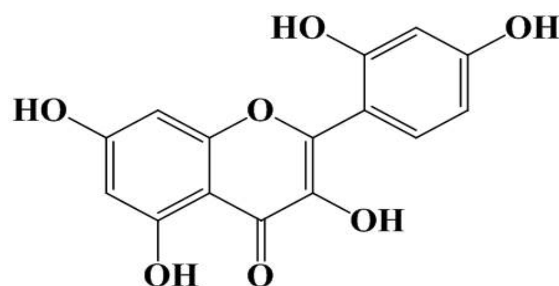
and aqueous solubility of isoflavones, and their rapid metabolizing rates prevent their efficient oral or intravenous administration. As a result scientists have focused their efforts on the development of effective nano-carriers of isoflavones aiming at the improvement of their solubility and further clinical applicability [96,97,98]. More specifically, studies on daidzein liquid formulations encapsulated in PAMAM and PPI dendritic nano-carriers showed the remarkable improvement of the aqueous solubility of the corresponding isoflavone. PPI dendritic molecules showed a more efficient daidzein loading capacity compared to that of PAMAM dendrimers, mainly due to the presence of the large number of hydrophobic internal cavities inside the PPI dendritic structure. Moreover, the release rates of daidzein were slower from the daidzein-loaded PPI dendrimers compared to that from the daidzein-loaded PAMAM dendritic molecules. However, PPI nano-formulations were far more toxic and less stable compared to PAMAM dendrimerson A549 and MCF-7 cells. Furthermore, the daidzein-loaded PAMAM-G3 nano-formulations presented a contiguous protective activity against H_2O_2-induced cytotoxicity on A549 and MCF-7 cells similar to that of free daidzein. As a result, daidzein-loaded PAMAM dendrimers have been proved an effective and safe choice in the design of daidzein delivery systems with improved bioavailability, prolonged daidzein delivery and release rates, and sustained bioactivity [99].

Scheme 12: Formula of Daidzein.

4.11. Morin

Morin, 2-(2,4-Dihydroxyphenyl)-3,5,7-trihydroxychromen-4-one **(Scheme 13)** constitutes a well-known polyhydroxy flavonol with various pharmaceutical and biological activities [100,101]. Morin possesses structural, physicochemical and therapeutic properties such as hydrophobicity, planarity, electrostatic, antioxidant and anti-amyloid aggregation activities [102], that depend on the changes of the surrounding environment. As a result, scientists have focused their efforts on the complete investigation of the interactions of morin with various biological molecules, such as PAMAM dendrimers, in order to fully understand the drug-dendrimer interactions and develop an effective dendritic drug delivery nano-formulation. Recent modeling docking and spectroscopic reports on the interactions of morin-loaded modified PAMAM dendrimers with a 25% surface attachment of N-(2-hydroxydodecyl) groups (PAMAM-C12 25%) confirmed the morin-(PAMAM-C12 25%) complex formation in the aqueous phase through hydrophobic, electrostatic, hydrogen bonds, and van der Waals forces. Morin presented three different types of binding sites with PAMAM-C12 25%, reinforcing the

view that morin encapsulation into PAMAM dendrimers can lead to the functionalization of efficient nano-formulations adequate for polyphenol delivery applications [103].



Scheme 13: Formula of Morin.

5. Summary

This chapter is a concise collection of data of natural polyphenol-dendrimer nano-formulations with potential therapeutic activities. Dendrimers can be produced with highly controllable properties such as shape, size, functionality, and architecture. Their diversified surface groups determine their targeting function and toxicity behavior. Moreover, dendrimers, as nano-carrier vehicles of globular structure, can improve the bioavailability and therapeutic efficacy of the encapsulated natural compounds, thus formulating an adequate natural polyphenol delivery system for the development of effective medicinal treatment against various diseases.

6. References

1. Jayaprakasha GK, Rao LJ, Sakariah KK. Antioxidant activities of curcumin, demethoxycurcumin and bisdemethoxycurcumin. Food Chemistry. 2006; 98: 720–724.

2. Amalraj A, Pius A, Gopi S, Gopi S, Biological activities of curcuminoids, other biomolecules from turmeric and their derivatives – A review. J. Tradit. Complement. Med. [Internet]. 2017; 7: 205-233.

3. Tiyaboonchai W, Tungpradit W, Plianbangchang P. Formulation and characterization of curcuminoids loaded solid lipid nanoparticles. Int. J. Pharm. [Internet]. 2007; 337: 299–306.

4. Williams P. Review of modern alkaloids. Structure, isolation, synthesis and biology. J. Nat. Prod. 2012; 75: 1261–1261.

5. Amirkia V, Heinrich M. Alkaloids as drug leads – A predictive structural and biodiversity-based analysis. Phytochem. Lett. 2014; 10: xlviii-liii.

6. Bilia AR, Isacchi B, Righeschi C, Guccione C, Bergonzi MC. Flavonoids loaded in nanocarriers: An opportunity to increase oral bioavailability and bioefficacy. Food Nutr. Sci. 2014; 5: 1212-1227.

7. Ross JA, Kasum CM. Dietary flavonoids: Bioavailability, metabolic effects, and safety. Annu. Rev. Nutr. 2002; 22: 19-34.

8. Yao LH, Jiang YM, Shi J, Tomás-Barberán FA, Datta N, Singanusong R, Chen SS. Flavonoids in food and their health benefits. Plant Foods Hum. Nutr. 2004; 59: 113-122.

9. Heim KE, Tagliaferro AR, Bobilya DJ. Flavonoid antioxidants: Chemistry, metabolism and structure-activity relationships. J. Nutr. Biochem. (2002); 13: 572-584.

10. Manach C, Williamson G, Morand C, Scalbert A, Rémésy C. Bioavailability and bioefficacy of polyphenols in humans. I. Review of 97 bioavailability studies. Am. J. Clin. Nutr. 2005; 81: 230-242.

11. Stahl W, van den Berg H, Arthur J, Bast A, Dainty J, Faulks RM, Gärtner C, Haenen G, Hollman P, Holst B, Kelly FJ, Polidori MC, Rice-Evans C, Southon S, van Vliet T, Viña-Ribes J, Williamson G, Astley SB. Bioavailability and metabolism. Mol. Aspects Med. [Internet]. 2002; 23: 39-100.

12. Tsolaki MN, Koutsouraki E, Katsipis GK, Myserlis PG, Chatzithoma MA, Pantazaki AA. Alternative anti-infective/anti-inflammatory therapeutic options for fighting Alzheimer's disease. 2017; 6: 1-153.

13. Narayan PS, Pooja S, Khushboo A, Diwakar T, Ankit S, Singhai A. A novel drug delivery system. Int. J. Pharm. Life Sci. [Internet]. 2010; 1: 382–388.

14. Mishra I. Dendrimer: a novel drug delivery system. JDDT. 2011; 1: 70–74.

15. Kaur D, Jain K, Mehra NK, Kesharwani P, Jain NK. A review on comparative study of PPI and PAMAM dendrimers. J. Nanopart. Res. 2016; 18: 146.

16. Pandey RP, Gurung RB, Sohng JK. Dietary sources, bioavailability and biological activities of naringenin and its derivatives. In book: Apigenin and naringenin: natural sources, pharmacology and role in cancer prevention. Publisher: Nova Science Publishers. Editors: Stacks NM. (2015).

17. Hawker CJ, Frechet JMJ. Preparation of polymers with controlled moleculararchitecture. A new convergent approach to dendritic macromolecules. J. Am. Chem. Soc. [Internet]. 1990; 112: 7638-7647.

18. Tomalia DA, Naylor AM, Goddard WA. Starburst dendrimers: molecular-level control of size, shape, surface chemistry, topology, and flexibility from atomsto macroscopic matter. Angew. Chem. Int. Ed. Engl. [Internet]. 1990; 29: 138-175.

19. Liu M, Fréchet JMJ. Designing dendrimers for drug delivery. Pharm. Sci.Technol. Today. 1999; 2: 393-401.

20. Kesharwani P, Jain K, Jain NK. Dendrimer as nanocarrier for drug delivery. Prog. Polym. Sci. [Internet]. 2014; 39: 268-307.

21. Gillies ER, Fréchet JMJ. Dendrimers and dendritic polymers in drug delivery. Drug Discov. Today. [Internet]. 2005; 10: 35-43.

22. Mignani S, Rodrigues J, Tomas H, Zablocka M, Shi X, Caminade AM, Majoral JP, Dendrimers in combination with natural products and analogues as anti-cancer agents. Chem. Soc. Rev. [Internet]. 2018; 47: 514-532.

23. Kesharwani P, Banerjee S, Gupta U, Mohd Amin MCI, Padhye S, Sarkar FH, Iyer AK. PAMAM dendrimers as promising nanocarriers for RNAi therapeutics. Mater. Today. [Internet]. 2015; 18; 565–572.

24. Kukowska-Latallo JF, Candido KA, Cao Z, Nigavekar SS, Majoros IJ, Thomas TP, Balogh LP, Khan MK, Baker JR Jr. Nanoparticle targeting of anticancer drug improves therapeutic response in animal model of human epithelial cancer. Cancer Res. [Internet]. 2005; 6512; 5317–5324.

25. Malik N, Evagorou EG, Duncan R. Dendrimer-platinate: a novel approach tocancer chemotherapy. Anticancer Drugs. 1999; 10: 767–776.

26. D. Luong, P. Kesharwani, R. Deshmukh, M.C.I. Mohd Amin, U. Gupta, K. Greish, Iyer AK. PEGylated PAMAM dendrimers: enhancing efficacy and mitigating toxicity for effective anticancer drug and gene delivery. Acta Biomater. 2016; 43: 14-29.

27. Milhem OM, Myles C, McKeown NB, Attwood D, D'Emanuele A. Polyamidoamine Starburst dendrimers as

solubility enhancers. Int. J. Pharm. [Internet]. 2000; 197: 239–241.

28. Devarakonda B, Hill RA, de Villiers MM. The effect of PAMAM dendrimer generation size and surface functional group on the aqueous solubility of nifedipine. Int. J. Pharm. 2004; 284: 133–140.

29. Devarakonda, B, Hill RA, Liebenberg W, Brits M, de Villiers MM. Comparison of the aqueous solubilization of practically insoluble niclosamide by polyamidoamine (PAMAM) dendrimers and cyclodextrins. Int. J. Pharm. 2005; 304: 193–209.

30. Goldberg DS, Vijayalakshmi N, Swaan PW, Ghandehari H. G3.5 PAMAM dendrimers enhance transepithelial transport of SN38 while minimizing gastrointestinal toxicity. J. Control. Release . 2011; 150: 318–325.

31. Mazzaferro S, Bouchemal K, Ponchel G. Oral delivery of anticancer drugs III: formulation using drug delivery systems. Drug Discov. Today. 2013; 18: 99-104.

32. Lu Y, Low PS. Folate-mediated delivery of macromolecular anticancer therapeutic agents. Adv. Drug Deliv. Rev. 2002; 54: 675–693.

33. Holm J, Hansen SI, Høier-Madsen M, Helkjaer PE, Nichols CW. Folate receptors in malignant and benign tissues of human female genital tract. Biosci. Rep. 1997; 17: 415–427.

34. Holm J, Hansen SI, Høier-Madsen M, Helkjaer PE, Bzorek M. Folate receptor in malignant effusions of ovarian carcinoma. APMIS [Internet]. 1995; 103: 663–670.

35. Pan J, Feng SS. Targeted delivery of paclitaxel using folate-decorated poly (lactide)-vitamin E TPGS nanoparticles. Biomaterials. 2008; 29: 2663–2672,

36. Mi Y, Liu Y, Feng SS. Formulation of Docetaxel by folic acid-conjugated d-α-tocopheryl polyethylene glycol succinate 2000 (Vitamin E TPGS2k) micellesfor targeted and synergistic chemotherapy. Biomaterials. 2011; 32: 4058–4066.

37. Gupta U, Agashe HB, Jain NK. Polypropylene imine dendrimer mediated solubility enhancement: effect of pH and functional groups of hydrophobes. J. Pharm. Pharm. Sci. [Internet]. 2007; 10: 358–367.

38. Hembade MJ, Kangane MR, Mansuk AG, Lukkad HR. Dendrimers: as promising nanocarriers for drug delivery. Int. J. Pharm. Arch. 2013; 2: 61–70.

39. Kesharwani P, Tekade RK, Jain NK. Generation dependent cancer targeting poly(propyleneimine) dendrimer. Biomaterials. 2014; 35: 5539–5548.

40. Bagre AP, Jain K, Jain NK. Alginate coated chitosan coreshell nanoparticles for oral delivery of enoxaparin: in vitro and in vivo assessment. Int. J. Pharm. 2013; 456: 31–40.

41. Jain K, Gupta U, Jain NK. Dendronized nanoconjugates of lysine and folate for treatment of cancer. Eur. J. Pharm. Biopharm. 2014; 87: 500–509.

42. Jain K, Verma AK, Mishra P, Jain NK Characterization and evaluation of Amphotericin B loaded MDP conjugated poly(propylene imine) dendrimers. Nanomedicine. [Internet]. 2015; 11: 705-713.

43. Kesharwani P, Banerjee S, Padhye S, Sarkar FH, Iyer AK. Parenterally administrable nano-micelles of 3, 4-difluorobenzylidene curcumin for treating pancreatic cancers. Colloids Surf. B Biointerfaces. 2015; 132: 138-145.

44. Padhye S, Banerjee S, Chavan D, Pandye S, Swamy KV, Ali S, Li J, Dou QP, Sarkar FH. Fluorocurcumins as cyclooxygenase-2 inhibitor: molecular docking, pharmacokinetics and tissue distribution in mice. Pharm. Res. 2009; 26: 2438–2445.

45. Padhye S, Yang H, Jamadar A, Cui QC, Chavan D, Dominiak K, McKinney J, Banerjee S, Dou QP, Sarkar FH. New difluoro Knoevenagel condensates of curcumin, their Schiff bases and copper complexes as proteasome inhibitors and

apoptosis inducers in cancer cells. Pharm. Res. 2009; 26: 1874–1880.

46. Luong D, Kesharwani P, Killinger BA, Moszczynska A, Sarkar FH,Padhye S, Rishi AK, Iyer AK. Solubility enhancement and targeted delivery of a potent anticancer flavonoid analogue to cancer cells using ligand decorated dendrimer nano-architectures. J. Colloid Interface Sci. [Internet]. 2016; 484: 33–43.

47. Yeung DKY, Leung SWS, Xu YC, Vanhoutte PM, Man RYK. Puerarin, an isoflavonoid derived from Radix puerariae, potentiates endothelium-independent relaxation via the cyclic AMP pathway in porcine coronary artery. Eur. J. Pharmacol. 2006; 552: 105–111.

48. Overstreet DH, Kralic JE, Morrow AL, Ma ZZ, Zhang YW, Lee DY. NPI-031G (puerarin) reduces anxiogenic effects of alcohol withdrawal or benzodiazepine inverse or 5-HT2C agonists. Pharmacol. Biochem. Behav. 2003; 75: 619–625.

49. Tao Q, Xiudi Z, Junhua W, Wanhui L, Youxin L, Kaoxiang S. Potential of poly (amidoamine) dendrimers as ocular carriers of puerarin based on human corneal epithelial cell line.2012 CRS Annual Meeting Abstracts.

50. Yavuz B, Pehlivan SB, Ünlü N. Dendrimeric systems and their applications in ocular drug delivery. Sci. World J. 2013; 2013: 732340.

51. Yao WJ, Sun KX, Liu Y, Liang N, Mu HJ, Yao C, Liang RC, Wang AP. Effect of poly(amidoamine) dendrimers on corneal penetration of puerarin. Biol. Pharm. Bull. 2010; 33: 1371-1377.

52. Yao C, Wang W, Zhou X, Qu T, Mu H, Liang R, Wang A, Sun K. Effects of poly (amidoamine) dendrimers on ocular absorption of puerarin using microdialysis. J. Ocul. Pharmacol. Ther. 2011; 27: 565-569.

53. Yao WJ, Sun KX, Mu HJ, Na L, Liu Y, Yao C, Rongcai L, Aiping W. Preparation and characterization of puerarin dendrimer complexes as an ocular drug delivery system. Drug Dev. Ind. Pharm. 2010; 36: 1027–1035.

54. Gu L, Wu ZH, Qi X, He H, Ma X, Chou X, Wen X, Zhang M, Jiao F. Polyamidomine dendrimers: an excellent drug carrier forimproving the solubility and bioavailability of puerarin. Pharm. Dev. Technol. [Internet]. 2013; 18: 1051–1057.

55. Akram M, Uddin S, Ahmed A, Usmanghani K, Hannan A, Mohiuddin E, Asif M. Curcuma longa and curcumin: A review article. Rom. J. Biol.-Plant Biol. [Internet]. 2010; 55: 65-70.

56. Manolova Y, Deneva V, Antonov L, Drakalska E, Momekova D, Lambov N. The effect of the water on the curcumin tautomerism: A quantitative approach. Spectrochim. Acta A. 2014: 132; 815–820.

57. Anand P, Kunnumakkara AB, Newman RA, Aggarwal BB. Bioavailability of curcumin: Problems ans promises. Mol. Pharm. 2007; 4: 807-818.

58. Burgos-Morón E, Calderón-Montaño JM, Salvador J, Robles A, López-Lázaro M. The dark side of curcumin. Int. J. Cancer. 2010; 126: 1771–1775.

59. Mollazade M, Nejati-Koshki K, Akbarzadeh A, Zarghami N, Nasiri M, Jahanban-Esfahlan R, Alibakhshi A. PAMAM dendrimers augment inhibitory effects of curcumin on cancer cell proliferation: possible inhibition of telomerase. Asian Pac. J. Cancer Prev. 2013; 14: 6925–6928.

60. H. Tang, C. J. Murphy, B. Zhang, Shen Y, Sui M, Van Kirk EA, Feng X, Murdoch WJ. Amphiphilic curcumin conjugate-forming nanoparticles as anticancer prodrug anddrug carriers: in vitro and in vivo effects.Nanomedicine. 2010; 5: 855–865.

61. Shindikar A, Singh A, Nobre M, Kirolikar S. Curcumin and resveratrol as promising natural remedies with nanomedicine approach for the effective treatment of triple negative breast cancer. J. Oncol. 2016; 2016.

62 . Flaig TW, Gustafson DL, Su L-J, Zirrolli JA, Crighton F, Harrison GS, Pierson AS, Agarwal R, Glodé LM. A phase

I and pharmacokinetic study of silybin-phytosome in prostate cancer patients. Invest. New Drugs. 2007; 25: 139–146.

63. Bosch-Barrera J, Sais E, Cañete N, Marruecos J, Cuyàs E, Izquierdo A, Porta R, Haro M, Brunet J, Pedraza S, Menendez JA. Response of brain metastasis from lung cancer patients to an oral nutraceutical product containing silibinin. Oncotarget. 2016; 7: 32006–32014.

64. Singh RP, Agarwal R. Cosmeceuticals and silibinin. Clin. Dermatol. 2009; 27(5); 479–484.

65. Kvasnicka F, Biba B, Sevcik R, Voldrich M, Kratka J. Analysis of the active components of silymarin. J. Chromatogr. A. 2003; 990: 239–245.

66 . Tedesco D, Tava A, Galletti S, Tameni M, Varisco G, Costa A, Steidler S. Effects of silymarin, a natural hepatoprotector, inperiparturient dairy cows. J. Dairy Sci. 2004; 87: 2239–2247.

67. Huang X, Wu Z, Gao W, Chen Q, Yu B. Polyamidoamine dendrimers as potential drug carriers for enhanced aqueous solubility and oral bioavailability of silybin. Drug Dev. Ind. Pharm. 2011; 37: 419–427.

68. Chávez G, Campos CH, Jiménez VA, Torres CC, Díaz C, Salas G, Guzmán L, Alderete JB. Polyamido amine (PAMAM)-grafted magnetic nanotubes as emerging platforms for the delivery and sustained release of silibinin. J. Mater. Sci. (2017); 52: 9269–9281.

69. Kim JB, Yu JH, Ko E, Lee KW, Song AK, Park SY, ShinI, HanW, Noh DY. The alkaloid berberine inhibits the growth of anoikis-resistant MCF-7 and MDA-MB-231breast cancer cell lines by inducing cell cycle arrest. Phytomedicine. 2010; 17: 436-440.

70. Chen W, Miao YQ, Fan DJ, Yang SS, Lin X, Meng LK. Bioavailability study of berberine and the enhancing effects of TPGS on intestinal absorption in rats. AAPS Pharm. Sci. Tech. 2011; 12: 705-711.

71. Gupta L, Sharma AK, Gothwal A, Khan MS,Khinchi MP, Qayum A, Singh SK, Gupta U. Dendrimer encapsulated and conjugated delivery of berberine: A novel approach mitigating toxicity and improving in vivo pharmacokinetics. Int. J. Pharm. 2017; 528: 88-99.

72. Pandith H, Zhang X, Thongpraditchote S, Wongkrajang Y, Gritsanapan W, Baek SJ. Effect of Siamweed extract and its bioactive component scutellarein tetramethyl ether on anti-inflammatory activity through NF-κB pathway. J. Ethnopharmacol. 2013; 147: 434–441.

73. Shadrack DM, Mubofu EB, Nyandoro SS. Synthesis of polyamidoamine dendrimer for encapsulating tetramethyl scutellarein for potential bioactivity enhancement. Int. J. Mol. Sci. 2015; 16: 26363–26377

74. Ikemoto S, Sugimura K, Yoshida N, Yasumoto R, Wada S, Yamamoto K, Kishimoto T. Antitumor effects of Scutellariae radix and its components baicalein, baicalin, and wogonin on bladder cancer cell lines. Urology. 2000; 55: 951–955.

75. Chen H, Gao Y, Wu J, Chen Y, Chen B, Hu J, Zhou J. Exploring therapeutic potentials of baicalin and its aglycone baicalein for hematological malignancies. Cancer Lett. 2014; 354: 5–11.

76. Li-Weber M. New therapeutic aspects of flavones: the anticancer properties of Scutellaria and its main active constituents Wogonin, Baicalein and Baicalin. Cancer Treat. Rev. 2009; 35: 57–68.

77. Chen Y, Minh Le V, Liu J, Angelov B, Drechsler M, Garamus VM, Willumeit-Romer R, Zou A, Baicalin loaded in folate-PEG modified liposomes for enhanced stability and tumor targeting. Colloids Surf. B: Biointerfaces. 2016; 140: 74–82.

78. Chen Y, Minh LV, Liu J, Angelov B, Drechsler M, Garamus VM, Willumeit-Römer R, Zou A. Baicalin loaded in folate-PEG modified liposomes for enhanced stability and tumor targeting. Colloids Surf. B Biointerfaces. 2016; 140: 74-82.

79. Pavan GM, Albertazzi L, Danani A. Ability to Adapt: Different Generations of PAMAM Dendrimers Show Different Behaviors in Binding siRNA. J. Phys. Chem. B. 2010; 114: 2667–2675.

80. Lv T, Yub T, Fang Y, Zhang S, Jiang M, Zhang H, Zhang Y, Li Z, Chena H, GaoY. Role of generation on folic acid-modified poly(amidoamine) dendrimers for targeted delivery of baicalin to cancer cells. Mater. Sci. Eng. C. 2017; 75: 182–190.

81. Silva MM, Santos MR, Caroço G, Rocha R, Justino G, Mira L. Structure-antioxidant activity relationships of flavonoids: a re-examination. Free Radic. Res. 2002; 36: 1219–27.

82. Hubbard GP, Wolffram S, Lovegrove JA, Gibbins JM. Ingestion of quercetin inhibits platelet aggregation and essential components of the collagen-stimulated platelet activation pathway in humans. J. Thromb. Haemost. 2004); 2: 2138–2145.

83. Beil W, Birkholz C, Sewing KF. Effects of flavonoids on parietal cell acid secretion, gastric mucosal prostaglandin production and Helicobacter pylori growth. Arzneimittelforschung. 1995; 45: 697–700.

84. Carluccio MA, Ancora MA, Massaro M, Carluccio M, Scoditti E, Distante A, Storelli C, De Caterina R. Homocysteineinduces VCAM-1 gene expression through NF-kappaB and NAD(P) Hoxidase activation: protective role of Mediterranean diet polyphenolic antioxidants. Am. J. Physiol. Heart Circ. Physiol. 2007; 293: H2344–2354.

85. Lee KM, Hwang MK, Lee DE, Lee KW, Lee HJ. Protective effect of quercetin against arsenite-induced COX-2 expression by targeting PI3K in rat liver epithelial cells. J. Agric. Food Chem. 2010; 58: 5815–5820.

86. Dajas F. Life or death: neuroprotective and anticancer effects ofquercetin. J. Ethnopharmacol. 2012; 143: 383–396.

87. Madaan K, Lather V, Pandita D. Evaluation of polyamidoamine dendrimers as potential carriers for quercetin, a versatile flavonoid. Drug Deliv. 2016; 23: 254-262.

88. Ruela-de-Sousa RR, Fuhler GM, Blom N, Ferreira CV, Aoyama H, Peppelenbosch MP. Cytotoxicity of apigenin on leukemia cell lines: implications for prevention and therapy. Cell Death Dis. [Internet]. 2010; 1: 1–11.

89. Chakravarthi S, Wen CF, Nagaraja HS. Apoptosis and expression of bcl-2 in cyclosporin induced renal damage and its reversal by beneficial effects of 4,5,7-Trihydroxyflavone. J. Anal. Bio-Sci. [Internet]. 2009; 32: 320–327.

90. Viola H, Wasowski C, Levi De Stein M, Wolfman C, Silveira R, Dajas F, Medina JH, Paladini AC. Apigenin, a component of Matricaria recutita flowers, is a central benzodiazepine receptors-ligand with anxiolytic effects. Planta Med. 1995; 61: 213–216.

91. Zbidah M, Lupescu A, Jilani K,Fajol A, Michael D, Qadri SM, Lang F. Apigenin-induced suicidal erythrocyte death. J. Agric. Food Chem. 2012; 60: 533–538.

92. Hawker CJ, Frechet JMJ. Preparation of polymers with controlled molecular architecture. A new convergent approach to dendritic macromolecules. J. Am. Chem. Soc. 1990; 112: 7638-7647.

93. Vinš P, Vermachová M, Drašar P, del Barrio M, Jarne C, Cebolla VL, de CózarA, Zangi R, Cossío FP. Size and branching effects on the fluorescence of benzylic dendrimers possessing one apigenin fluorophore at the core. Tetrahedron. 2013; 69: 10361-10368.

94. Sun M-Y, Ye Y, Xiao L, Rahman K, Xia W, Zhang H. Daidzein: A review of pharmacological effects. Afr. J. Tradit. Complement. Altern. Med. 2016; 13: 117.

95. Ahmed T, Javed S, Tariq A, Budzyńska B, D'Onofrio G, Daglia M, Nabavi SF, Nabavi SM. Daidzein and its effects on brain. Curr. Med. Chem. 2017; 24: 365-375.

96. Xavier CR, Silva APC, Schwingel LC, Borghetti GS, Koester BL, Mayorga P, Teixeira HF, Bassani VL. Improvement of genistein content in solid genistein/beta-cylcodextrin complexes. Quim. Nova. 2010; 33: 587–590.

97. Stancanelli R, Mazzaglia A, Tomamasini S, Calabro ML, Villari V. The enhancement of isoflavones water solubility by complexation with modified cyclodextrins: a spectroscopic investigation with implications in the pharmaceutical analysis. J. Pharm. Biomed. Anal. 2007; 44: 980–984.

98. Lee SH, Kim YH, Yu HJ, Cho NS, Kim TH, Kim DC, Chung CB, Hwang YI, Kim KH. Enhanced bioavailability of soy isoflavones by complexation with beta-cyclodextrin in rats. Biosci. Biotechnol. Biochem. 2007; 71: 2927–2933.

99. Zhao C, Wang Y, Su Y, Zhang H, Ding L, Yan X, Zhao D, Shao N, Ye X, Cheng Y. Inclusion complexes of isoflavones with two commercially available dendrimers: Solubility, stability, structures, release behaviors, cytotoxicity, and anti-oxidantactivities. Int. J. Pharm. 2011; 421: 301-309.

100. Makris DP, Rossiter JT. Hydroxyl free radical-mediated oxidative degradation of quercetin and morin: a preliminary investigation. J. Food Comp. Anal. 2002; 15: 103–113.

101. Choi BC, Choi JS, Han HK. Altered pharmacokinetics of paclitaxel by the concomitant use of morin in rats. Int. J. Pharm. [Internet]. (2006); 323(1-2); 81–85.

102. Noor H, Cao P, Raleigh DP. Morin hydrate inhibits amyloid formation by islet amyloid polypeptide and disaggregates amyloid fibers. Protein Sci. 2012; 21: 373–382.

103. Zhang H, Cao J, Wang Y. Spectroscopic and molecular modeling studies of the interaction between morin andpolyamidoamine dendrimer. Luminescence. 2014; 29: 573–578.