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# Feature

## Interactions between modified fullerenes and proteins in cancer nanotechnology

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**Fullerenes have numerous properties that fill the gap between small molecules and nanomaterials. Several types of chemical reaction allow their surface to be ornamented with functional groups designed to change them into 'ideal' nanodelivery systems. Improved stability, and bioavailability are important, but chemical modifications can render them practically soluble in water. 'Buckyball' fullerene scaffolds can interact with many biological targets and inhibit several proteins essential for tumorigeneses. Herein, we focus on the inhibitory properties of fullerene nanomaterials against essential proteins in cancer nanotechnology, as well as the use of dedicated proteins to improve the bioavailability of these promising nanomaterials.**

**Keywords: fullerene; protein interactions; protein corona; enzyme inhibitors; cancer nanotechnology; nanomedicine**



### Introduction

Development of the derivatization reactions of fullerenes heralded a new era in medicinal chemistry. Specific functionalization allows greater effectiveness in creating designed fullerene nanomaterials (DFNs) with excellent water solubility, biocompatibility, and potential for applications as nanodelivery systems for drugs.[1] The three main types of modification of buckyballs can be classified as: the formation of hydroxyfullerenes; Bingel–Hirsch cyclopropanations forming mono- and hexakisadducts; and Prato reaction products (i.e., pyrrolidinofullerenes).[2]

Several interactions of DFNs with biomolecules have been described and verified *in vitro* and *in vivo*. For example, the interactions of buckyballs with nucleic acids (DNA and several types of RNA) rely on the formation of complexes between positively charged fullerenes and negatively charged phosphonic groups, and have led to applications in transfection protocols studied in rodent models.[3] The specific interactions of fullerene nanomaterials with proteins were observed in early biomedical studies (using HIV-1 protease,[4] human serum albumin,[5] and lysozymes[6] as the main targets). An extraordinary proof of concept for interactions was the discovery of a specific antifullerene monoclonal antibody.[7] More importantly, it allowed for extended investigation of the cellular fate, and the targets of those nanoparticles (NPs) opened a route to a more direct approach. Interestingly, current reports reveal several diverse applications of fullerene derivatives in nanomedicine, which can be categorized as: (i) photodynamic therapy of cancer/photoinactivation of microbes; (ii) small molecule/nucleic acid delivery; (iv)


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antioxidants/neurodegenerative disease; and (iv) MRI/PET contrast agents.[8–14] However, no drug candidate based on a fullerene scaffold has entered clinical trials, apart from dermatological studies to test the anti-wrinkle property of fullerene-$C_{60}$ in humans.[15]

One of the biggest challenges is to understand the nature of interactions between DFNs and proteins. Complex interactions between fullerenes and proteins can be categorized into three main subgroups: π–π stacking (between fullerene $sp^2$ carbons and aromatic residues of proteins); van der Waals (between the $C_{60}$ cage and protein surface); and hydrophobic (i.e., nonpolar solvation).[16] Interestingly, the larger structure of $C_{70}$ fullerene enables stronger interactions with more amino acids, which create its binding pocket.[17] Calvaresi and coworkers designed an algorithm that quantitatively investigates the interaction of $C_{60}$ and the surface of each protein from the desired test set from the Protein Database (PDB), which led to identification of new protein targets that could interact with $C_{60}$.[18]

Here, we focus on the interactions between DFNs and proteins from the perspective of cancer nanotechnology. The interactions of fullerene derivatives with DNA/RNA are based on cationic complex formation (i.e., aminofullerenes–anionic nucleic acids) and, hence, are not discussed further here.[11] A more detailed target-related approach to interactions is necessary because many reports have shown only the cytotoxic effect of fullerene nanomaterials. In addition, determining the exact protein targets and mechanisms of action has not yet been described in detail. It has been postulated that the biological effects of DFNs are dependent upon the interaction between fullerene and the protein upon administration and their intratumoral levels (Figure 1). DFNs can be incorporated with proteins (e.g., lysozymes and/or albumin) *ex vivo* before administration to reach the desired solubility. Alternatively, DFNs can interact with serum proteins so that a protein corona is formed around the fullerene NPs. Each mechanism can lead to a different (or identical) interaction with cancer-related proteins. Moreover, direct inhibition of cancer-related enzymes is also possible. These possibilities highlight several questions: how is the mechanism of action of DFNs related to the delivery method? Is it possible to have protein-unbound DFNs in tissues? Which protein-based delivery results in optimal anticancer effects?

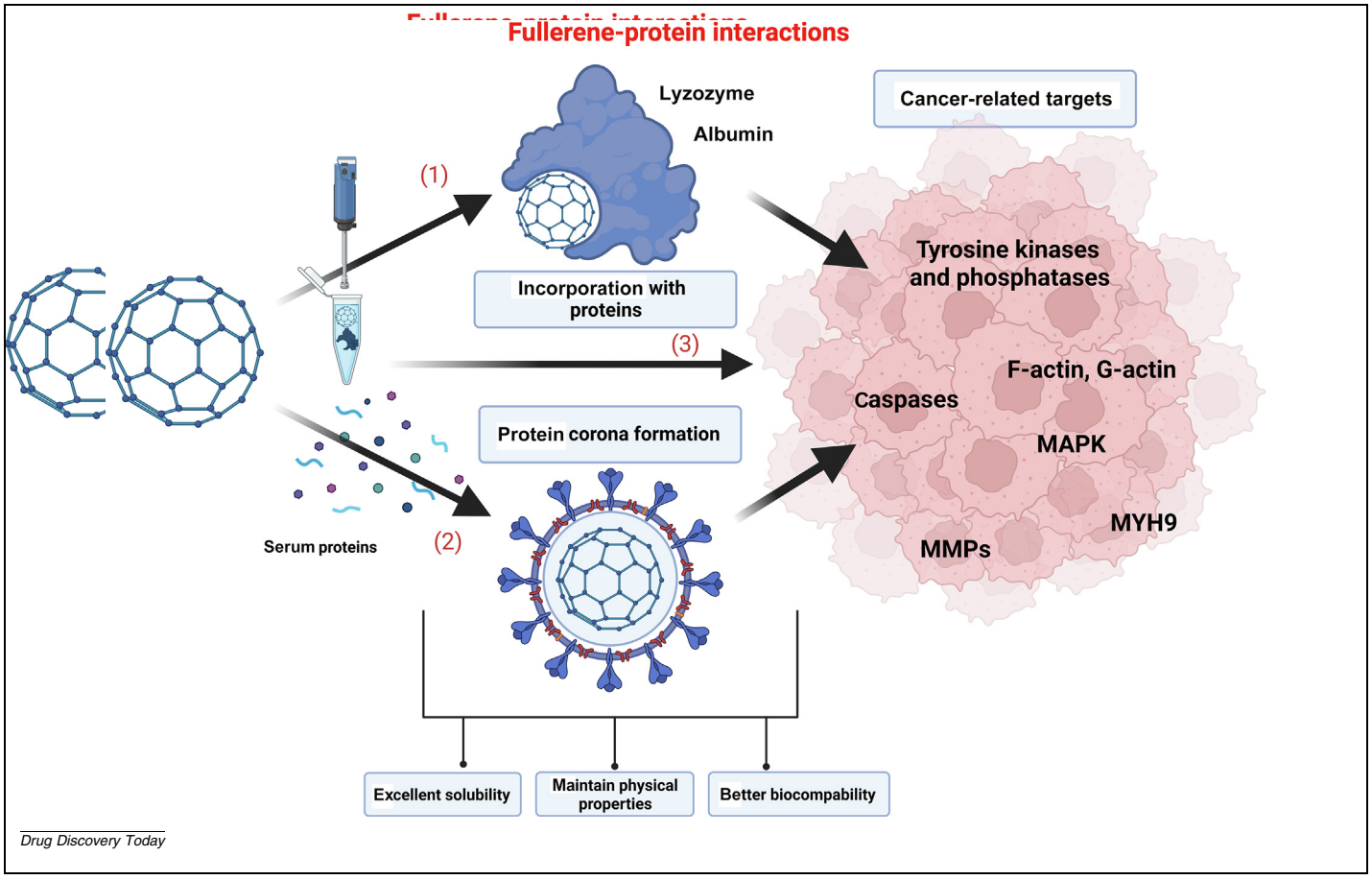


**FIGURE 1**

Schematic of fullerene–protein interactions. Possible interactions of fullerene nanomaterials with proteins: (1) solubilization through interaction between a designed fullerene nanomaterial (DFN) and protein (lysozyme or albumin); (2) formation of a protein corona adsorbed on the surface of buckyballs as a result of interactions with serum proteins; and (3) direct interaction between the DFN and cancer-related proteins. Created with BioRender (BioRender.com). Abbreviations: MAP, mitogen-activated protein kinase; MMP, matrix metalloproteinase; MYH9, myosin heavy chain 9.

### Complexes between fullerenes and proteins

The interactions of fullerenes with peptides can be viewed from the perspective of creating a specific complex between a fullerene cage and a specific fragment of a protein (pockets, gaps, or even crevices), which can bind fullerene derivatives of different sizes and shapes. This can be a controlled or uncontrolled process. In a controlled process, selected proteins (albumin or lysozymes) are used to dissolve the carbon nanomaterial in an aqueous environment, resulting in a fullerene–protein complex with improved pharmacological features. Conversely, if modified fullerene nanomaterials are administered to cell cultures or directly to a living organism, a protein corona (hard or soft) is formed, the composition of which cannot be fully predicted.

Pristine fullerenes are highly hydrophobic in that their bulk form is almost insoluble in biologically relevant environments. Derivatization is not always helpful in overcoming this problem, but it can increase protein-binding properties considerably. Calvaresi and colleagues described a method for improving fullerene solubility by forming complexes with selected proteins (mainly serum albumin and lysozymes) and these complexes were used as a novel type of photosensitizer in anti-cancer therapies.[19] The fullerene scaffold interact with proteins via guest–host interactions whereby the binding pocket of the protein binds the hydrophobic fullerene scaffold via interactions (π–π stacking, hydrophobic, surfactant-like, or charge–π). In general, the advantage of this bioconjugation approach is the monodispersed nature of the formed complex, which prevents deactivation of excited electronic states by surrounding fullerene particles.[16] Their study with lysozymes demonstrated that fullerene particles interacted with lysozymes in a 1:1 ratio, which prevents the formation of undesirable aggregates (Figure 2).[20] Additionally, Figure 3 provides a schematic of the interactions between fullerenes and proteins.

The affinity of a fullerene for a protein is the driving force for most of the activities mentioned above. The formation of a protein corona is not a new concept in NPs but, in the case of fullerenes, cannot be controlled and designed via specific chemical functionalization. In biological environments (e.g., extracellular liquids or blood), NPs make contact with various proteins to form a corona on their surface.[21] Notably, this is not restricted to animal models, and permits investigation in simplified *in vitro* environments. The corona determines the biological activity of the engineered nanomaterial and, thus, influences its associated biological properties. In addition, formation of a protein corona can influence cellular uptake of the DFN. For instance, internalization of the fluorescently labeled fullerene nanomaterial $C_{60}$serPF was sensitive to various inhibitors, which suggested multiple pathways of uptake (clathrin-mediated endocytosis, caveolae-mediated endocytosis, and micropinocytosis).[22]

In-depth analysis of the literature revealed detailed proteomic studies of protein coronas for various carbon nanomaterials, including carbon nanotubes and graphene oxide.[23,24] However, proteomic data describing the detailed composition of hard and soft protein coronas in the case of fullerene nanomaterials are lacking. Recently, Wu and coworkers described physicochemical studies of the formation of protein coronas on fullerene nanocomplexes, which induced further aggregation of nanocomplexes and demonstrated that the secondary structure of the studied proteins changed after binding to a $C_{60}$-nanocomplex.[25] Formation of a protein corona on the surface of glycofullerenes also changed the inhibitory properties of fullerene nanomaterials against non-receptor tyrosine kinases.[26]

The development of synthetic methods has led to a significant development in the preparation of water-soluble fullerene nanomaterials, which can be generally classified as hydroxyfullerenes, (methano)-fullerene acids, and aminofullerenes. The structures of most common anti-cancer fullerenes are depicted in Figure 4 and a tabular summary of the molecular targets for each fullerene class is provided in Table 1.

### Enzyme inhibition by fullerene nanomaterials

#### *Tyrosine kinases and phosphatases*

Tyrosine kinases and phosphatases are often overexpressed in cancer, and enable the progression and survival of cancer cells.[27] Thus, receptor tyrosine kinases have been major targets for cancer specific drugs for more than two decades. However, such a treatment often is associated with tumor resistance and relapse.[28] The first suggestion of direct inhibition of tyro-



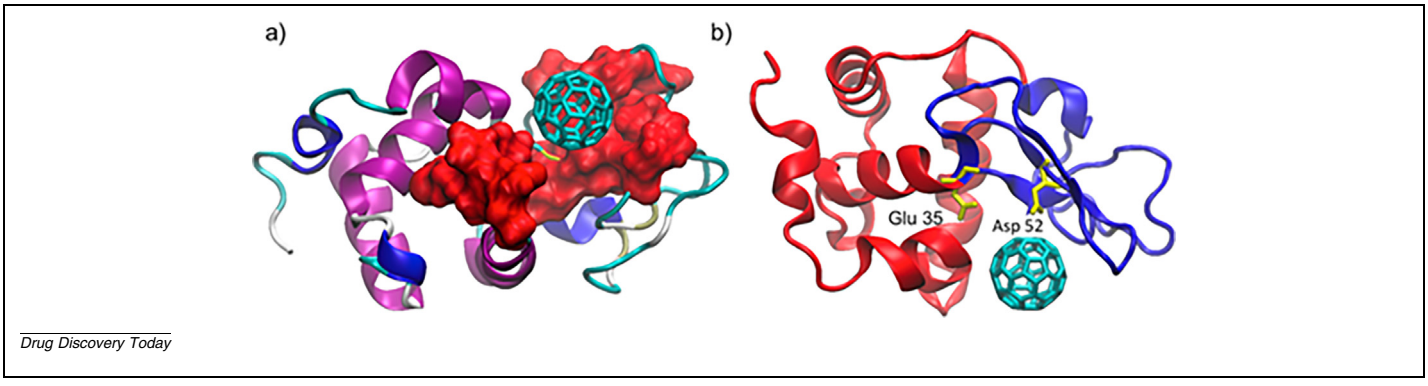


**FIGURE 2**

Identification of the $C_{60}$ binding pocket. **(a)** Docking of $C_{60}$ in the lysozyme structure; the red area corresponds to the residues undergoing the largest chemical shift changes according to nuclear magnetic resonance (NMR) measurements; **(b)** lysozyme R (red) and β domains (blue). The active site residues (Glu35 and Asp 52), crucial for the catalytic activity of the enzyme, are shown in yellow. Reproduced, with permission, from[20].

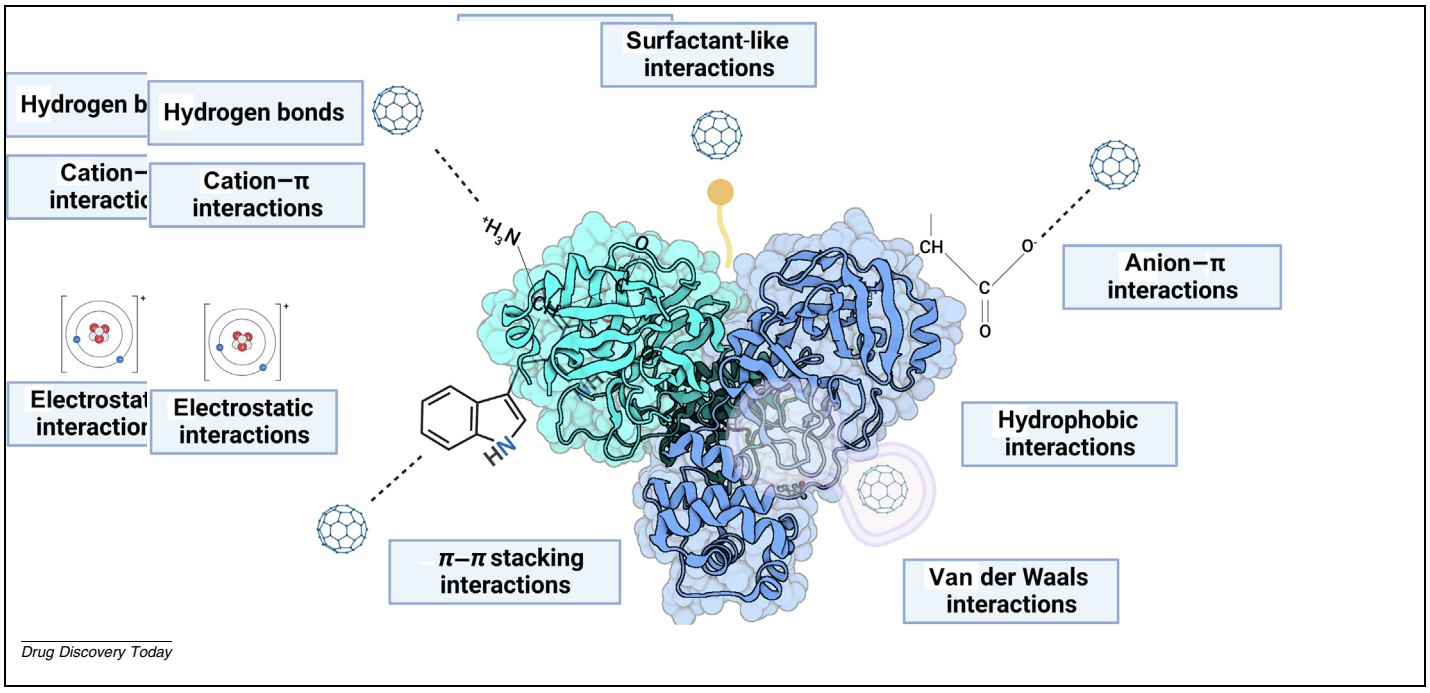


**FIGURE 3**
Schematic of the different types of interaction between fullerenes and proteins. Created with BioRender (BioRender.com).

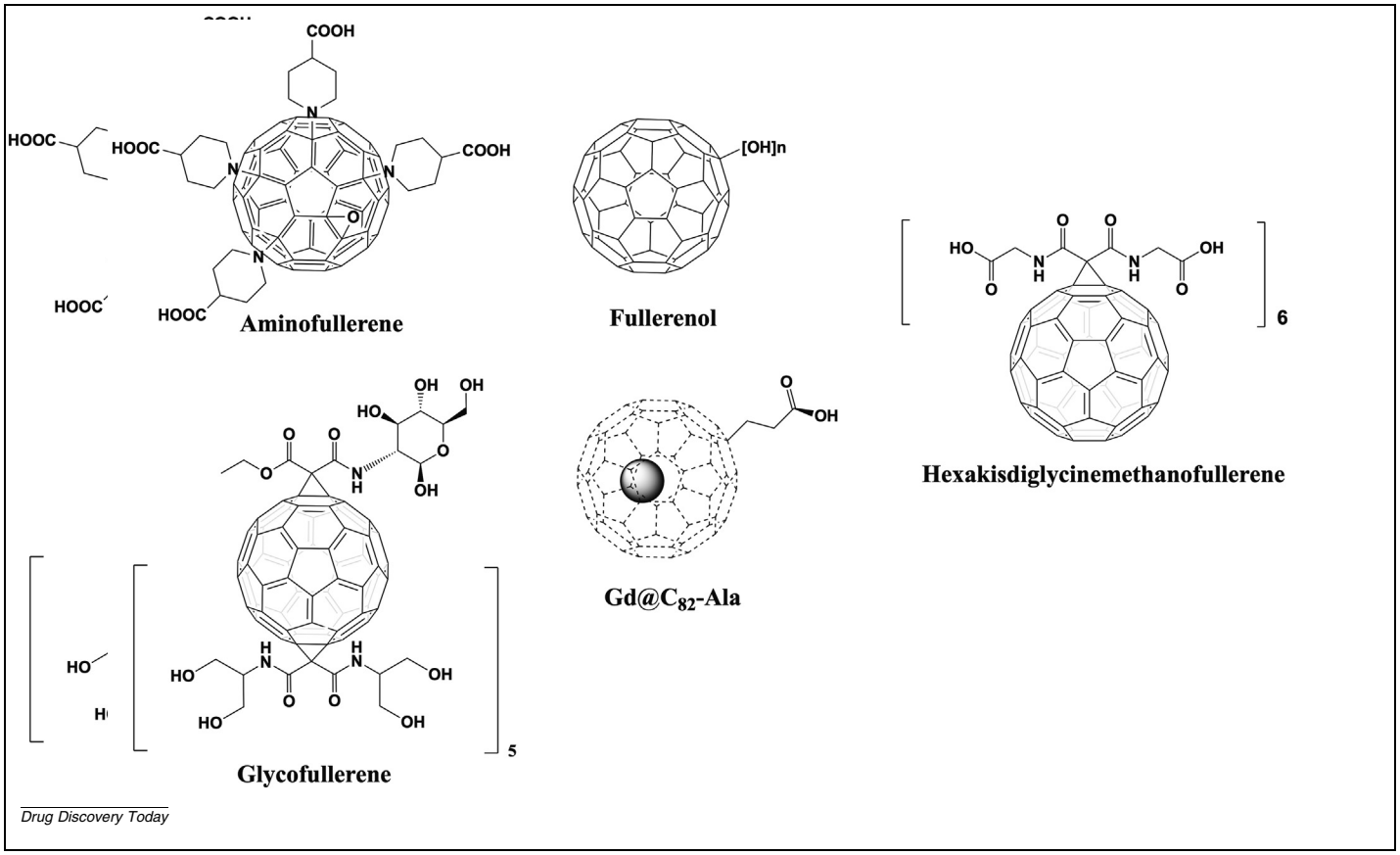


**FIGURE 4**
Structures of common anti-cancer fullerene nanomaterials.

**TABLE 1**

**Examples of protein targets inhibited by fullerene nanomaterials.**

| Fullerene class | Therapeutic agent | Target | Refs |
|---|---|---|---|
| Aminofullerenes | $C_{70}$-EDA | MYH9 | 36 |
| | Tetra[4-(amino)piperidin-1-yl]$C_{60}$ epoxide TAPC4 | Hsp90β, MYH9 | 37 |
| | $C_{60}(NH_2)_{30}$ | CD45 | 44 |
| | Pyridinium derivatives | Caspase-3/7 | 39 |
| | *Bis*-pyridinium fullerene | MEK-ERK | 33,34 |
| | 1,10,10-tetramethyl [60]fullerenodipyrrolidinium diiodide | Caspase-9 | 40 |
| Fullerenols | $C_{60}(OH)_{30}$ | CD45 | 44 |
| | $Na_4[C_{60}(OH)_{30}]$ | PTP1B | 30 |
| | [60/70]fullerenols | F-actin and G-actin | 52,54 |
| | Glycofullerenes | FynA, BTK proteins | 26 |
| Gadofullerenes | $Gd@C_{82}(OH)_{22}$ | MMP-2, MMP-9 | 50,51 |
| Carboxyfullerenes | Glycine-derived fullerene | BTK proteins | 32 |
| | Fulleropyrrolidine derivatives | CD45 | 30,43 |
| Pristine $C_{60}$ | Oral fullerene tablets | p53, NF-κB, STAT3 | 47 |
| Miscellaneous | Miscellaneous | p38- and ERK-MAPK, p65 protein | 46 |

sine kinases by DFNs was made in 1998, but relevant research has since been abandoned. In seminal work, Lu and coworkers observed that polyhydroxylated fullerene (considered to be a trapper of free radicals) exerted antiproliferative activity through cytosolic protein kinase.[29] Later, enzymatic studies by Kobzar and colleagues suggested that DFNs be considered a new class of inhibitors of protein tyrosine phosphatases (PTPs).[30] The evaluated compounds were potent inhibitors of cluster of differentiation (CD)45, showing a half-maximal inhibitory concentration in the high-nanomolar range and with inhibitory activity against PTP1B and other phosphatases. A study on Jurkat cells by Ritter and coworkers revealed that inhibition of phosphorylation of protein tyrosine could be one of the photocytotoxic effects of $C_{60}$.[31] Discovery of selective inhibitors of tyrosine kinases provided several efficacious small molecules for clinical practice and revolutionized the pharmaceutical market. However, inhibitors of these enzymes at the nanometer level have been reported only sporadically. Serda and colleagues described the inhibitory activity of sugar derivatives of glycofullerenes to be similar to that of non-receptor kinase inhibitors that target proto-oncogene tyrosine-protein kinase Fyn (Fyn A) and Bruton's tyrosine kinase (BTK).[26] Interestingly, formation of a fullerene–protein corona was an important factor in inhibition and changing the selectivity of the fullerene. Synthesized DFNs were found to be nontoxic against healthy cells, inducing autophagy and disrupting the redox balance in pancreatic cancer cells. Computational studies on glycine-derived fullerene (HDGF) demonstrated that HDGF could bind the active site of BTK and interact directly with Cys 481, Arg 525, and Tyr 551, residues that are crucial for the activity of BTK.[32] Furthermore, Sumi and collaborators demonstrated that a *bis*-pyridinium fullerene derivative induced the apoptosis of human chronic myelogenous leukemia-derived (K562) cells via downregulation of expression of breakpoint cluster region protein (BCR-ABL) proteins and T315I-mutated BCR-ABL in a reactive oxygen species (ROS)-dependent manner.[33,34]

### *Myosin heavy chain 9*

Myosin heavy chain 9 (MYH9) is a cytoplasmic protein that controls epithelial–mesenchymal transition (EMT) and cell motility. A meta-analysis of large-scale clinical trials showed MYH9 to be overexpressed in various malignancies and to correlate with a poor prognosis. Interestingly, MYH9 can regulate the phenotypes of cancer stem cells, worsening tumor prognosis.[35] In addition, downregulation of MYH9 can be associated with decreased expression of proteins crucial for cancer development, such as Snail, Vimentin, E-Cadherin, SOX, CD44, and OCT4. Recent studies on an ethylenediamine (EDA) derivative of $C_{70}$ have referred to its antineoplastic properties and further impact on metastasis.[36] Those studies demonstrated that $C_{70}$-EDA binds to the C-terminal part of MYH9. After cell uptake, $C_{70}$-EDA accumulates in lysosomes and mitochondria, and binds cytoplasmatic MYH9. Blockade of the C terminus prevents protein transport to the cell edge, which indicates that this aminofullerene impacts the cellular distribution of MYH9, but not its protein expression. Huo and coworkers revealed that a synthesized aminofullerene targeted MYH9 and HSP90 directly.[37] They investigated the anti-cancer mechanism of action of their DFN, which revealed that it inhibited expression of cyclin D1, which led to arrest of the cell cycle in G0/G1. The synthesized aminofullerene achieved high efficacy *in vivo*, inhibiting the proliferation and metastasis of melanoma cells.

### *Caspases*

Caspases are specific proteases with a crucial part in programmed cell death. Therefore, modulation/inhibition of their activity could be important for therapy of cancer and neurodegenerative diseases. However, caspases can also regulate cellular proliferation and genomic instability. This broad spectrum of actions highlights the risks of insufficient modulation of caspases and possibility of drug resistance development.[38] Yasuno and colleagues synthesized a series of novel pyridine derivatives of $C_{60}$-fullerene. Examination of their effect on leukemic (HL60) cells revealed that such derivatives had strong antiproliferative effects, including drug-resistant variants.[39] Interestingly, this DFN activated caspase-3 and caspase-7, causing condensation of nuclear chromatin and inducing apoptosis. Other studies of cationic pyrrolidinium fullerenes

reported apoptosis induction resulting from activation of caspase-9 via suppression of protein kinase B in primary effusion lymphoma (PEL) cells.[40]

### *Tyrosine phosphatase*

PTPs have a vital role in the enzymatic dephosphorylation of tyrosine residues in proteins that have undergone phosphorylation, thereby exerting an influence on signaling transduction similar to that of protein tyrosine kinases (PTKs).[41] Tyrosine phosphorylation is controlled by PTPs, which can function as tumor suppressors.[42] Using enzymatic assays, several water-soluble fullerene derivatives have been identified as PTP (e.g., CD45, PTP1B, TC-PTP, and SHP2) inhibitors, and their inhibition mode studied using molecular docking.[30,43,44] Molecular-docking calculations of the pristine $C_{60}$ scaffold and hydroxyfullerene derivatives showed them to be docked into the space between the D1 and D2 domains of CD45.

### *p38, mitogen-activated protein kinase and nuclear factor-κB pathways*

Mitogen-activated protein kinase (MAPK) has important oncogenic roles in a broad spectrum of tumors (including increase of cancer proliferation, survival, and metastasis), and is associated with poor response to treatment. Despite the high impact of MAPK activation, results from clinical trials have not been promising because of the presence of alternative pathways and feedback loops.[45] Nuclear factor (NF-κB) has been identified as one of the links between resistance therapy pathways and anti-cancer treatment failure. Various strategies to inhibit NF-κB have been applied without poor success, possibly beause of multiple upstream and downstream effectors. The hydroxyfullerene $C_{60}(OH)_{22}$ has been reported to influence the metastasis of murine breast cancer (4 T1) cells.[46] This DFN inhibits the EMT by blocking cytokine release and impacting the p38 and MAPK signaling pathways. $C_{60}(OH)_{22}$ blocks the p38 and extracellular signal-regulated kinase (ERK)–MAPK signaling pathways and phosphorylation of p65 protein, which is necessary for activating NF-κB in malignant brown adipose-derived stem cells. However, in normal cells, it influences only the p38–MAPK signaling pathway. Interestingly, oral fullerene tablets can act directly on, and reduce the inflammatory state at, colorectal tumor sites in mice. This newly designed DFN scavenged ROS, prevented mutations of wild-type p53, and inhibited activation of the NF-κB pathway.[47]

### *Matrix metalloproteinases*

Matrix metalloproteinases (MMPs) are zinc-dependent endopeptidases responsible for degradation of extracellular matrix (ECM) proteins (i.e., collagen and laminin). High expression of MMPs in tumor cells aid ECM remodeling and release of membrane-bound growth factors to create the microenvironment for tumorigenesis.[48] Three strategies are popular for MMP activity modulation in cancer: suppression of MMPs translation and/or transcription; inhibition of MMPs enzymatic activity; and blocking of MMPs activation. Despite much research, small molecular and very selective MMP inhibitors are difficult to identify.[49] Gadofullerene ($Gd@C_{82}$) is a type of endohedral DFN that contains a gadolinium atom enclosed in a cage comprising 82 carbon atoms. The hydroxylated version of gadofullerene, $Gd@C_{82}(OH)_{22}$, in the form of NPs has been evaluated as an antineoplastic agent that activates the immune system, remodels the ECM, and influences angiogenesis.[50,51] The main targets for inhibition are MMP-2 and MMP-9 via allosteric modulation exclusively at the ligand site.

### *F-actin and G-actin proteins*

Actin is involved in cytoskeleton formation, cell signaling, cytokinesis, and cell motility. Qin *et al.* reported that fullerenols interfere with the dynamic assembly of actin to inhibit the invasion and migration of cancer cells.[52] One crucial step would be to block the EMT in cancer cells. This transition can be regulated by actin-binding proteins (e.g., CFL1 and SATB1).[53] In a mouse model, a DFN obstructed the spread of breast cancer cells through blood vessels and their ability to establish new colonies in the lung. The reduced capacity of treated cells to adhere to surfaces might result from interference by fullerenols with rearrangement of the actin cytoskeleton and modified intracellular distribution of integrin. This action is achieved as a result of modification of the equilibrium of F-actin and G-actin and remodeling of the actin cytoskeleton.

In addition, hydroxyfullerenes reduced the number of actin fibers as well as the number and length of filopodia. These results show that fullerenes can inhibit the migration and invasiveness of cancer cells by remodeling the actin skeleton of these cells and changing the intracellular distribution of integrins. Other work by Qin *et al.* revealed that fullerenols bind directly to the F-actin surface, thereby inhibiting bundling and relevant cell behaviors.[54]

### *Receptors*

In the case of receptor–fullerene interactions, the amount of published data available is significantly lower compared with that relating to enzymes. In work by Ren *et al.*,[55] possible binding sites and modes of action of fullerene derivatives to β2 adrenergic receptors were presented. However, no results from experiments on isolated receptors or larger systems were provided. The potential interactions of fullerenes with potassium channels have also been explored.[56,57] Interestingly, Kraszewski *et al.* demonstrated that pristine fullerenes or their aggregates can interact with both the extracellular and transmembrane regions of proteins.[57] Furthermore, studies by Calvarezi *et al.* indicated that there might be multiple binding sites for fullerenes on channel proteins.[58] From the perspective of this review, studies of the interaction of fullerenes with toll-like receptors (TLRs) appear to be more significant. Such interactions have been observed for other carbon nanostructures, such as nanotubes, as well as for fullerene derivatives.[59,60] TLRs are a group of receptors associated with innate immunity.[61] Their activity is crucial for nonspecific defense mechanisms against bacterial and viral threats.[62] However, there have been reports suggesting that TLRs and the associated activation of inflammatory states also have a role in tumor development[63] and that blocking them could be an effective anticancer therapeutic approach.[64]

## Concluding remarks

Fullerene NPs are biocompatible and have low toxicity. Their stability and easy functionalization make them ideal vehicles for drug delivery. Increasing numbers of studies have shown that DFNs can interact with various proteins. Importantly, these interactions can involve adsorption onto

the protein surface, insertion into the hydrophobic pockets of proteins, and modulation of protein conformation. However, crucial features must be established. Theoretical and experimental studies should be undertaken to ascertain the composition of fullerene–protein coronas. Molecular-docking approaches could be used to find other proteins for fullerene solubilization. The mechanism of inhibition must be determined each time an interaction with a specific protein is observed. Regardless of the increasing number of reports on enzyme inhibition, whether a DFN can interact directly with active centers competing with substrates remains unknown. The interaction of fullerenes with proteins is an exciting area of research with potential applications in drug delivery and other fields. However, more research is needed to fully understand the benefits and risks of these interactions, and to develop safe and efficacious fullerene-based technologies.

### Declaration of interest

The authors declare no conflict of interest.

## Data availability

No data was used for the research described in the article.

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