Fullerenes in Medicinal Chemistry and their Biological Applications

N. Tagmatarchis* and H. Shinohara

Nagoya University, Chemistry Department, Nagoya 464-8602, Japan

Abstract: The isolation and preparation ten years ago of the fullerenes in bulk quantities, sparked off a truly remarkable interdisciplinary research activity, encompassing diverse fields of chemistry, physics, materials science and medicinal chemistry. Pharmaceutical and biological studies have revealed that fullerene-based compounds exhibit various types of biological activity either *in vitro* or *in vivo* and are discussed in this review.

INTRODUCTION

The discovery of fullerenes by Kroto *et al*. in 1985 [1] and their synthesis in the bulk by Kraetschmer *et al.* [2, 3] five years later, sparked off a truly remarkable interdisciplinary research activity. The unique structure of fullerenes, all-carbon closed-cage molecules, has attracted considerable attention to diverse scientific fields ranging from materials science to medicinal chemistry. In this review, we will discuss the applications of fullerenes and their derivatives in medicinal chemistry and biology. However, some of their general physical and chemical properties should be addressed in the beginning as they shed light to understand already established biological applications and predict new ones.

SOLUBILITY

The solubility of fullerenes in polar solvents is very limited [4, 5]. The large number of carbons and the hydrophobicity of the closed structures are the main reasons for the absence of solubility in water and/or other polar media. To overcome this disadvantage several methods have been considered: (i) organic functionalisation of the carbon cage allows the synthesis of numerous fullerene derivatives with better solubility in physiological media (e.g. fullerenol, a polyhydroxylated fullerene with an excellent solubility in water [6]) – as a trend, the higher the number of the polar groups added, the greater the water solubility of the fullerene derivative [7-14], (ii) suspension and/or aggregation mixtures of fullerenes [15] (e.g. by adding small volumes of water/tetrahydrofuran into saturated aromatic solutions of fullerenes [16]) and (iii) incorporation of fullerenes into water soluble super- and supra-structures (e.g. calixarenes, surfactants, cyclodextrines) [16-24]. Therefore, with the above already established techniques, the applications of fullerenes in medicinal chemistry have been significantly increased accordingly with their solubility in physiological media.

*Address correspondence to this author at the Nagoya University, Chemistry Department, Nagoya 464-8602, Japan; Tel: ++81-52-7893660; Fax: ++81-52-7891169; E-mail: nikos@nano.chem.nagoya-u.ac.jp

UV-VISIBLE ABSORPTION

The band-gap energy between the highest occupied molecular orbital (HOMO) and the lowest unoccupied molecular orbital (LUMO) for the pristine [60] fullerene is only 1.8 eV [25, 26]. This is reflected to its UV-Vis absorption spectrum where the onset of the spectrum – typically a measure of the HOMO-LUMO energy band-gap of fullerenes – lies below 700 nm wavelengths.

Upon UV irradiation the first excited singlet states of fullerenes are formed [27-30]. Intersystem crossing (ISC) to the triplet states is the major decay channel for the deactivation of the excited singlet states with very high quantum yields for C_{60} and C_{70} [31]. The so-formed triplet states can efficiently be quenched by molecular oxygen to generate large amounts of singlet oxygen [27-30]. The sensitization of singlet oxygen by fullerenes strongly relates them with some physiologically important oxidative damages as well as photodynamic damages of biological systems.

DEOXYRIBONUCLEIC ACID (DNA) CLEAVAGE

Supercoiled pBR322 DNA was cleaved, when incubated under UV light irradiation but not in dark, by the water-soluble fullerene derivatives **1** and **2** [32]. Singlet oxygen modifies guanosines by either [4+2] or [2+2] cycloadditions to the five-membered imidazole ring of the purine base. This modification enhances the alkaline hydrolysis rate of the phosphate diester bond in DNA. Consequently, as a response of DNA cleavage, cytotoxicity of the fullerene species was occurred. This was the case for derivative **1** as confirmed with *in vitro* experiments on tumoral HeLa S3 cells. As a conclusion, the above results clearly indicate that photo-excited C_{60} strongly interacts with living cells either directly or through some chemical mediator.

Oligonucleotides can be used to target single-stranded nucleic acids such as mRNA or even double-helical DNA. Their binding can block translation of the target mRNA (*antisense strategy*) or transcription of the targeted gene by triple helix formation (*antigene strategy*). Consequenly and most importantly, attachment of reactive species to oligonucleotides can potentially induce irreversible reactions

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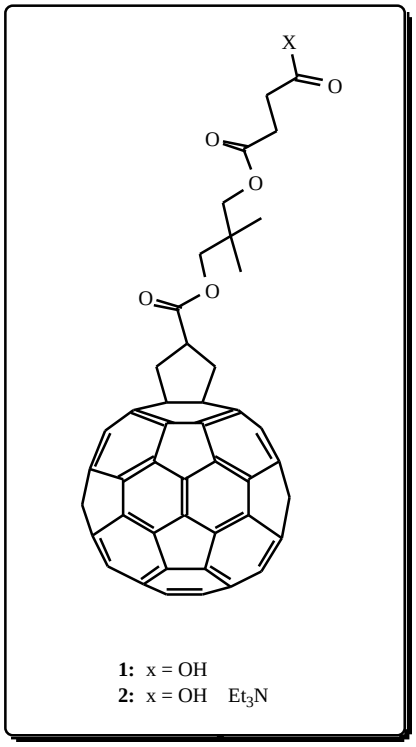
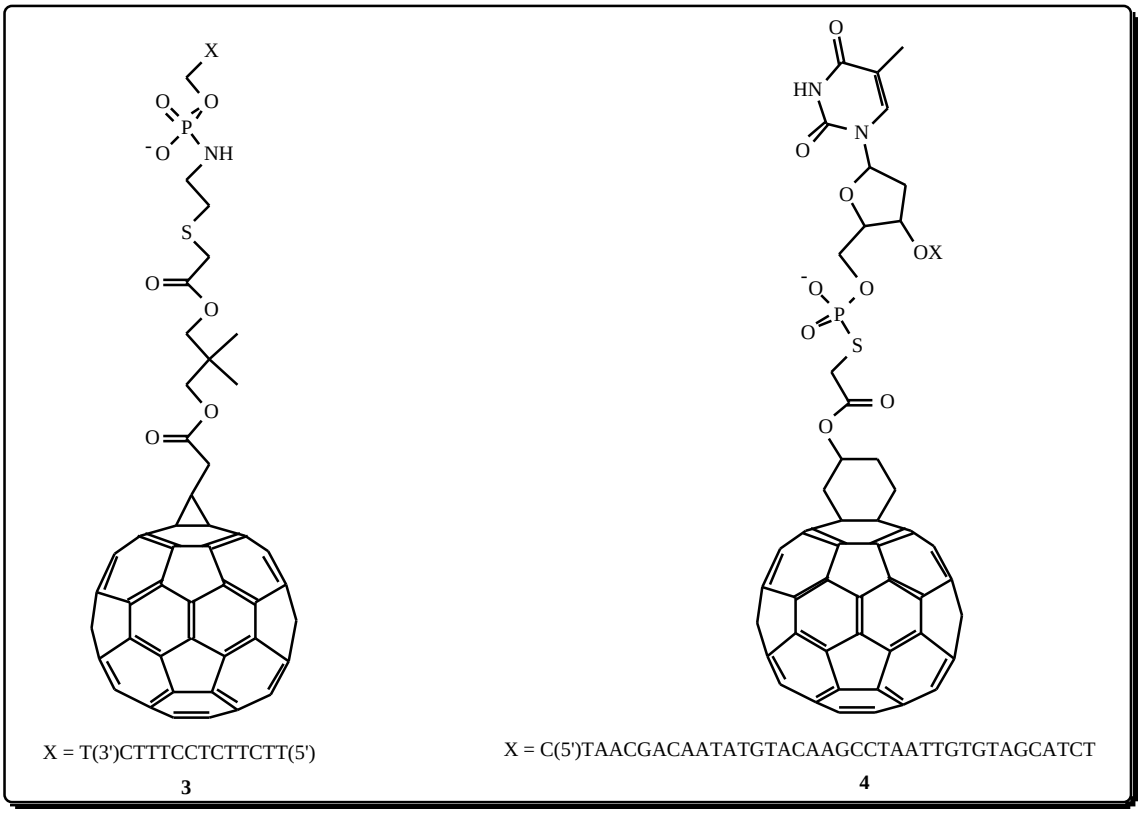


1: x = OH
2: x = OH Et_3N

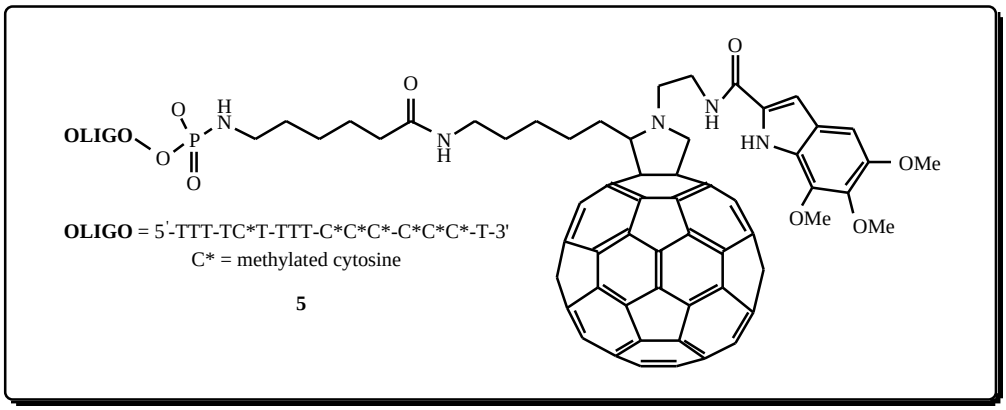
in the target sequence [33, 34]. Hybrid fullerene-DNA oligonucleotides **3** and **4** were synthesized and found to selectively cleave a 182 base-pair fragment at guanine residues. Moreover, hybrid **3** was shown not only to cleave DNA at guanine residues near the fullerene moiety of the hybrid but also to bind single- or double-stranded DNA or even other forms [35, 36]. These results indicated that hybrids fullerene-DNA oligonucleotides could be suitable photoprobes for the investigation of gene transcription and mRNA translation. The strong oxidative properties of the so-formed singlet oxygen were considered in the first place to be responsible for the exhibited damages of these fullerene derivatives. However, the possible intermediacy of singlet oxygen was examined for compound **4** by comparing its reactivity with a known hybrid that sensitizes the formation of singlet oxygen, namely eosin-oligonucleotide [36]. Since singlet oxygen has longer lifetime in deuterated environments [37], the experiment was performed in D_2O instead of H_2O and indeed eosin-oligonucleotide cleavage was found to be more efficient, presumably because of the longer singlet oxygen life time and more importantly consistent with the `singlet oxygen mechanism`. In sharp contrast, the fullerene-deoxyoligonucleotide hybrid exhibited the same rate of cleavage of DNA in both solvents, D_2O and H_2O. Moreover, sodium azide (a singlet oxygen quencher) was found to inhibit the eosin-oligonucleotide DNA cleavage but not that of fullerene-oligonucleotide. These findings clearly suggest that in the light-induced fullerene-oligonucleotide cleavage, singlet oxygen is not the active species. Most likely, the mechanism should involve a single electron transfer followed by further hydrolytic degradation of the affected guanosines [36].

Very recently, the synthesis of a novel functionalized fullerene derivative **5** having a DNA minor groove binder and an oligonucleotide chain was reported [38]. It has



X = T(3')CTTTCCTCTTCTT(5')
3

X = C(5')TAACGACAATATGTACAAGCCTAATTGTGTAGCATCT
4

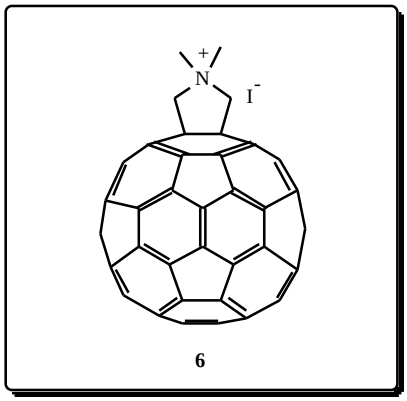


OLIGO = 5'-TTT-TC*T-TTT-C*C*C*-C*C*C*-T-3'
C* = methylated cytosine

5

already been shown that coupling of fullerenes to an intercalator or a minor groove binder leads to higher affinity and specificity towards target DNA [39]. However, results on duplex and triplex helices DNA formation and structure-activity relationship (SAR) studies are currently lacking.

The synthesis of hybrids materials of the type fullerene-DNA allows transmission electronic microscopy (TEM) imaging of deoxyribonucleic acid without the use of any heavy metal. Recently, compound **6** has been shown to bind



6

to DNA, through strong electrostatic interactions with the phosphate groups, and provides excellent contrast for imaging individual DNA molecules [40].

PHOTODYNAMIC THERAPY

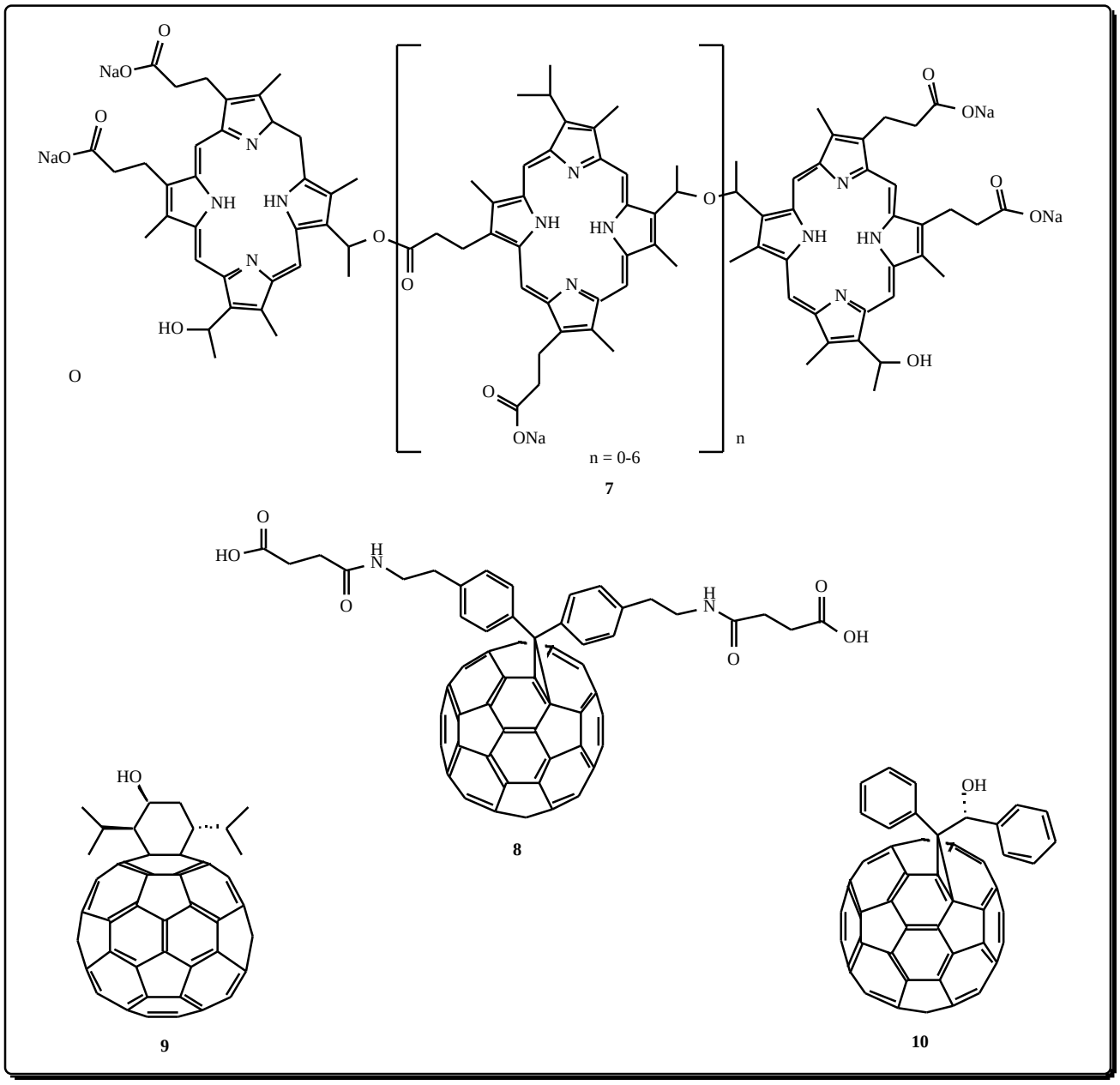
Considerable interest has been paid in photodynamic therapy of biological systems soon after the approval of the first applicable drug, the porphyrin compound **7**, namely *photofrin*. Its action is based both on the photosensitization of singlet oxygen at wavelengths of light that penetrate tissues as well as the localization of the very hydrophobic drug in tumor cells, which are generally hydrophobic tissues. Since C_{60} and C_{70} fullerenes do not absorb effectively in the visible region and longer wavelengths their potential use as *in-vivo* sensitizers could be considered only if an addend was appended to their skeleton acting as a `light-harvesting` antenna. Indeed, this pathway was considered and reported recently by Schuster *et al*. [41]. In that study, C_{60} was linked to a porphyrin; its fluorescence spectrum showed strong quenching of the porphyrin singlet excited state by the attached fullerene [41].

In another report, fullerene C_{60} conjugated with polyethylene glycols showed strong cytotoxicity to L929 cells upon visible light irradiation [42]. Also, cytochrome-c was reduced in the presence of light and the above fullerene-polyethylene derivatives. Moreover, addition of superoxide dismutase suppressed the reduction of cytochrome-c. Taken these findings together, superoxide formation was considered to be the active species responsible for the damage.

In a recent study, the role that the substituents of the fullerene skeleton play on the formation of singlet oxygen was examined. As it was found, the efficiency of singlet oxygen production was independent of the nature of addends but decreased significantly when the number of addends was increased [43]. Finally, it is worthwhile mentioning that the best advantage of fullerenes in their potential application to photodynamic therapy is the remarkable stability towards singlet oxygen.

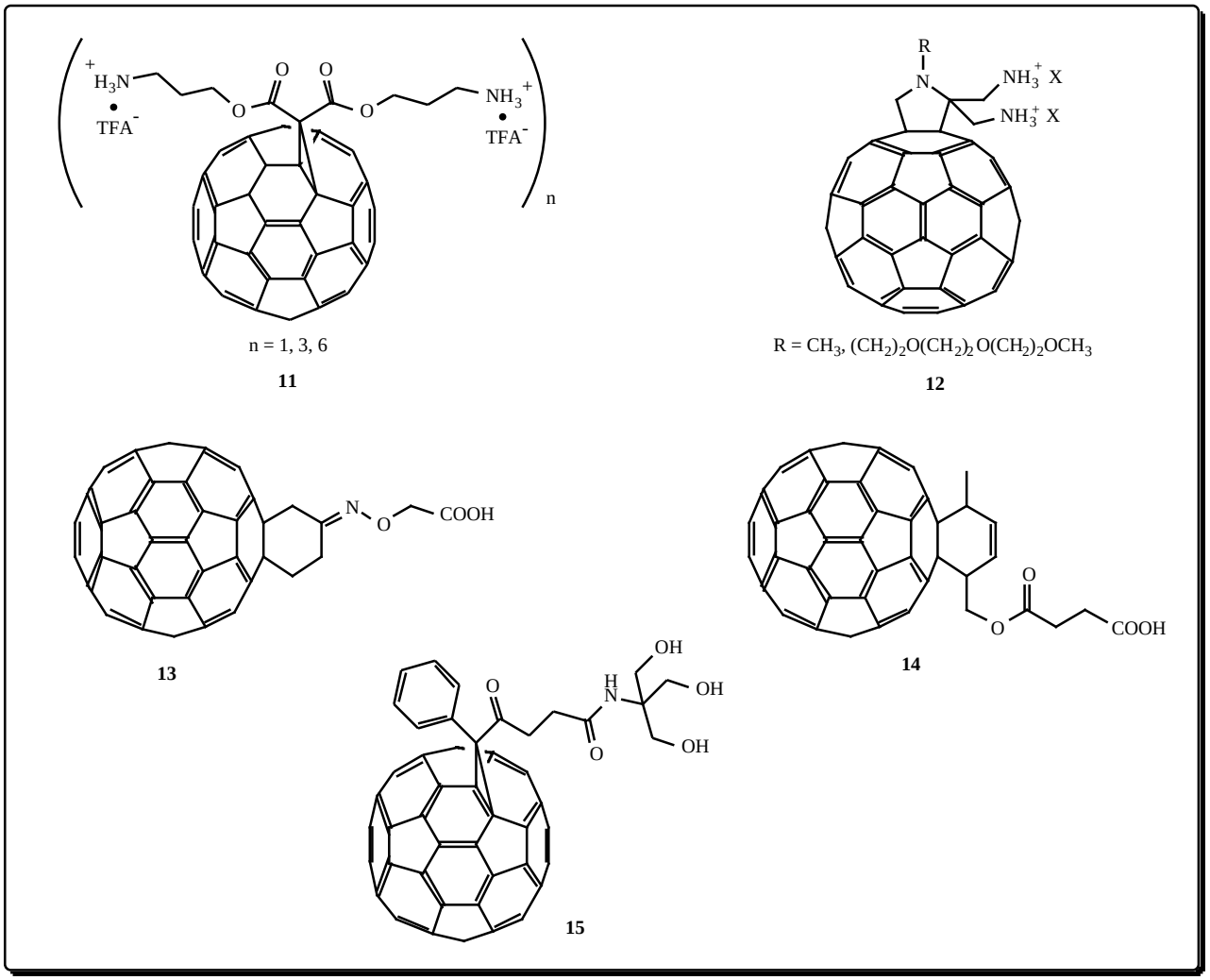
ENZYMATIC INHIBITION AND RECEPTOR BINDING

One of the first applications of fullerenes in medicinal chemistry was reported in 1993 by Friedman *et al.* using molecular modeling [44]. The authors proposed that C_{60} derivatives can be inhibitors of the HIV-1 protease due to their steric and chemical complementarity with the active site of the enzyme – an open-ended cylindrical hydrophobic cavity with a diameter of about 10 A for the central spherical extension. Inside the cavity, aminoacids residues Asp 25 and Asp 125 catalyze the hydrolysis of the substrate. Water-soluble fullerene derivative **8** was found to fit tightly into the active hydrophobic site of the HIV-1 protease and exhibit rather strong Van der Waals interactions with it, resulting in the inhibition of the human immunodeficiency virus [45]. Although the best peptide-based HIV-1 protease inhibitors are effective in the low nanomolar range and the best nonpeptide inhibitors in the high nanomolar range, the K_i value of 5.3 _M for compound **8** could be further decreased by evaluation of hypothetical fullerene-based derivatives docked into the active site [46]. Indeed, compounds **9** and



10, which were designed by the above sequence and accordingly synthesized, showed better anti-HIV-1 activity with K_i values of 103 and 150 nm, respectively. To further increase the anti-HIV-1 protease activity of fullerenes, derivatization at specific positions of the cage with groups (which may give electrostatic and/or hydrogen bond interactions with the two aminoacids residues Asp 25 and Asp 125 that catalyze the hydrolysis of the substrate) should be considered. Very recently, based on the above logical plan, two different classes of water-soluble amino fullerene were synthesized. Compounds **11** and **12** may exhibit interesting anti-HIV activity owing to the presence of ammonium groups that they share strategically located on the cage surface [47, 48]. As the presence of addends on the fullerene cage that interact with the two aminoacids residues of the enzyme has been found to be of great importance for its inhibition, several fullerene derivatives were synthesized and pharmacologically evaluated [49]. Among them, derivatives **13, 14** and **15** were found to be the most active, when examined as dimethylsulfoxide-water emulsions against HIV protease [49, 50].

In addition, a series of reports have been appeared in the literature discussing the inhibition of various other enzymes by fullerenes. In general, the unique characteristics of fullerenes including hydrophobicity, elecrophilicity and high reduction potential have been envisioned to be responsible for the mechanisms of enzymes inhibition. It has been shown that C_{60} can occupy the empty space at the interface of two subunits of glutathione-S-transferase and allosterically change the binding site of the enzyme [51]. Also, fullerenol, a polyhydroxylated fullerene, was found to inhibit

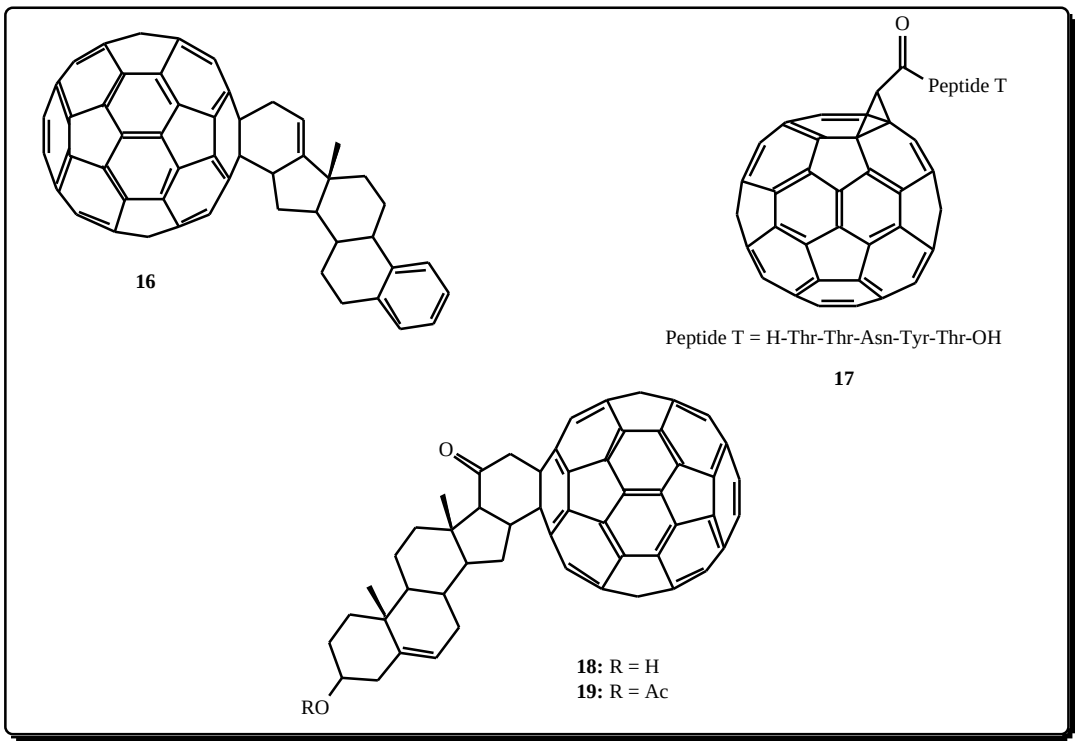


microsomal cytochrome P_{450}-dependent monooxygenases as well as mitochondrial oxidative phosphorylation [52]. The majority of P_{450}-dependent monooxygenase system is associated with endoplasmatic reticulum of the liver, while mitochondria carry out a variety of biochemical processes the most important of which is oxidative phosphorylation. Addition of the polyhydroxylated fullerene to rat liver mitochondria, resulted in a dose-dependent inhibition of adenosine 5'-diphosphate (ADP)-induced uncoupling and mitochondrial adenosine triphosphatase (ATPase) activity with an IC_{50} value of 7.1 _M. Moreover, it has exhibited noncompetitive and mixed-type inhibition in benzo[_]pyrene hydroxylation and 7-ethoxycoumarin O-deethylation respectively.

Very recently, it was reported that C_{60} monomalonate adducts can selectively inactivate the neuronal nitric oxide synthase isoform in a manner completely preventable by the concurrent presence of superoxide dismutase and catalase [53]. The inactivation was found to be irreversible by dilution as well as dependent on time, fullerene concentration and turnover. As a result, the fullerene`s interaction with neuronal nitric oxide synthase distorts its structure as to dissociate the formation of reactive oxygen intermediates (including superoxide anion and hydrogen peroxide) which finally, via a unique mechanism, inactivate neuronal nitric oxide synthase.

Compound **16**, a fullerene-estrone hybrid, exhibited estrogenic activity binding to estradiol receptor with a K_d value of 40 _M [54]. The fullerene-peptide hybrid **17**, exhibited activity in a CD4 receptor-mediated human monocyte chemotaxis assay [55]. The unique spherical structure of fullerenes may be envisaged as fitting the hydrophobic cleft often characterizing the structures of proteins and enzymes. As a result, when the cleft corresponds to the active site of an enzyme and the intermolecular interactions with the fullerenes are strong enough, a significant, although nonselective, inhibitory effect may be expected.

Two novel fullerene steroids **18** and **19** were synthesized and some preliminary studies on their effect on sarcoplasmatic reticulum (SR) Ca^{2+}-ATPase and survival of human lung adenocarcinoma cancer A_{459} cells were performed [56]. Compound **18** was found to decrease both the ATP hydrolysis and Ca^{2+} uptake activity of SR Ca^{2+}-ATPase and the inhibitions were concentration-dependent.



Finally, the effects of C_{60} solubilized with polyvinylpyrrolidine on central nervous system (CNS) receptor response in various tissues of guinea pig and rat have been studied pharmacologically [57]. There was observed no effect on receptor responses of several neurotransmitters in guinea pig ileum (e.g. acetylocholine, L-isoprenoaline and serotonin- mediated responses were unaffected by the fullerene at 4 mM concentration). As a consequence, C_{60} has no direct effects or even antagonistic properties toward drug receptors, however, sub-chronic exposure of C_{60} decreased responsiveness. The latter was argued to be due to a change in post-receptor processes [57].

ELECTRON-TRANSFER AND MEMBRANE EFFECTS

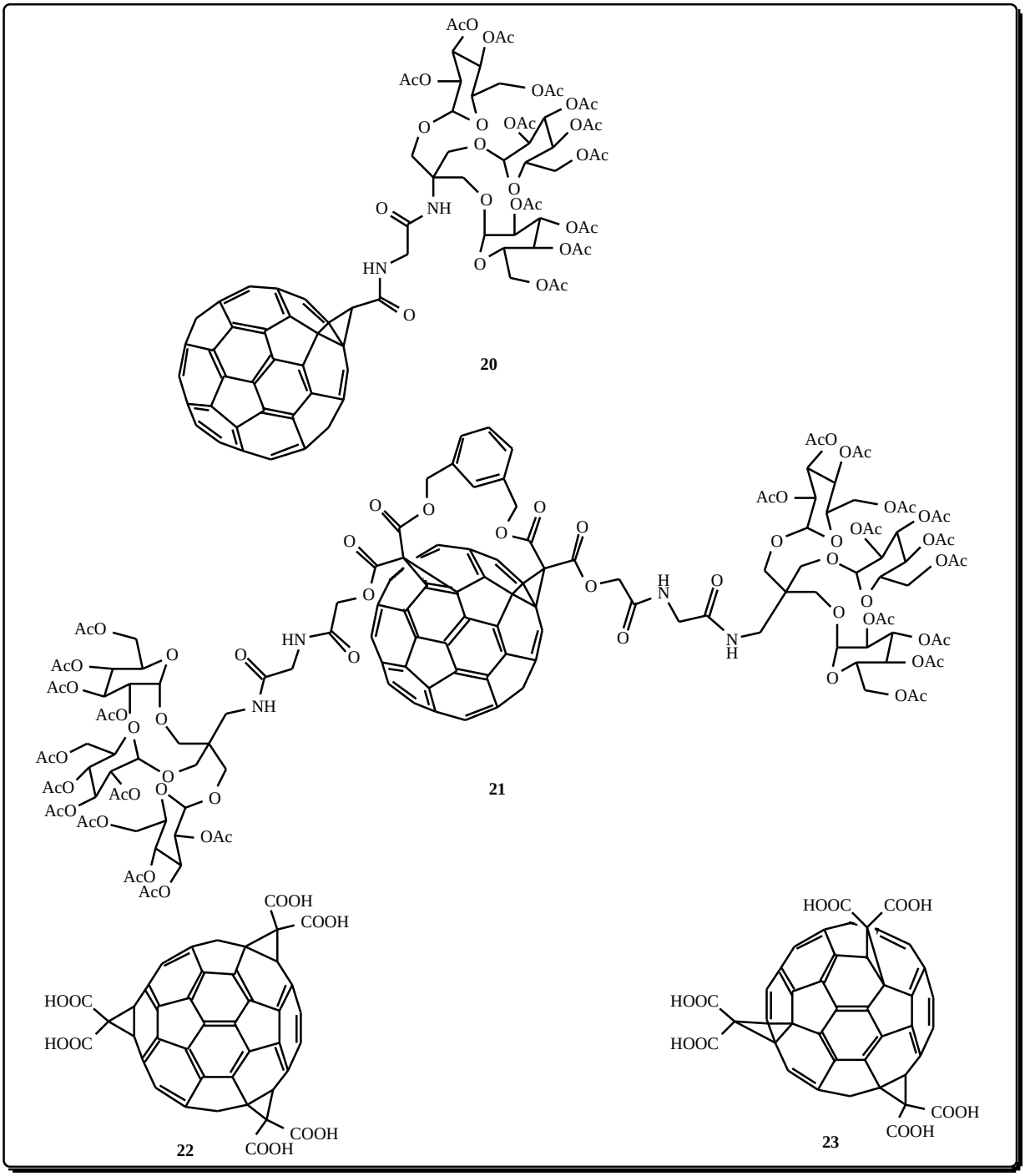
As fullerenes are excellent electron acceptors [58], there have been numerous studies upon possible electron-transfer abilities of these materials in biological systems. As one of the most intriguing applications, C_{70} was found to mediate electron transport through a lipid bilayer system [59]. Two possible mechanisms were considered: there is an electron transport via `electron hopping` between fullerene molecules in the membrane, or a current could result by the reduction of C_{70} near the boundary, diffusion of the fullerene-radical anion across the bilayer and finally release of the electron on the other side of the membrane. In another interest application, glycosylated fullerene layers have been reported that can be potentially used as glycoprotein biosensors [60]. The amphiphilic C_{60}-dendrimer conjugates **20** and **21** form stable, ordered monomolecular Langmuir layers at the air-water interface. The bulky glycodendron headgroups were very effective in suppressing fullerene aggregation, resulting in reversible compression and expansion behavior.

CELL SIGNALING AND APOPTOSIS

Examination of fullerene derivatives in cortical neuronal cultures grown in the absence of glial cells determines whether or not they are effective agents against apoptotic forms of cells death. While the mechanism by which transforming growth factor _ (TGF-__ a 25-kDa dimeric protein) induces apoptosis is not yet clear, its effects are mediated by antioxidants. Two regioisomers of trismalonic acid adduct to C_{60}, compounds **22** and **23**, were found to modify the apoptotic signaling by TGF-_ in human hepatoma cells [61]. This anti-apoptotic activity is correlated with their ability to eliminate TGF-b-generated reactive oxygen species. Regioisomer **22** was found to be more potent in protecting cells from apoptosis than isomer **23**. A plausible explanation for the different selectivity of the two regioisomers is that compound **22** possesses a bipolar structure with all hydrophilic moieties on one side of the fullerene cage, whereas isomer **23** has hydrophilic groups around the equator. This high hydrophobic moment of **22,** supported as well by fluorescence-quenching results, should facilitate its interaction with the cell membrane and its efficient capture of free radicals [61]. In another study, the effects of trisadducts **22** and **23** on apoptosis induced by the lipophilic second messenger ceramide were examined [62].

NEUROPROTECTIVE ANTIOXIDANTS

The contribution of oxidative damage to neurological conditions may be especially prominent for the reliance of the brain on aerobic metabolism, its rich content of unsaturated fatty acids and because the nervous system has limited ability to regenerate damaged tissues. There is



evidence that reactive oxygen species (e.g. superoxide, hydroxyl radicals, nitric oxide etc) can cause oxidative damage to cellular components [63]. Fullerenes can highly scavenge free radicals (`radical sponge`) and possess strong antioxidant properties [64]. As a result, fullerenes can inhibit the chain reaction of lipid peroxidation by scavenging intermediate peroxyl radicals. This would stop the peroxyl radicals from attacking adjacent fatty acid chains or membrane proteins. Due to a combination of these properties fullerene derivatives **22** and **23** have been found to act as neuroprotective agents in the central nervous system [65]. In cell culture experiments they rescued cortical neurons from a broad range of insults. Regioisomer **23** was found to inhibit excitotoxic death of cultured cortical neurons induced by exposure to N-methyl-D-aspartate (NMDA) and _-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA). Moreover, it was found to reduce apoptotic neuronal death induced by either serum deprivation or exposure to $A_{\_1\text{-}42}$ protein and was shown robust neuroprotection in a number of other cell culture models of neurological disease including Parkinson`s [65].

ENDOHEDRAL METALLOFULLERENES – DRUG DESIGN AND MRI CONTRAST AGENTS

Incorporation of metal atoms inside the empty space of fullerene cages results in a novel family of nanostructured materials, namely endohedral metallofullerenes [66]. One of the most promising applications of metallofullerenes lies in the field of nuclear medicine. Endohedral metallofullerenes could potentially provide a unique alternative to chelating compounds because of their resistance to metabolism and their high kinetic stability. The major disadvantage of the current radiopharmaceutical drugs that contain chelated radioisotopes of metals for imaging is their *in vivo* kinetic instability, which can allow the release of small quantities of toxic radiometals [67, 68]. Once the toxic heavy radioactive metal is placed inside the fullerene cage, it will have no chance to escape out. Biodistribution studies of radioactive $^{166}Ho@C_{82}(OH)_x$ in mice have been performed and verified the theory that endohedral metallofullerenes can act as radiotracers for *in vivo* studies. Among the most important findings is that endohedral metallofullerol $^{166}Ho@C_{82}(OH)_x$ has a blood pool residency time of over an hour with nearly total clearance from blood shortly thereafter, localized in liver - most likely unmetabolized there but with continued slow excretion - and was not acutely toxic *in vivo* at the doses tested [69].

Magnetic resonance imaging (MRI) is supposed to be amongst the best medical techniques used for the diagnostic examination of human patients. MRI produces images by observing the relaxation of water protons within the body in response to an applied transverse magnetic field pulse. However, sometimes the additional use of paramagnetic materials namely contrast agents, is also needed to increase the image resolution in cases such as the imaging of metastatic tumors [70]. Water soluble gadolinium endohedral metallofullerenes, $(Gd@C_{82})(OH)_x$, have been shown to be novel magnetic resonance imaging (MRI) contrast agents [71, 72].

CONCLUSION AND PERSPECTIVES

An effort was made to gather and present an up-to-date of the major and promising biological applications of fullerenes in medicinal chemistry. In addition to this mini-review, two well-written reviews exist and complement each other [73, 74].

Of the numerous fullerene compounds that have been synthesized and biologically evaluated in different laboratories worldwide, yet none of them are on the pharmaceutical market. However, there are several possible fullerene-based compounds that might be at the edge of commercialization. In a patent, a fullerene-coated cell-culture glassware was reported [75]. It was also reported that DNA and proteins were bound to surfaces in the following order: nitrocelloulose > fullerene-coated glass > glass > polystyrene [76].

Another potential application would take advantage of the UV absorbing ability of fullerenes to prepare sunscreen cosmetics containing fullerenes with UV protection effects using oils as dissolving agents. In addition, the use of water-soluble fullerenes as photosensitizers for photodynamic therapy has already been patented [77].

Concluding, the impact of fullerenes in medicinal chemistry will be enhanced only if both their toxicity is sufficiently suppressed and their solubilisation in physiological media reaches high values.

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