A Cationic [60] Fullerene Derivative Reduces Invasion and Migration of HT-29 CRC Cells in Vitro at Dose Free of Significant Effects on Cell Survival

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Abstract: Nanomaterials with unique characteristics exhibit favorable therapeutic and diagnostic properties, implying their enormous potential as biomedical candidates. C_{60} has been used in gene- and drug-delivery, as imaging agents, and as photosensitizers in cancer therapy. In this study, the influences of a cationic functionalized fullerene on cellular behavior of human colorectal cancer cell line (HT-29) were investigated. Results indicated that HT-29 treated with the studied compound showed a lower sensitivity but a significant impairment in migration and invasion by interfering with the activities of matrix metalloproteinases (MMP-2 and 9). The presence of fullerene also altered the capacity of adhesion-related proteins to perform their activity, thereby inducing dramatically adverse effects on the cell physiological functions such as cell adhesion. Thus, our study suggests that this compound is a new potential anti-metastatic effector and a therapeutic component for malignant colorectal cancer.

Keywords: Fullerene; Cancer; Anti-metastatic drug; Nanomedicine

Introduction

Current status of anticancer chemotherapy of solid malignant tumors indicates the necessity for agents active against tumor metastases. Metastasis, the spread of a primary tumor from its initial location to distant localities, is the main cause of death in cancer patients. Colorectal cancer (CRC) can lead to metastasization and represents one of the leading causes of cancer-related mortality [1]. Its progression to a highly advanced, metastatic stage (mCRC) still decreases the overall 5-year survival to less than 8-10% [2].

The rapid development of nanotechnology and its applications has allowed for a wide variety of nanoparticles to provide a broad range of opportunities in multidisciplinary fields and particularly in medicine, for clinical therapy and diagnosis [3].

Recent reports show that carbon nanomaterials, in particular fullerenes, inhibit various angiogenic signalling pathways and, therefore, can be potentially used in anti-angiogenic therapy [4-7]. Based on these properties, we might expect that fullerenes can have significant effects on tumour metastases either preventing their formation or inhibiting their growth. In this context it might be interesting to note that the endohedral metallofullerenol $Gd@C_{82}(OH)_{22}$ has been demon-

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strated to interfere with the neoplastic growth, as well as with tumour metastasis, in a mouse cancer model, with almost no toxicity to normal cells *in vivo* and *in vitro* [8]. Also the water-soluble pristine (unmodified) C_{60} inhibits the transplantable malignant Lewis lung carcinoma growth and metastasis in C57Bl/6J male mice [7]. Limited knowledge exists on the capacity of the fullerenes to interfere with tumor invasion and the mechanism involved in the fullerene anti-metastatic effect remains to be elucidated. To explore this further, the effect of fullerene on key steps of tumor metastasis, including cell adhesion, migration and invasion, was investigated.

Our previous studies demonstrated that a cationic fullerene derivative ($C_{60}+$), a fulleropyrrolidinium salt, was able to effectively inhibit tumor cell proliferation *in vitro* [9] and, recently published data from our laboratory, obtained by RNA-sequencing on MCF7 cells [10], indicated that the expression profile of several proteins involved in cell-cell adherence junctions was altered when treated by $C_{60}+$. Therefore, the question if compound $C_{60}+$ plays a role in the regulation of migration and adhesion of HT-29 colorectal cell line naturally arose.

The present study examined the hypothesis that derivative $C_{60}+$ participates in colorectal cancer cell invasion. Cells treated with it exhibited significant impairment in a series of preliminary migration and adhesion assays *in vitro*. Thus, compound $C_{60}+$ may be a new potential anti-tumor effector and therapeutic component for malignant colorectal cancer.

Experimental

The synthesis and the characterization of the fullerene derivative $C_{60}+$ was performed as previously described [9,11].

The human colorectal carcinoma cell line were purchased from the ECACC N° 86012803 (HT-29). HT-29 was maintained in RPMI-1640. The culture medium was supplemented with 10% (v/v) fetal bovine serum (FBS), penicillin (100 U/mL), streptomycin (100 µg/mL), and L-glutamine 2 mM; cells were grown at 37°C in a 95% air and 5% CO_2 humidified incubator.

HT-29 were harvested by trypsinization and plated into 96-well culture plates at approximately 1.5×10^4 per well. After incubated for 24 h, different concentrations of $C_{60}+$ (1, 5, 10, 25 and 40 µM) dissolved in culture medium were added to each well. Then the samples were incubated 48 h at 37°C in the humidified atmosphere (5% CO_2). The colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay was performed to assess the metabolic activity of cells treated as described above. 20 µl stock MTT (5 mg/mL) were added to each well, and cells

were then incubated for 4 h at 37°C. Cells were lysed with isopropanol HCl 0.04 N. Absorbance was measured at 540 and 630 nm using a microplate reader (Automated Microplate Reader EL311, BIOTEK® Instruments, Vermont, USA). All measurements were done in six replicates, and at least three independent experiments were carried out.

A 96-well plate was pre-coated with fibronectin, collagen I, collagen IV, Poly-L-lysine, laminin (Sigma-Aldrich, St. Louis, USA) and Matrigel™ (20 µg/ml) for 4 h at 37°C or 4°C overnight and subsequently blocked with PBS-BSA 0.1% (w/v) for 15 min at 37°C. Sub confluent tumor cells were treated with $C_{60}+$ (25 µM) for 48 h and then grown in serum-free medium for additional 24 h. Cells were trypsinized with 1 mM EDTA, resuspended in serum free medium with 0.1% BSA for 30 min at room temperature to ensure re-expression of integrins on the cell surface and seeded in the plate (5×10^4 cells/well). HT-29 were allowed to attach to each substrate for 1 h at 37°C and subsequently fixed with trichloroacetic acid 10% (v/v) for 1 h at 4°C and stained with sulphorodamine B 0.4% (w/v). The absorbance was read at 570 nm and related to the adhesion rate.

Effects of the $C_{60}+$ to inhibit cancer cells motility were tested performing a conventional Boyden chamber assay.

HT-29 cells, sown 72 h before, were left in serum-free medium containing 0.1% bovine serum albumin (BSA) for 24 h after being treated with $C_{60}+$ (25 µM) for 48 h, at 37°C. At the end of the treatment, 5×10^4 cells were sown in 200 µL of serum-free medium containing 0.1% BSA, in the upper side of a polyvinylpirrolidone-free polycarbonate filter (6.5-mm diameter and 8-µm pore size) set in a Transwell® cell culture chamber (Corning Costar Italia, Milan, Italy) in triplicate. The lower compartment was filled with the appropriate culture medium, supplemented with 10% FBS. Plates were left in the incubator for 72 h, at 37°C, 5% CO_2, 100% relative humidity. At the end of the incubation, cells that had not invaded were mechanically removed from the upper surface of the filter by wiping them with a cotton bud. Cells that had migrated to the lower surface were fixed with 1.1% glutaraldehyde for 15 min, washed with deionised water and air-dried. Transwells® were then stained with 0.1% crystal violet in 200 mM borate buffer, pH 9.0 for 20 min at room temperature. After washings with deionised water and complete drying, the dye was dissolved in 10% acetic acid and the absorbance was read at 590 nm by a SpectraCount spectrophotometer (Packard Bell, Meriden, CT, USA).

Invasive capability was measured in a Transwell® cell culture chamber (Corning Costar Italia, Milan, Italy) according to a method modified from Albini et al. [12]. In brief, the surface of a polyvinylpirrolidone-free polycarbonate filter (6.5-mm diameter and 8-µm pore

size) was coated with 30 μg per 50 μL of Matrigel® (Beckton Dickinson, Bedford, MA, USA) diluted in DMEM and air-dried overnight at room temperature. The filters were reconstituted with DMEM immediately before use. HT-29 cells, sown 72 h before, were left in serum-free medium containing 0.1% bovine serum albumin (BSA) for 24 h after to being treated with $C_{60}+$ (25 μM) for 48 h, at 37°C. At the end of the treatment, cells were harvested with trypsin–EDTA, and 5×10^4 cells were sown in 200 μL of serum-free medium containing 0.1% BSA, in the upper compartment chamber, in triplicate. The lower compartment was filled with the appropriate culture medium, supplemented with 10% FBS. Plates were left in the incubator for 96 h, at 37°C, 5% CO_2, 100% relative humidity. At the end of the incubation, the plate was processed as described in the migration assay.

To perform gelatin zymography, supernatant was collected from sub confluent cell culture treated for 48 h with $C_{60}+$ and left 24 h in serum-free medium. Proteins present in the supernatant were concentrated by centrifugation and quantified using Nano Drop spectrophotometer. 180 μg of total mixture of proteins were separated on 10% polyacrylamide gel containing 0.06% gelatin (Sigma-Aldrich, St. Louis, USA). Then the gel was washed for 30 minutes using 2.5% Triton X-100 solution to remove SDS and then incubated in an activating buffer (50 mm pH 7.5 TRIS, 5 mM $CaCl_2$, 0.2 M NaCl) for 20 h at 37°C. Gelatinase activity was demonstrated by gel staining with 0.5% Commasie blue and destaining with 30% methanol solution and 10% acetic acid. To assess activity, the stained gel was photographed and analyzed using ImageJ software.

Data were subjected to computer-assisted analysis by Graph Pad Instat 3 and statistical significance or regression analysis, were reported in the text.

Results and discussion

Fullerene derivative $C_{60}+$ inhibited HT-29 proliferation

The effects of $C_{60}+$ (Fig. 1(a)) on the viability of HT-29 cells after 48 h challenge with concentrations ranging from 1 to 40 μM are reported in Fig. 1(b). $C_{60}+$ caused a statistically significant reduction of cell viability (about 40% inhibition), as determined by the MTT test only at the highest dose tested, being the other dosages only marginally capable to influence HT-29 cell growth during the experiment. This result is consistent with unpublished data showing different degrees of cytotoxicity of this fullerene derivative *in vitro* on a number of tumor cell lines: MCF7 cells were particularly sensitive to the cytotoxic effects [9], whereas other cell lines, e.g. MDA-MB231, were much less sensitive, as reported here for HT-29 cells. The scenario depicted by

our RNA-seq analysis suggested that $C_{60}+$ induced an alteration of the gene expression targeting mTOR signaling at different levels in MCF7 cell line [10]. Among the many factors potentially determining an inhibition of mTOR signaling, we proposed a decrease of available cellular energy as the most plausible. We here hypothesize that the mechanism of action of $C_{60}+$ in the HT-29 cell line could be the same, given that HT-29 represents a cell line with high *in vitro* invasive and *in vivo* metastatic behavior.
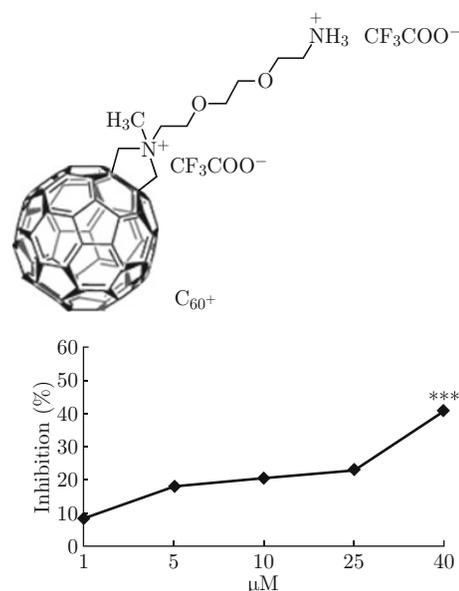

Fig. 1 (a) Chemical structure of fullerene derivative $C_{60}+$; (b) Effect of $C_{60}+$ on proliferation of Colon Cancer Cell Line (HT-29). The cells were treated for 48 h at different concentrations. Each value represent the mean of 3 separate experiments, each with at least three independent samples, ± S.D. (***) $p < 0.01$

Cell adhesion analysis

Cell adhesion to the extracellular matrix (ECM) is an important step that regulates different cellular functions including cell migration and proliferation, differentiation, and tissue organization [13]. This event is mediated by cell surface receptors, the integrins, α and β chain heterodimers with short cytoplasmic tails, the enzymatic activity of which activates a variety of intracellular signalling pathways [14]. Integrins were shown to be involved in the processes of cancer cell invasion and metastasis [15], and, most notably, the integrin-ECM interactions were also demonstrated to have a role for cell survival and resistance to chemotherapy in many types of solid cancers, including colon cancer [16].

In order to mimic the detachment of metastatic cells from the primary mass and their adhesion in a secondary site, we performed a study where HT-29 cells

were exposed to $C_{60}+$ at 25 μM for 48 h, then harvested from the plate and allowed to adhere to different substrates components of the ECM such as laminin, fibronectin and collagens. Data reported in Table 1 show that $C_{60}+$ causes a similar inhibition of the adherence ability of HT-29 cells independently whether the substrate used was a component of the ECM, an unspecific substrate (poly-L-lysine) or no substrate at all. However, in all the cases, the HT-29 cells, after such treatment, showed a marked reduction (60-70% inhibition) of their ability to adhere as compared to untreated cells.

Table 1   Inhibitory potential of $C_{60}+$ to the interaction between HT-29-ECM substrates. After treatment with $C_{60}+$, cells were seeded on 96-well plate functionalized with fibronectin, collagen I and IV, laminin, poly-L-lysine and Matrigel (20 μg/mL) and left to adhere 1 h at 37°C with 5% CO_2. Unbound cells were removed while adhered cells were fixed, stained with sulphorodamine B and absorbance read at 570 nm. Results obtained for each substrate are expressed as percentage of adhesion inhibition of treated HT-29, considering the untreated HT-29 adhesion value as 100%

Substrate	% Inhibition of Adhesion by $C_{60}+$
No substrate	78
Poli-L-lys	69
Laminin	63
Fibronectin	66
Collagen I	68
Collagen IV	62

This effect might be related to a number of hypotheses. Integrins might be activated by extracellular events, aside from the intracellular signaling events [17], such as ligand binding, divalent cation concentration, mechanical stress, all of them potentially perturbated by the fullerene treatment. However it must be taken into account also the possible effect of this compound on cell viability that, although not leading to cell death, might keep the treated cells to a lower stage of "biological activity".

Cell migration, invasion and zymography assays

Migration and invasion are two essential steps of the pathological events leading to cancer metastasis. We studied cell migration using a conventional Boyden chamber assay (Fig. 2(a)).

HT-29 cells, treated with $C_{60}+$ for 48 h, were left to migrate for 72 h from the upper chamber through the insert, following the chemotactic signal (serum) coming from the lower side of the chamber. Data reported in Fig. 2(a) shows a pronounced (80%) and statistically significant reduction of the migration ability of the treated cells as compared to control constituted by untreated HT-29 cells.

Similar effects were obtained when we studied the invasion process. In this case, cells were seeded on a layer of matrigel previously deposed on the Boyden chamber grid and evaluated after 96 h. Compound $C_{60}+$ inhibited more than 75% the capacity of the treated HT-29 cells to cross the matrigel barrier and to accumulate in the lower compartment of the chamber (Fig. 2(b)).
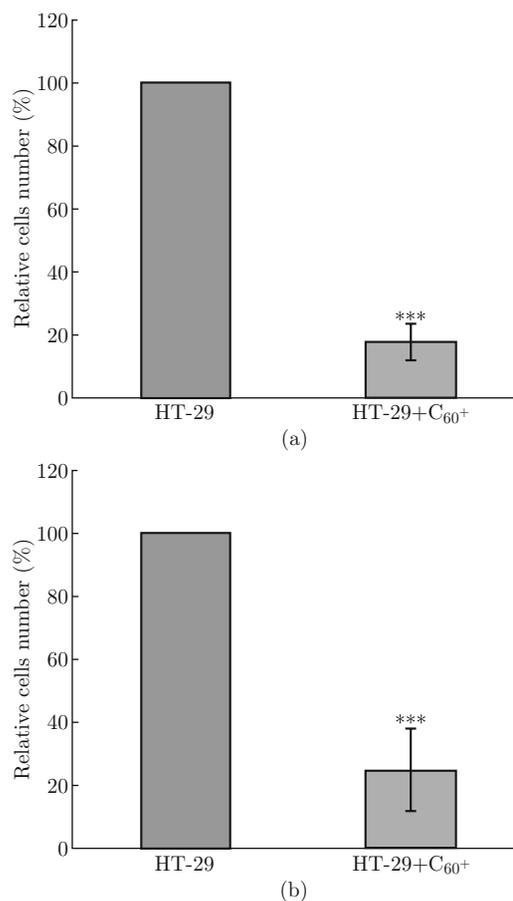

Fig. 2   (a) Boyden chamber assay: HT-29 cells were treated with $C_{60}+$ for 48 h and then seeded in a Boyden chamber and left to migrate for 72 h. At the end of this time, cells that migrated were fixed, stained with crystal violet and the absorbance read at 590 nm. Statistical analysis was performed with ANOVA Unpaired t test-One tail t test (∗∗∗) $p < 0.0001$ *vs* control (HT-29). (b) Invasion assay: HT-29 treated 48 h with $C_{60}+$ were seeded on the upper side of inserts functionalized with matrigel and were left invade 96 h. At the end of incubation, cells on the lower side of the inserts were fixed, stained with crystal violet and the absorbance read. Statistical analysis was performed with ANOVA Unpaired t test-One tail t test (∗∗∗) $p < 0.0001$ *vs* HT-29.

The compared analysis of the effects of $C_{60}+$ on migration and invasion suggests that this compound does not reduce the capacity of HT-29 cells to degrade the ECM. In fact, it seems that the effects of the considered fullerene derivative on these cells might consist of a more general reduction of the cells ability to move or a

reduced capacity to respond to the chemotactic signal, operated by the serum present in the lower wall. On the contrary, the treated cells have a reduced gelatinolytic ability, as shown by a study of MMP2 and MMP9 activity, performed on the supernatant obtained by the HT-29 cells exposed to $C_{60}+$ at 25 μM for 48 h (38-42% inhibition, Fig. 3), suggesting that the treated cells have a lower capacity to degrade the ECM.
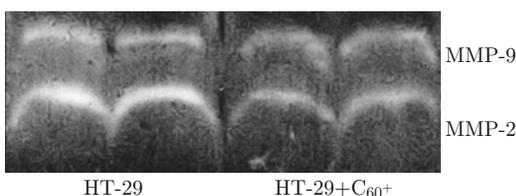

Fig. 3   Gelatinolytic activity of MMP-2 and MMP-9 was evaluated in HT-29 cells treated with $C_{60}+$ at 48 h.

The activation of the zymogen form of MMP2 (pro-MMP2) is a cell-surface event that is mediated by members of the membrane-type (MT) subfamily of MMPs as MT1-MMP and by the tissue inhibitor of metalloproteinase (TIMP-2), a member of the family of MMP inhibitors [18,19]. It has been demonstrated that this inhibitor directly interacts with MMP2 or through MT1-MMP/TIMP-2 complex formation reducing the enzymatic activity of MMP2 [20-22].

Recently we found that $C_{60}+$ increases 4-folds the expression level of TIMP-2 after 24 and 48 h of treatment [10] in the human breast cancer cell line MCF7. This result could suggest a similar effect of this compound also on HT-29 colon cancer cell line and a consequent reduced gelatinolytic activity of MMP2 detected after treatment of cells with fullerene $C_{60}+$.

Moreover the complex pro-MMP2 and TIMP-2 is able to inhibit other MMPs such as MMP9 through the formation of a ternary complex pro-MMP2-TIMP-2-MMP9 [23-25]. This data suggest that the reduced MMP9 activity detected in treated HT-29 cells, could be due to the increased level of TIMP-2 after $C_{60}+$ treatment and its modulation on MMP9 activity via MMP2 involvement.

Conclusions

The *in vitro* study of fullerene derivative $C_{60}+$ on the ability of HT-29 cells to migrate and invade, events that mimic important steps of the process of metastasis formation, showed promising effects. The compound, in fact, inhibited the capacity of HT-29 cells to re-adhere, to migrate and to invade and these effects were, at least in part, associated with the decrease of the gelatinolytic activity of the treated cells. The treated HT-29 cells did not present significant differences in the adherence process in presence of the various ECM substrates and

this seems to rule out an effect of $C_{60}+$ on cell integrins. Rather, it seems that $C_{60}+$ might act on a more generalized pathway responsible for multiple effects of the metastatic capacity of the cells.

Anyhow, these data, although preliminarily and considering the role attributed to phenomena such as adhesion and migration for the process of metastasis formation suggest the ability of this fullerene derivative to interfere with metastases of solid tumors, in agreement with other data already reported in the literatures [7,8].

Acknowledgements

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