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Research Paper

Modulation of Tumor Necrosis Factor-mediated Cell Death by Fullerenes

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Purpose. The fullerene (C_{60}/C_{70} mixture—$C_{60/70}$) nanocrystalline suspension prepared by solvent exchange method using tetrahydrofyran (THF/$nC_{60/70}$) and polyhydroxylated $C_{60/70}$ [$C_{60/70}(OH)_n$] were compared for their ability to modulate cytotoxicity of the proinflammatory cytokine tumor necrosis factor (TNF).
Materials and Methods. TNF-induced cytotoxicity was assessed in L929 fibrosarcoma cells by crystal violet assay. The type of cell death (apoptosis/necrosis), production of reactive oxygen species, mitochondrial depolarization and caspase activation were determined by flow cytometry using the appropriate reporter dyes.
Results. THF/$nC_{60/70}$ augmented, while $C_{60/70}(OH)_n$ reduced the cytotoxicity of TNF. The numbers of cells undergoing apoptosis/necrosis, as well as of those displaying the activation of apoptosis-inducing enzymes of caspase family, were respectively increased or reduced by THF/$nC_{60/70}$ or $C_{60/70}(OH)_n$. The antioxidant *N*-acetylcysteine and mitochondrial permeability transition inhibitor cyclosporin A each partly blocked the cytotoxic action of TNF, indicating the involvement of oxidative stress and mitochondrial dysfunction in the TNF cytotoxicity. Accordingly, THF/$nC_{60/70}$ or $C_{60/70}(OH)_n$ potentiated or suppressed, respectively, TNF-triggered oxidative stress and mitochondrial depolarization.
Conclusion. The ability of different fullerene preparations to modulate TNF-induced oxidative stress and subsequent cell death suggests their potential value in the TNF-based cancer therapy or prevention of TNF-dependent tissue damage.

KEY WORDS: apoptosis; fullerene; necrosis; oxidative stress; tumor necrosis factor.

INTRODUCTION

Because of the unique chemical and physical properties that enable interaction with living cells, fullerenes (C_{60}) and their derivatives are promising candidates for many biomedical applications (1). The electrochemical features that provide reactivity with cell-damaging reactive oxygen species (ROS) such as superoxide ($O_2^{\bullet-}$) and hydroxyl (•OH) radicals, make water-soluble fullerene derivatives behave as "free radical sponges." Accordingly, water-soluble fullerenes display a protective effect in various experimental models of ROS-dependent cell death (2–6), and the polyhydroxylated fullerenes, known as fullerols or fullerenols, are particularly efficient in that respect (7,8). Somewhat surprisingly, it has recently been shown that pure C_{60} brought into water by means of solvent extraction forms water-stable crystalline aggregates (nano-C_{60} or nC_{60}), which, in contrast to fullerol, generate reactive oxygen and kill various cell types in a ROS-dependent manner (9–12). While the mechanisms underlying this different behavior of water soluble vs. nanocrystaline C_{60} are still to be revealed, it has been proposed that derivatization of the fullerene core in order to provide water solubility, might somehow decrease its prooxidant and increase the antioxidant capacity (9,10). On the other hand, as nC_{60} prepared by detergent solubilization or long-term mixing in water does not display ROS-dependent cytotoxicity (13,14), it has been suggested that the observed ROS generation by solvent exchange-prepared nC_{60} could actually result from the interaction of C_{60} with the relatively small amount (<10%) of otherwise nontoxic residual organic solvent (tetrahydrofuran) that remains trapped in nC_{60} crystalline lattice (12,15,16).

Tumor necrosis factor (TNF, previously known as TNF-α) is a pleiotropic proinflammatory cytokine produced by activated macrophages, as well as by several other cell types, including lymphocytes, fibroblasts, and hepatocytes (17). Many cell types are targets for TNF action due to an extremely broad expression of TNF receptors TNF-R1 and TNF-R2 (18). One of the most prominent and most explored biological actions of TNF is its fairly selective cytotoxic activity on tumor cells (19), making it a plausible candidate for anticancer treatment, particularly against surgically unresectable and chemotherapy- or radiation therapy-resistant tumors (20). However, the clinical use of TNF has been hampered by the

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ABBREVIATIONS: DHR, dihydrorhodamine; FITC, fluorescein isothiocyanate; MPT, mitochondrial permeability transition; PI, propidium iodide; ROS, reactive oxygen species.

resistance of many tumors to TNF-mediated death (21), as well as because of the severe side-effects of systemic TNF administration, such as hypotension and hepatotoxicity (22,23). Due to systemic toxicity, TNF therapy is limited to sophisticated locoregional drug-delivery systems in patients with some types of organ-confined solid tumors (24). TNF is also involved in liver damage during sepsis, viral hepatitis, alcoholic hepatitis, ischemia-reperfusion liver injury, fulminant hepatic failure and chemical-induced hepatotoxicity (25,26), which is consistent with its ability to cause TNF-R1-dependent hepatocyte damage *in vitro* and *in vivo* (27). Therefore, exploring the mechanisms for the enhancement of TNF-mediated tumor cell killing and decreasing its toxicity against normal tissues is of paramount importance for successful development of the TNF-related anticancer or anti-inflammatory therapeutic strategies.

As the cytotoxic action of TNF depends on its ability to trigger ROS production in target cells (28–30), ROS-modulating agents such as fullerenes are potential candidates for the interference with TNF-mediated cell death. Accordingly, it has been demonstrated that malonic acid C_{60} derivatives (carboxyfullerenes) can protect human peripheral blood mononuclear cells from oxidative stress-dependent apoptosis induced by combination of TNF and cycloheximide (31). However, the effects of other fullerene derivatives on TNF-induced cell death have not been investigated to date. In the present study, we show that nanocrystalline and polyhydroxylated fullerenes differently modulate the TNF toxicity towards L929 mouse fibrosarcoma cell line, which has been widely used as a model system for exploring TNF-induced cell death.

MATERIALS AND METHODS

Preparation of Fullerenes

For the preparation of fullerene colloid in water we used C_{60}/C_{70} extract of carbon soot (79% C_{60}, 20% C_{70}, 1% higher-order fullerenes) produced by arc discharge (32). C_{60}/C_{70} colloid was produced by evaporating tetrahydrofuran (THF) from a mixture of water and molecularly dispersed C_{60}/C_{70} in THF (Sigma, St. Louis, MO), as previously described (33). The concentration of C_{60}/C_{70} suspension in water, referred to as THF/nC$_{60/70}$, was determined from the absorption spectrum and adjusted to 10 μg/ml. Briefly, 1ml of fullerene water suspension was mixed with equal amount of 2% NaCl solution, and toluene (2 ml) was added. The mixture was sonicated for a few minutes and then set in the dark until the aqueous and toluene phases separated. The upper toluene phase, containing fullerene in molecular form, was withdrawn carefully, and the absorbance at 335 nm was measured and compared with referent spectrums. Polyhydroxylated fullerene, referred to as $C_{60/70}(OH)_n$ (n = 2–24), was prepared as previously described (34) from the same C_{60}/C_{70} extract of carbon soot used for the THF/nC$_{60/70}$ preparation. Both THF/nC$_{60/70}$ and $C_{60/70}(OH)_n$ were characterized by dynamic light scattering, UV-Vis and Fourier transform infrared spectroscopy as previously described (11). A schematic representation of the structure of the two fullerene derivatives is given in Fig. 1. Both fullerene preparations were stored at 4°C until used for experiments. Since we did not assess the ratio of C_{60} and C_{70} in THF/nC$_{60/70}$ and $C_{60/70}(OH)_n$, only one batch of each fullerene preparation was used in the experiments presented, in order to avoid the possible influence of changes in C_{60}/C_{70} ratio in different batches. Nevertheless, it should be noted that similar results were obtained with different batches of both THF/nC$_{60/70}$ and $C_{60/70}(OH)_n$.

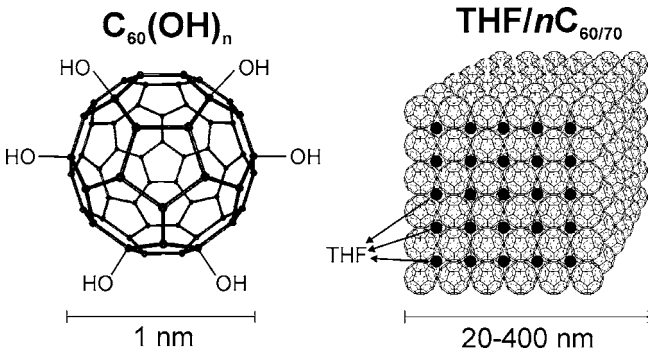
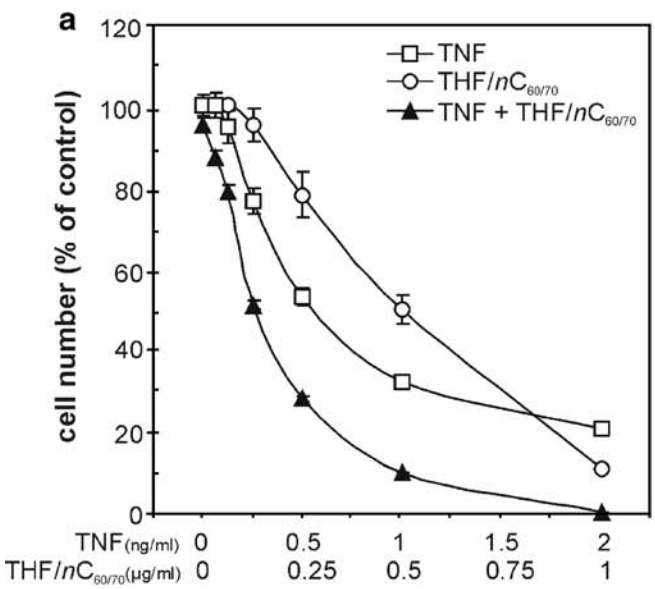


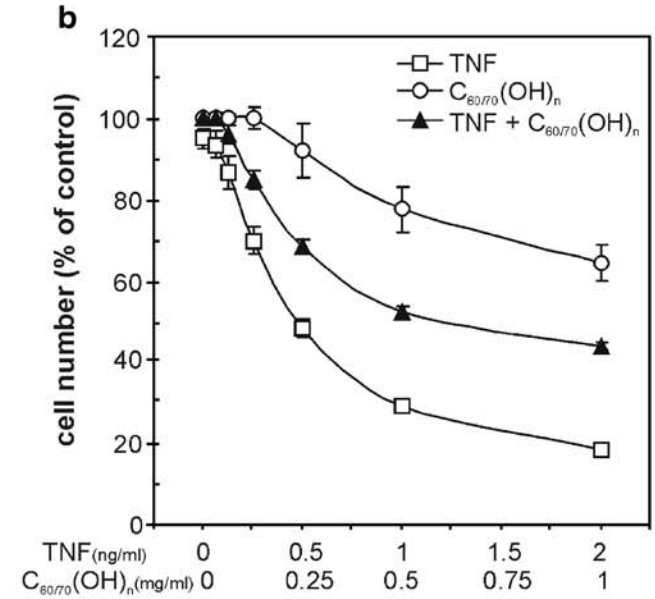
Fig. 1. Schematic representation of the structure of $C_{60}(OH)_n$ (n = 2–24) and THF/nC$_{60/70}$. For clarity, only six C_{60} molecules are presented per one side of THF/nC$_{60/70}$ crystal, while C_{70} molecules are not depicted. The size of THF/nC$_{60/70}$ nanoparticles used in the present study was approx. 100 nm.

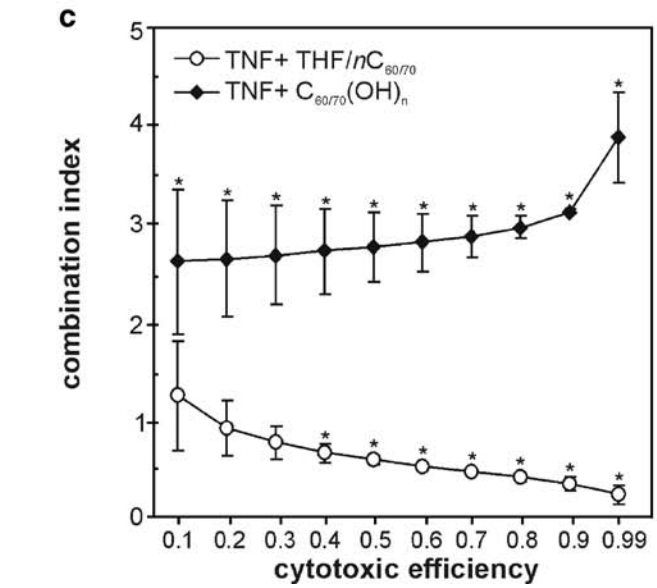
Cells and Cell Cultures

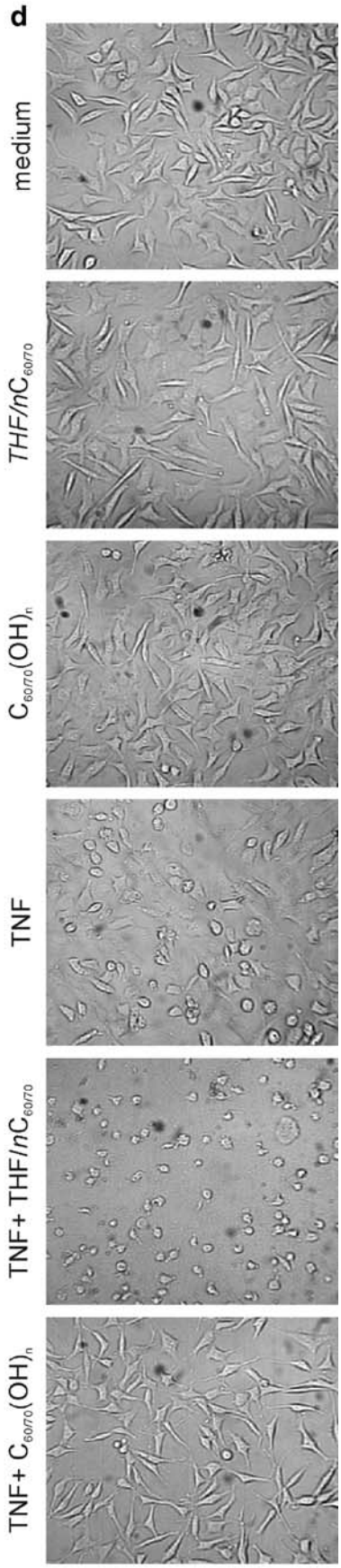
The mouse fibrosarcoma cell line L929 was obtained from the European Collection of Animal Cell Cultures (Salisbury, UK), while the human glioma cell line U251 was kindly donated by Dr. Pedro Tranque (Universidad de Castilla-La Mancha, Albacete, Spain). The primary macrophages were obtained from peritoneal cavities of C57/BL6 mice (Institute for Biological Research, Belgrade) as previously described (35), and in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the United States National Institutes of Health. The tumor cells were seeded in flat-bottom 96-well (1×10^4 cells/well) or 6-well (2×10^5 cells/well) cell culture plates (Sarstedt, Newton, NC) for the cell viability assessment or flow cytometry analysis, respectively. Peritoneal macrophages were seeded in 96-well plates at 1×10^5 cells/well. After being rested for 24h, cell cultures were washed and incubated with TNF alone or in combination with THF/nC$_{60/70}$ or $C_{60/70}(OH)_n$. U251 cells were treated with cisplatin or exposed to hyperthermia (43°C for 1h), in the presence or absence of fullerene preparations. The cells were incubated at 37°C in a humidified atmosphere with 5% CO_2, in a HEPES (20 mM)-buffered RPMI 1640 cell culture medium (Sigma, St. Louis, MO) supplemented with 5% fetal calf serum, 2 mM L-glutamine, 50 μM 2-mercaptoethanol, 10 mM sodium pyruvate, and 100 IU/ml penicillin and streptomycin (all from Sigma). Working

Fig. 2. Fullerene preparations differently modulate TNF-mediated cytotoxicity. **a**, **b** L929 cells were incubated with different concentrations of TNF, THF/nC$_{60/70}$ or $C_{60/70}(OH)_n$ alone or in different combinations, and the cell number was assessed after 24 h. The results are mean values of triplicates from a representative of three experiments. **c** The Chou–Talalay combination index was calculated and the data are given as mean ± SD values from three independent experiments (*$p < 0.05$ denotes values significantly different from 1). **d** The photomicrographs of the cells treated for 24 h with TNF (0.5 ng/ml), in the presence or absence of THF/nC$_{60/70}$ (125 ng/ml) or $C_{60/70}(OH)_n$ (250 μg/ml).


a
cell number (% of control)
TNF
THF/nC60/70
TNF + THF/nC60/70
TNF(ng/ml) 0 0.5 1 1.5 2
THF/nC60/70(μg/ml) 0 0.25 0.5 0.75 1


b
cell number (% of control)
TNF
C60/70(OH)n
TNF + C60/70(OH)n
TNF(ng/ml) 0 0.5 1 1.5 2
C60/70(OH)n(mg/ml) 0 0.25 0.5 0.75 1


c
combination index
TNF+ THF/nC60/70
TNF+ C60/70(OH)n
cytotoxic efficiency


d
medium
THF/nC60/70
C60/70(OH)n
TNF
TNF+ THF/nC60/70
TNF+ C60/70(OH)n

solutions of THF/nC$_{60/70}$ were prepared by addition of the appropriate amounts of 10-fold concentrated cell culture medium and deionized water, while C$_{60/70}$(OH)$_n$ was dissolved directly in the cell culture medium.

Cell Viability Determination

For the assessment of cell viability we used a crystal violet assay, which is based on the inability of dead cells to remain adherent to cell culture plastic (36). After incubation, cells were washed with PBS to remove dead, non-adherent cells. The remaining adherent, viable cells were fixed with methanol and stained with 1% crystal violet solution at room temperature for 10min. The plates were thoroughly washed with water, and crystal violet was dissolved in 33% acetic acid. The absorbance of dissolved dye, corresponding to the number of viable cells was measured in an automated microplate reader at 570nm. The results are presented as relative to the control value (untreated cells).

Analysis of Apoptotic and Necrotic Cell Death

The type of cell death (apoptotic or necrotic) was analyzed by double staining with annexin V-FITC and propidium iodide (PI), in which annexin V bound to the early apoptotic cells with exposed phosphatidylserine, while PI labeled the late apoptotic and necrotic cells with the membrane damage. Staining was performed according to the instructions by the manufacturer (BD Pharmingen, San Diego, CA), and flow cytometric analysis was conducted on a FACSCalibur flow cytometer (BD). The percentage of apoptotic (annexin$^+$/PI$^-$) and necrotic (annexin$^+$/PI$^+$) cells was determined using CellQuest Pro software.

Detection of Caspase Activation

Activation of caspases was measured by flow cytometry after labeling the cells with a cell-permeable, FITC-conjugated pan-caspase inhibitor (ApoStat; R&D Systems, Minneapolis, MN) according to the manufacturer's instructions. The increase in green fluorescence (FL1) is a measure of caspase activity within individual cells of the treated population. The results are expressed as % of cells containing active caspases.

Measurement of Intracellular ROS Production

Intracellular production of ROS was determined by measuring the intensity of green fluorescence emitted by redox-sensitive dye dihydrorhodamine 123 (DHR; Invitrogen, Paisley, UK), which was added to cell cultures (2 μM) at the beginning of treatment. At the end of incubation, cells were detached by trypsinization, washed in PBS, and the green fluorescence (FL1) of DHR-stained cells was analyzed using a FACSCalibur flow cytometer.

Assessment of Mitochondrial Membrane Potential

The mitochondrial depolarization was assessed using DePsipher (R&D Systems), a lipophilic cation susceptible to the changes in mitochondrial membrane potential. It has the property of aggregating upon membrane polarization forming an orange-red fluorescent compound. If the potential is disturbed, the dye can not access the transmembrane space and remains or reverts to its green monomeric form. The cells were stained with DePsipher as described by the manufacturer, and the green monomer and the red aggregates were detected by flow cytometry. The results were presented as a green/red fluorescence ratio (geomean FL1/FL2), the increase of which reflects mitochondrial depolarization.

Mathematical Analysis of Synergism/Antagonism

To analyze the type (additive, synergistic or antagonistic) of fullerene interaction with TNF in inducing tumor cell death, cells were treated with each agent alone and their appropriate combinations. The cell viability was assessed using a crystal violet assay. The values of combination index, reflecting additive (= 1), synergistic (<1) or antagonistic interactions (>1), were calculated according to the method based on the median-effect principle of Chou and Talalay (37).

Statistical Analysis

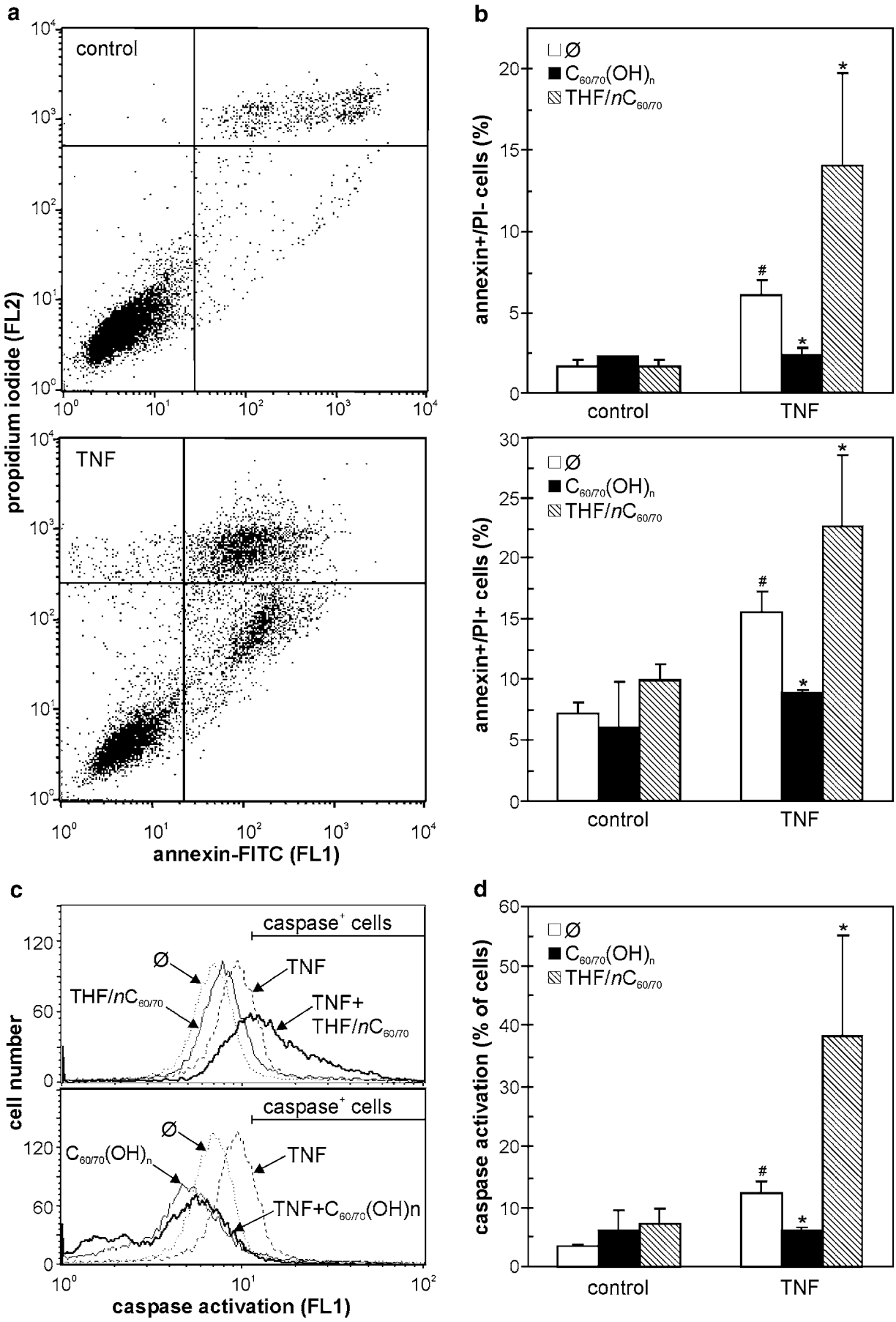
The statistical significance of the differences was analyzed by t-test or ANOVA followed by the Student–Newman–Keuls test. The value of $p < 0.05$ was considered significant.

RESULTS

THF/nC$_{60/70}$ and C$_{60/70}$(OH)$_n$ Differently Affect TNF-mediated Cytotoxicity

To assess the influence of THF/nC$_{60/70}$ and C$_{60/70}$(OH)$_n$ on TNF cytotoxicity, we treated L929 cells with different doses of fullerene preparations and TNF, separately or in combination. In accordance with our previous results (11), crystal violet assay showed that both fullerene preparations were cytotoxic, but THF/nC$_{60/70}$ was at least three orders of magnitude more potent than its hydroxylated counterpart (Fig. 2a,b). Interestingly, while combination of THF/nC$_{60/70}$ and TNF was more toxic than each agent alone (Fig. 2a), addition of C$_{60/70}$(OH)$_n$ clearly reduced the cytotoxic effect of TNF (Fig. 2b). Accordingly, the combination index calculated by Chou–Talalay approach was >1 throughout 0.1–0.99 efficiency range for combination of C$_{60/70}$(OH)$_n$ with TNF, and <1 for combination of THF/nC$_{60/70}$ and TNF (efficiency range 0.4–0.99), thus confirming antagonistic and synergistic interactions, respectively (Fig. 2c). For further investigation we used TNF at the concentration of 0.5 ng/ml, which displayed suboptimal cytotoxicity and therefore allowed direct comparison of the opposite effects of nontoxic THF/nC$_{60/70}$ and C$_{60/70}$(OH)$_n$ doses (125 and 250 μg/ml,

Fig. 3. The effects of fullerene preparations on TNF-induced apoptosis, necrosis and caspase activation. **a**, **b** L929 cells were incubated with TNF (0.5 ng/ml), in the absence or presence of THF/nC$_{60/70}$ (125 ng/ml) or C$_{60/70}$(OH)$_n$ (250 μg/ml). Apoptosis, necrosis (**a**, **b**) and caspase activation (**c**, **d**) were analyzed by flow cytometry after 18 h. The representative dot plots and histograms are presented in (**a**, **c**), while the data in (**b**, **d**) are mean ± SD values from three independent experiments ($^{\#}p < 0.05$ refers to untreated cells; $^{*}p < 0.05$ refers to cells treated with TNF alone).


a
control
TNF
propidium iodide (FL2)
annexin-FITC (FL1)
b
Ø
C60/70(OH)n
THF/nC60/70
annexin+/PI- cells (%)
annexin+/PI+ cells (%)
control
TNF
c
caspase+ cells
Ø
TNF
THF/nC60/70
TNF+ THF/nC60/70
C60/70(OH)n
TNF+C60/70(OH)n
cell number
caspase activation (FL1)
d
caspase activation (% of cells)

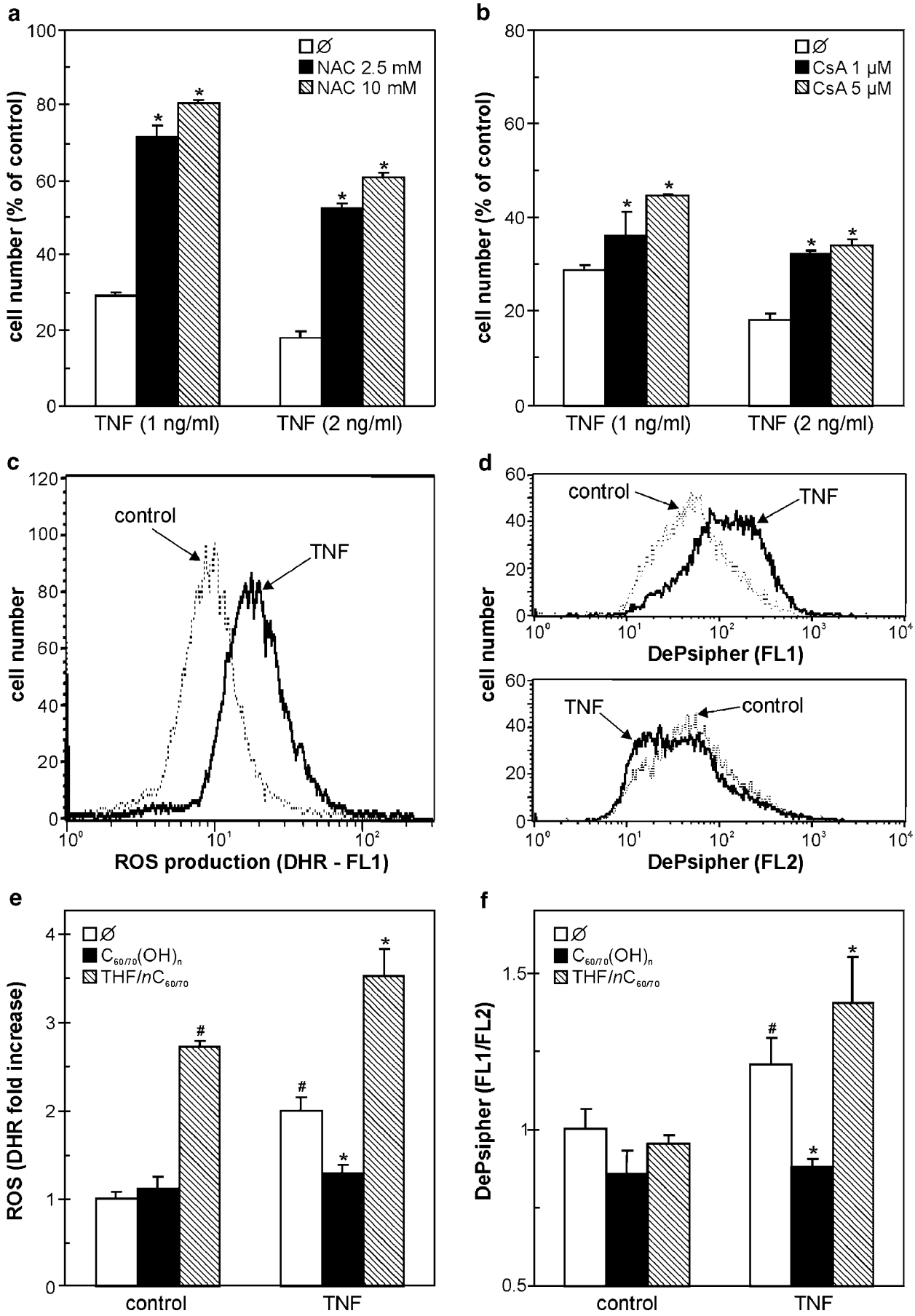

a
cell number (% of control)
Ø
NAC 2.5 mM
NAC 10 mM
TNF (1 ng/ml)
TNF (2 ng/ml)
b
cell number (% of control)
Ø
CsA 1 μM
CsA 5 μM
TNF (1 ng/ml)
TNF (2 ng/ml)
c
cell number
control
TNF
ROS production (DHR - FL1)
d
cell number
control
TNF
DePsipher (FL1)
TNF
control
DePsipher (FL2)
e
ROS (DHR fold increase)
Ø
C60/70(OH)n
THF/nC60/70
control
TNF
f
DePsipher (FL1/FL2)
Ø
C60/70(OH)n
THF/nC60/70
control
TNF

Fig. 4. The effects of fullerene preparations on TNF-induced ROS production and mitochondrial depolarization. **a**, **b** L929 cells were treated with TNF, in the absence or presence of *N*-acetylcysteine (NAC) (**a**) or cyclosporin A (CsA) (**b**), and the cell number was determined after 24 h. (**c**–**f**) L929 cells were incubated with TNF (0.5 ng/ml), in the absence (**c**, **d**) or presence (**e**, **f**) of THF/nC$_{60/70}$ (125 ng/ml) or C$_{60/70}$(OH)$_n$ (250 μg/ml). ROS production (DHR) (**c**, **e**) and mitochondrial depolarization (DePsipher) (**d**, **f**) were analyzed by flow cytometry after 8 h of incubation. The representative histograms are presented in (**c**, **d**), while the data in (**e**, **f**) are mean ± SD values from three independent experiments ($^{\#}p < 0.05$ refers to untreated cells; $^{*}p < 0.05$ refers to cells treated with TNF alone).

respectively) on TNF-mediated cell death. In accordance with the cytotoxicity data, the TNF-treated cells lost their polygonal morphology and became smaller, with granular appearance and poorly defined margins (Fig. 2d). While neither THF/nC$_{60/70}$ nor C$_{60/70}$(OH)$_n$ alone were able to cause significant changes in L929 cell morphology, both agents significantly affected morphological changes induced by TNF treatment (Fig. 2d). Namely, the cells treated with TNF and THF/nC$_{60/70}$ became completely round and detached from the surface of cell culture wells, which is consistent with the synergistic induction of cell death. On the other hand, the cells treated with TNF and C$_{60/70}$(OH)$_n$ mainly retained normal polygonal morphology characteristic for healthy, untreated cells, thus confirming the protective effect of polyhydroxylated fullerenes.

The Effects of Fullerene Preparations on TNF-induced Apoptosis/Necrosis and Caspase Activation

The treatment with suboptimal dose of TNF led to a significant increase in numbers of cells displaying early apoptotic (annexin$^+$/PI$^-$) and late apoptotic/necrotic (annexin$^+$/PI$^+$) phenotype, as revealed by flow cytometric analysis (Fig. 3a,b). The TNF-mediated apoptosis/necrosis was associated by significant activation of apoptosis-executing enzymes caspases in target cells (Fig. 3c,d). In accordance with the cytotoxicity data obtained in a crystal violet assay (Fig. 2a,b), the addition of THF/nC$_{60/70}$ markedly increased the numbers of both early apoptotic and late apoptotic/necrotic cells in TNF-treated L929 cultures, while C$_{60/70}$(OH)$_n$ clearly reduced TNF-triggered apoptosis/necrosis (Fig. 3b). In addition, although unable to significantly activate caspases if applied alone, THF/nC$_{60/70}$ caused synergistic potentiation of TNF-induced caspase activation in L929 cells (Fig. 3c,d). On the other hand, TNF-mediated caspase activation was completely blocked by simultaneous addition of C$_{60/70}$(OH)$_n$ (Fig. 3c,d). Therefore, the opposite effects of THF/nC$_{60/70}$ and C$_{60/70}$(OH)$_n$ on TNF cytotoxicity were associated with the enhancement and suppression, respectively, of TNF-induced caspase activation and apoptotic/necrotic cell death.

The Effects of Fullerene Preparations on TNF-induced ROS Production and Mitochondrial Depolarization

We next investigated the interference of THF/nC$_{60/70}$ and C$_{60/70}$(OH)$_n$ with the intracellular events responsible for TNF-induced cell death. A well-known antioxidant agent *N*-acetylcysteine and cyclosporine A, an inhibitor of mitochondrial depolarization, both partially improved the viability of TNF-treated L929 cells (Fig. 4a,b), thus indicating the involvement of oxidative stress and subsequent mitochondrial failure in TNF cytotoxicity. Accordingly, treatment with TNF led to a significant production of ROS and loss of mitochondrial membrane potential, as demonstrated by the fluorescence of redox-sensitive dye DHR (Fig. 4c) and increase in green vs. red fluorescence of mitochondrial-binding dye DePsipher (Fig. 4d). THF/nC$_{60/70}$ itself increased intracellular amount of ROS to a certain extent, while the cells treated with both THF/nC$_{60/70}$ and TNF produced more ROS than in response to each agent alone (Fig. 4e). In contrast, C$_{60/70}$(OH)$_n$ did not cause intracellular accumulation of ROS and completely prevented the TNF-triggered oxidative stress (Fig. 4e). Similar pattern of action of the two C$_{60/70}$ preparations was observed when TNF-induced mitochondrial depolarization was monitored in L929 cells. At the concentrations used, fullerene preparations did not significantly affect mitochondrial membrane potential in L929 cells (Fig. 4f). The TNF-mediated mitochondrial depolarization, however, was markedly enhanced by THF/nC$_{60/70}$ and efficiently prevented by C$_{60/70}$(OH)$_n$ (Fig. 4f). Therefore, the opposite effects of nC$_{60}$ and C$_{60}$(OH)$_n$ on TNF cytotoxicity could partly stem from their ability to augment and reduce, respectively, TNF-triggered ROS generation and subsequent mitochondrial depolarization.

Synergistic Cytotoxicity of TNF and THF/nC$_{60/70}$ Confers Selectivity Towards Tumor Cells

It has previously been reported that THF-prepared nC$_{60}$ at high doses is toxic to various types of transformed and primary cells, including mouse peritoneal macrophages (12). Therefore, we wanted to examine whether combination of subtoxic doses of THF/nC$_{60/70}$ and TNF could provide selective cytotoxicity toward TNF-sensitive L929 cells, without damaging TNF-resistant macrophages. Neither THF/nC$_{60/70}$ nor TNF alone at subtoxic concentrations were able to markedly reduce viability of L929 cells or peritoneal macrophages (Fig. 5a,b). However, while combination of THF/nC$_{60/70}$ and TNF exerted synergistic toxicity toward L929 cells (Fig. 5a), the viability of primary macrophages was not significantly affected (Fig. 5b). It therefore appears that cytotoxicity of THF/nC$_{60/70}$ could be selectively targeted to TNF-sensitive tumor cells by combining non-cytotoxic doses of THF/nC$_{60/70}$ with TNF.

Distinct Regulation of Cell Death by Fullerenes is not Specific for Cytotoxic Stimulus or Cell Type

Finally, we investigated whether the observed distinct effects of THF/nC$_{60/70}$ and C$_{60/70}$(OH)$_n$ are restricted to TNF-induced death of L929 cells, or they could be relevant for other cytotoxic stimuli and cell types as well. To that effect, we used human glioma cell line U251 treated with the classic anticancer drug cisplatin or hyperthermia (1h at 43°C), both known for their ability to induce oxidative stress-mediated cell death (38,39). Accordingly, both treatments significantly reduced the cell number in U251 glioma cultures (Fig. 6a–c). Consistent with the results obtained with TNF-treated L929 cells, THF/nC$_{60/70}$ or C$_{60/70}$(OH)$_n$ potentiated or inhibited, respectively, both cisplatin- and hyperthermia-mediated kill-

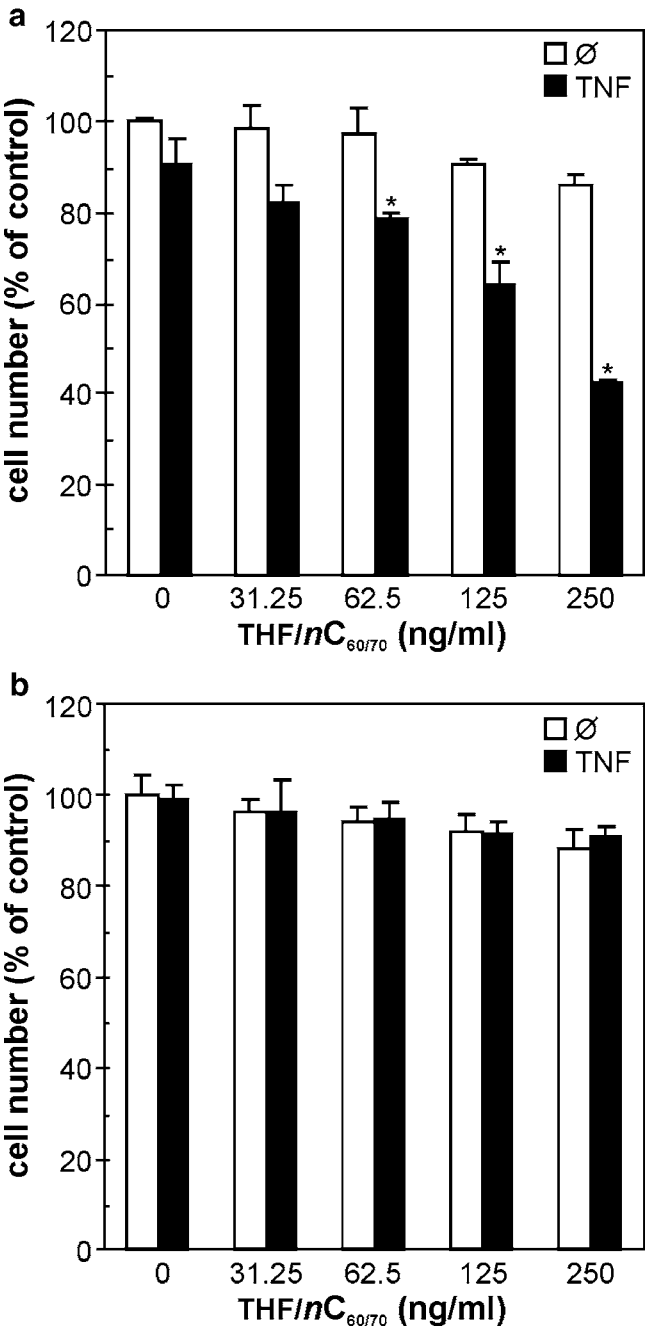


Fig. 5. Synergistic cytotoxicity of TNF and THF/$nC_{60/70}$ confers selectivity towards tumor cells. L929 cells (**a**) or peritoneal macrophages (**b**) were incubated with or without TNF (125 pg/ml) and/or different concentrations of THF/$nC_{60/70}$, and the cell number was assessed after 24 h. The data are mean ± SD values of triplicates from a representative of three independent experiments (*$p < 0.05$ refers to cells exposed to TNF or THF/$nC_{60/70}$ alone).

ing of glioma cells (Fig. 6a–c). Similar results were obtained with cisplatin- or hyperthermia-exposed L929 cells (data not shown).

DISCUSSION

The present report clearly demonstrates the ability of two different fullerene preparations, nanocrystalline $C_{60/70}$

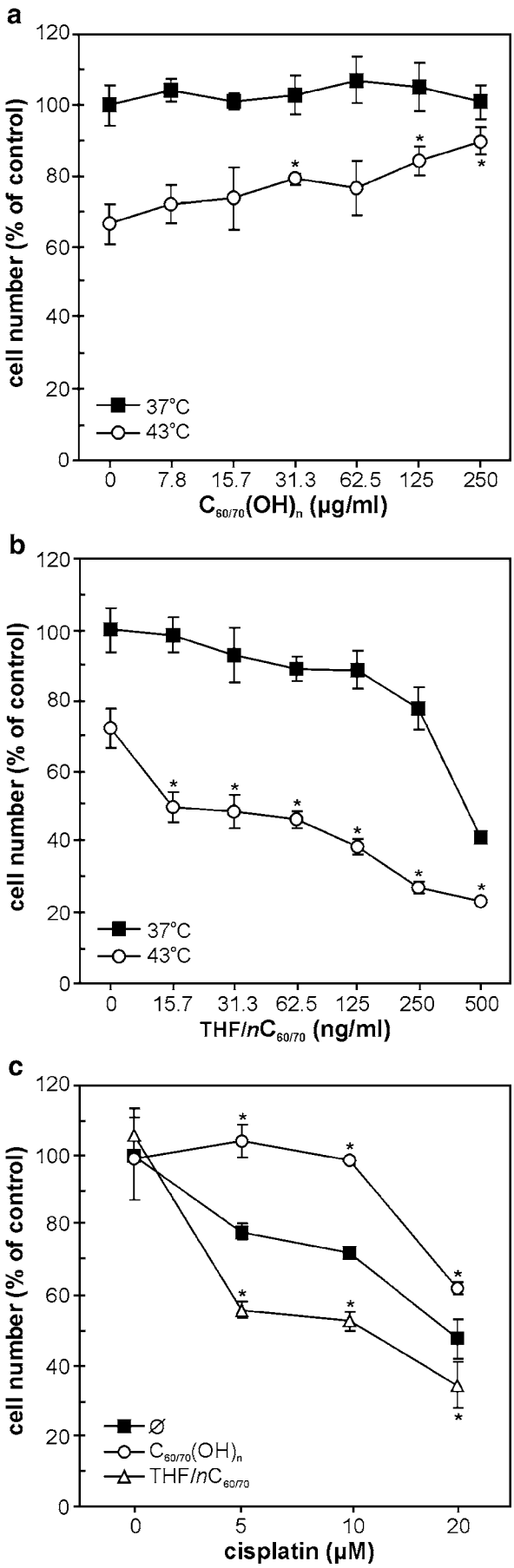


Fig. 6. The effects of fullerene preparations on hyperthermia- or cisplatin-induced death of U251 glioma cells. **a**, **b** U251 cells were exposed to hyperthermia (1 h at 43°C) in the presence or absence of $C_{60/70}(OH)_n$ (**a**) or THF/$nC_{60/70}$ (**b**). **c** U251 cells were incubated with cisplatin, in the absence or presence of $C_{60/70}(OH)_n$ (250 μg/ml) or THF/$nC_{60/70}$ (125 ng/ml). **a–c** The data are mean ± SD values of triplicates from a representative of three independent experiments (*$p < 0.05$ refers to cells exposed to hyperthermia or TNF alone).

(THF/$nC_{60/70}$) and polyhydroxylated $C_{60/70}$ [$C_{60/70}(OH)_n$], to augment and antagonize, respectively, the cytotoxicity of the main proinflammatory cytokine TNF. The mechanisms underlying the observed effects possibly involved the interaction of fullerene preparations with TNF-induced oxidative stress and subsequent mitochondrial depolarization in target cells (Fig. 7). It should be noted that the UV absorbance spectra of the two fullerene preparations did not change in the presence of TNF (Harhaji *et al.*, unpublished), thus arguing against the possibility that the physico-chemical interaction between fullerene particles and TNF was involved in the observed effects. This was further confirmed by the failure of fullerenes to interfere with some other TNF-induced responses, such as expression of inducible nitric oxide synthase in the TNF-resistant C6 glioma cell line (Harhaji *et al.*, unpublished). While cytotoxic and cytoprotective properties of THF-prepared nanocrystalline and polyhydroxylated fullerenes have previously been observed, this is the first report describing their ability to modulate TNF-triggered cell death. It is important to note that the colloidal fullerene suspension used in the present study was prepared from the mixture of C_{60} (80%) and C_{70} (20%). Nevertheless, our previous studies performed with nanocrystals made from pure C_{60} and C_{60}/C_{70} mixture (11,12), as well as preliminary results with pure C_{70} (Markovic *et al.*, unpublished), suggest that THF/nC_{60} and THF/nC_{70} might behave similarly, at least with regard to their ability to exert ROS-dependent cytotoxicity.

Apoptosis and necrosis are two main cell death modes with distinct morphological appearance and profoundly different implications for the surrounding tissues (40, 41). The ability of TNF to induce apoptosis or necrosis has been a matter of controversy, since different groups reported that TNF-mediated L929 cell killing proceeds preferentially through apoptotic (42–44) or necrotic pathway (29, 30, 45), possibly depending on the L929 subclone used. In some studies, however, both apoptotic (internucleosomal DNA fragmentation and/or phosphatydilserine exposure) and necrotic changes (cell membrane damage) were observed in the same cell culture following the TNF treatment (46–48), thus leading some researchers to coin the terms such as "atypical apoptosis" and "necrapoptosis" (49,50). This is consistent with the appearance of two distinct cell populations in TNF-treated L929 cells in our experiments, showing the characteristics of either apoptotic (phosphatydilserine exposure on the intact cell membrane) or necrotic cell death (the loss of cell membrane integrity). Interestingly, the modulation of TNF toxicity by THF/$nC_{60/70}$ or $C_{60/70}(OH)_n$ was associated with enhancement or suppression, respectively, of both apoptosis and necrosis, indicating that the fullerenes might affect some intracellular event that is shared by both cell death pathways. It should be noted, however, that we could not exclude the possibility that the cell population with membrane damage in our experiments actually consisted of late apoptotic, rather than genuinely necrotic cells, in which case the fullerene interference with cell death would be limited to apoptosis induction.

Recent data indicate that process called mitochondrial permeability transition (MPT), which precedes the loss of mitochondrial membrane potential, could be a converging point for various cell death signals, including those triggered by TNF (50,51). The MPT pore opening and mitochondrial depolarization are associated with the subsequent release of small molecules such as cytochrome c, leading to activation of apoptosis-initiating cysteine proteases caspases. However, such perturbation of mitochondrial function may ultimately culminate not only in apoptosis, but also necrosis, or the mixture of both, depending on cellular ATP level and other modifying factors (50,51). The loss of mitochondrial membrane potential induced by TNF treatment in our study, as well as cytoprotective effect of an MPT blocker cyclosporin A, indeed support the involvement of mitochondria in TNF toxicity toward L929 cells. Moreover, our data indicate that the ability of THF/$nC_{60/70}$ and $C_{60/70}(OH)_n$ to modulate TNF-dependent apoptosis/necrosis might rely on the interference with TNF-induced mitochondrial depolarization, which is consistent with the protective effect of carboxyfullerenes on TNF + cycloheximide-induced mitochondrial dysfunction in human peripheral blood mononuclear cells (31). This may also explain the observed fullerene-mediated modulation of caspase activation, having in mind the role of mitochondria-released factors in initiation of caspase cascades and subsequent apoptosis. However, our results that fullerene-mediated potentiation (THF/$nC_{60/70}$) and inhibition [$C_{60/70}(OH)_n$] of caspase activation were respectively associated with increased

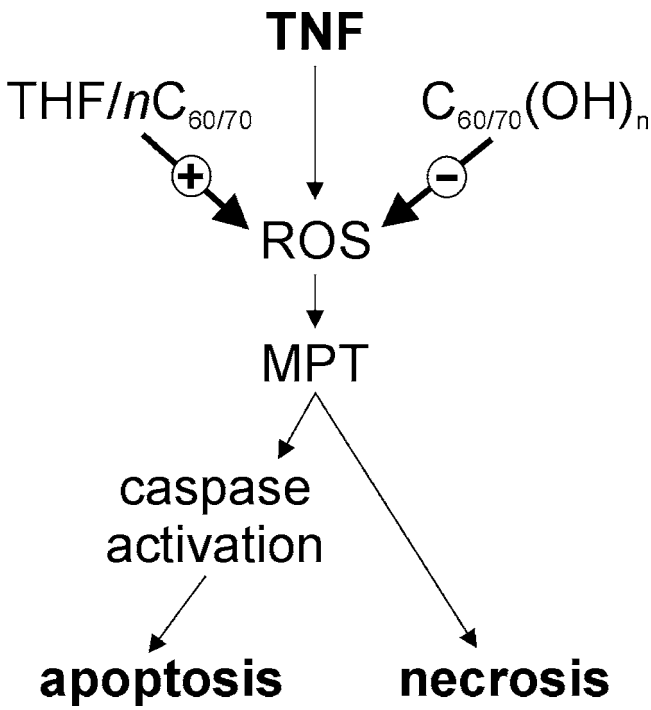


Fig. 7. The mechanisms of TNF cytotoxicity modulation by fullerene preparations. TNF induces caspase-dependent apoptosis and caspase-independent necrosis, presumably through ROS-dependent mitochondrial permeability transition (MPT). THF/$C_{60/70}$ and $C_{60/70}(OH)_n$ could increase or decrease, respectively, TNF-triggered apoptotic and necrotic cell death by modulating ROS production and the subsequent onset of MPT.

and reduced TNF cytotoxicity, might seem inconsistent with the findings that caspase inhibition in TNF-treated L929 cells actually enhances their sensitivity to necrosis and augments cell death (29,30,45). A conceivable explanation for this apparent discrepancy is that the caspase-modulating effects of the two fullerene preparations, unlike those of the selective caspase inhibitors, are positioned upstream of caspase activation, presumably at the mitochondrial level. In that way, fullerenes might affect a broad spectrum of cell death-associated events, including those related to necrotic cell death pathway, and exert effects different from those of selective caspase inhibition.

There is also a question of the mechanisms underlying the interference of the fullerenes with TNF-induced mitochondrial dysfunction. It has been known that the excessive ROS generation could cause MPT pore opening, while, in turn, the collapse of the mitochondrial membrane potential triggers an increase in ROS generation by the electron transfer chain, thus creating a vicious circle that leads to further mitochondrial and cellular injury (52). Indeed, the results of several studies (28–30), including our experiments with the antioxidant agent *N*-acetylcysteine, strongly support the involvement of oxidative stress in the cytotoxic action of TNF. Moreover, the finding that ROS generation precedes TNF-triggered depolarization of mitochondrial membrane points to an oxidative stress as a primary cause for mitochondrial damage in TNF-treated cells (53). Accordingly, the cytoprotective effect of polyhydroxylated fullerenes was associated with the reduction of TNF-triggered ROS production. Having in mind the well-known ability of fullerols to act as "free radical sponges" (1,7,8,11), it is conceivable to assume that this effect was due to a "passive" scavenging of ROS, rather than to an "active" interference with TNF-induced generation of reactive oxygen. On the other hand, nontoxic dose of THF/nC$_{60/70}$ itself significantly increased intracellular amount of ROS and enhanced TNF-induced ROS generation. However, the effects of TNF and THF/nC$_{60/70}$ on ROS generation were only additive, thus questioning the role of oxidative stress in the synergistic cytotoxicity of the two agents. Moreover, it should be noted that, despite the fairly lower ability for the oxidative stress induction in comparison with THF/nC$_{60/70}$, TNF was much more efficient in causing ROS-dependent mitochondrial depolarization resulting in cell demise. A plausible explanation for these discrepancies could be that TNF and THF/nC$_{60/70}$ might induce reactive oxygen intermediates that are different and/or produced at distinct intracellular sites. Namely, while TNF treatment leads to production of superoxide by mitochondria and subsequent mitochondrial dysfunction (53,54), solvent exchange-prepared C$_{60}$ nanocrystals apparently generate singlet oxygen (15,55) and accumulate mainly at the surface of cell membrane, causing its damage through lipid peroxidation (9,10).

CONCLUSION

The findings of the present study might be relevant for investigation of fullerenes as possible anticancer or cytoprotective therapeutics. A synergistic enhancement of TNF cytotoxic action by THF/nC$_{60/70}$ could be exploited in order to reduce the toxicity of each agent alone, while still enabling efficient killing of TNF-sensitive tumor cells. As an example of such an approach, we have demonstrated how combining the noncytotoxic concentrations of TNF and THF/nC$_{60/70}$ can confer selectivity toward L929 fibrosarcoma cells without harming primary macrophages. On the other hand, fullerene-mediated protection from TNF cytotoxicity might be potentially useful in preventing TNF-mediated tissue damage associated with TNF-based therapy or various inflammatory/autoimmune conditions. While polyhydroxylated fullerenes, because of the high retention in bone tissues (56), may not be likely drug candidates for the cytoprotective therapy, our data nevertheless support development of other eliminable fullerene derivatives, such as malonic acid derivatives, for such purpose. Finally, we have shown that the observed effects of the two fullerene preparations are not restricted to cytotoxic action of TNF, but that they could also pertain to different oxidative stress-dependent cell death paradigms, such as cisplatin- or hyperthermia-mediated cytotoxicity. Our study therefore warrants further investigation of potential usefulness of fullerenes as either prooxidant/cytotoxic or antioxidant/cytoprotective therapeutics.

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