Center of Analysis and Testresearch[1], Department of Pharmacology and Toxicology[2], East China University of Science and Technology, Shanghai, P.R. China

Cytotoxicity of a newly synthesized nitroxide derivative of 4-ferrocenecarboxyl-2,2,6,6-tetramethylpiperidine-1-oxyl in high metastatic lung tumor cells

Y. Wu[1], W. Tang[2], C. L. Li[2], J. W. Liu[2], L. D. Miao[1], J. Han[1], M. B. Lan[1]

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Prof. M. B. Lan, Center of Analysis and Testing, East China University of Science and Technology, 130 Meilong Road, Shanghai 200237, P.R. China
minbolan@ecust.edu.cn

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Low molecular weight nitroxides are widely used as electron paramagnetic resonance (EPR)-detectable spin labels in the biological and pathological areas. A novel nitroxide derivative, 4-ferrocenecarboxyl-2,2,6,6-tetramethyl piperidine-1-oxyl (FC-TEMPO), was synthesized for evaluating the effects of spin label compounds on tumor cells and firstly its biological effects on tumor and normal cells were evaluated. The cytotoxicity of FC-TEMPO in the high metastatic lung carcinoma cells (95-D) showed that it markedly inhibited the viability of cancer cells in a dose-dependent manner, while it was less toxic to a normal human cell line. Further studies found that FC-TEMPO suppressed the growth of tumor cells by induced apoptosis through activating caspase-3 but not caspase-8 which was proved by caspase inhibitors, and the cell cycle arrest at G_1 phase. Moreover, the concomitant increase of superoxide dismutase (SOD) and catalase (CAT) activity was observed. Taken together, these results might provide a base for further anticancer investigations of nitroxides and their potential pharmacological applications.

1. Introduction

Low molecular weight nitroxides are stable free radicals widely used as electron paramagnetic resonance (EPR)-detectable spin labels in the biological and pathological areas (Lai et al. 1980; Mader et al. 1992; Brasch et al. 1983; Baker et al. 1997). Studies indicate that some piperidine nitroxides as antioxidants can exert cytoprotective action against oxidative stress induced by radiation (Mitchell et al. 1991), cytotoxic drugs (Monti et al. 1996), and hyperoxia-induced brain damage (Howard et al. 1996). However, this class of nitroxide compounds can also exert pro-oxidative and cytotoxic effects, in particular on cancer cells, depending on the specific microenvironment and the time/dose of cells exposure to drugs (Voest et al. 1986; Gariboldi et al. 1998). Piperidine nitroxides suppressed tumorigenesis as a cell proliferation modifier and apoptosis inducer (Suy et al. 2005; Gariboldi et al. 2003). Furthermore, the toxic effects on tumor cells could be due to the presence or structure of substituent(s) at position 4 of the piperidine nitroxide radical (Koceva-Chyla et al. 2000; Metodiewa et al. 2000, 1999). Moreover, some anticancer drugs with this nitroxide substituent possessed less toxic and more active than their parent drugs (Sosnovsky and Rao 1991).
Taking into account the role of substituent(s) of the piperidine nitroxides in the cytotoxic effects of nitroxides on cancer cells, a ferrocene group was considered to be introduced at position 4 of the piperidine nitroxide radical because some studies indicated that ferrocene derivatives had the potential anticancer activity (Popova et al. 1993; Tamura and Miwa 1997). Moreover ferrocene derivatives can be oxidized to ferrocenium cation radical. There was evidence to show that the reduction of ferrocenium ions *in vivo* generated active oxygen radicals such as hydroxyl, which was responsible for cancer cells damage through the formation of radical metabolites (Osella et al. 2000).
In this study, a novel piperidine nitroxide derivative FC-TEMPO was synthesized and developed (Lan et al. 2005). The cytotoxicity of this compound was detected on highly metastatic lung tumor cells (95-D). Results showed that it markedly inhibited the viability of 95-D cells in a dose-dependent manner, while it was less toxic to the corresponding normal cell line. Further studies found that FC-TEMPO could exert cytotoxic effects by induction of apoptosis through activating caspase-3 and cell cycle arrest at G_1 phase.

2. Investigations and results

2.1. Cytotoxicity of FC-TEMPO in 95-D and HLF cells

FC-TEMPO suppressed the tumor cell growth in a dose dependent manner, which was detected by the assay of MTT [3-(4,5-dimethylthiazo l-2-yl)-2,5-diphenyltetrazolium bromide] reduction and lactate dehydrogenase (LDH) release. The growth inhibition effects of FC-TEMPO and two substrates on 95-D cells are shown in Fig. 1A. About 50%, 70% percent of tumor cells were suppressed under the treatment of FC-TEMPO at 390, 520 μM for 48 h, respectively.

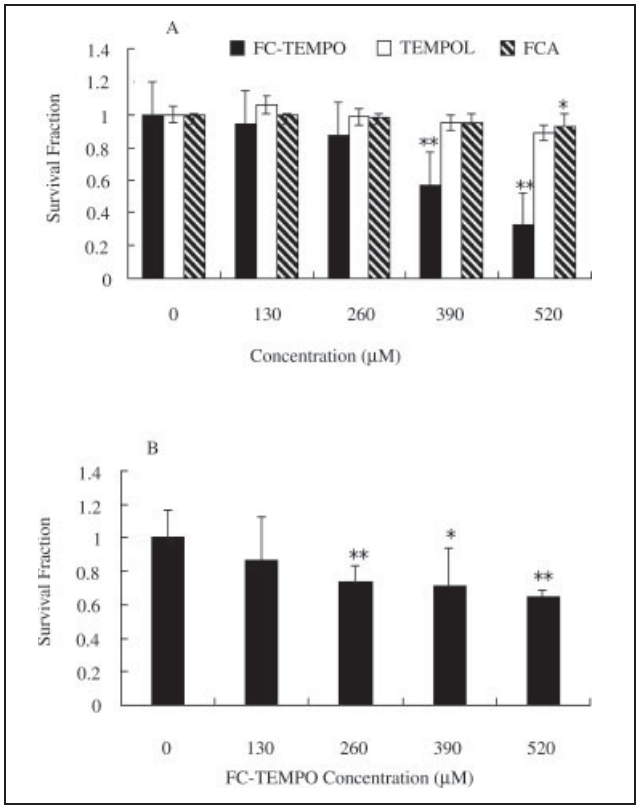


Fig. 1: Growth inhibition effects of TEMPOL, FCA and FC-TEMPO on 95-D cells (A) and FC-TEMPO on HLF cells (B). 1×10^5/ml cells after 48 h exposure to indicated concentrations of compounds. The MTT test was used to assess the effects of compounds on cells growth. Results shown are representative data of three individual studies. Asterisks indicate significant difference from control (*) $p < 0.05$, (**) $p < 0.01$, (***) $p < 0.001$

According to the data shown in Fig. 1A, other conclusions can be drawn: (a) ferrocenecarboxylic acid (FCA) and 4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPOL) had little effect on cell viability. (b) FC-TEMPO possessed obvious cytotoxicity for 95-D cells in dose-dependent manner. These indicated that the inhibition ratio of FC-TEMPO was higher than its two substrates under the same conditions. Moreover, the cytotoxicity of FC-TEMPO on human normal lung fibroblast (HLF) cells was less than on tumor cells based on the data shown in Fig. 1B, which shows that the percent of viable normal cells was over 70% at the 390 μM. The effects of FCA and TEMPOL on HLF cells were similar to those of FC-TEMPO (data not shown).

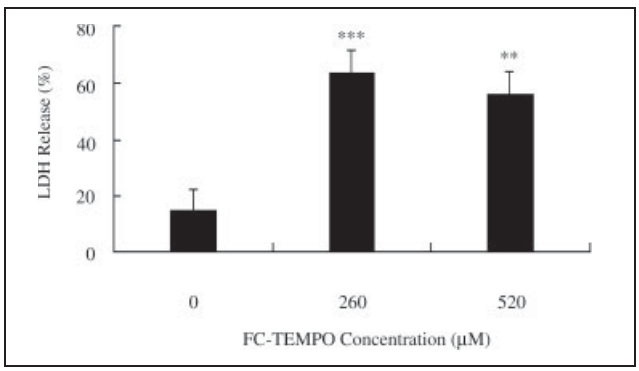


Fig. 2: LDH release from 95-D cells treated by FC-TEMPO at concentrations of 0 μM, 260 μM, 520 μM. Cells were analyzed as described in "Experimental". Results were normalized to 100% cells death caused by sonication. The results are shown as mean ± SD. Asterisks indicate significant difference from control (*) $p < 0.05$, (**) $p < 0.01$, (***) $p < 0.001$

LDH is a metabolic enzyme in cytosol. Under the stress environment, LDH could be releases to the extracellular medium if the cell membrane is disturbed. Fig. 2 showed that FC-TEMPO stimulated the release of LDH from the cytosol of tumor cells to the extracellular medium. About a 3-fold increase in LDH activity was observed at both 260 μM and 520 μM for cell exposure to drugs 48 h compared with the control. There was the significant difference of LDH release between the control and treated cells. But the release did not exhibit a difference between 260 μM and 520 μM.

2.2. Induction of apoptosis through activating caspase-3 and cell cycle arrest at G_1 phase

Induction apoptosis of tumor cells is often responsible for the growth inhibition. In this experiment, the induced apoptosis in tumor cells was detected as showed in Fig. 3. Apoptosis cells induced by FC-TEMPO at the concentra-

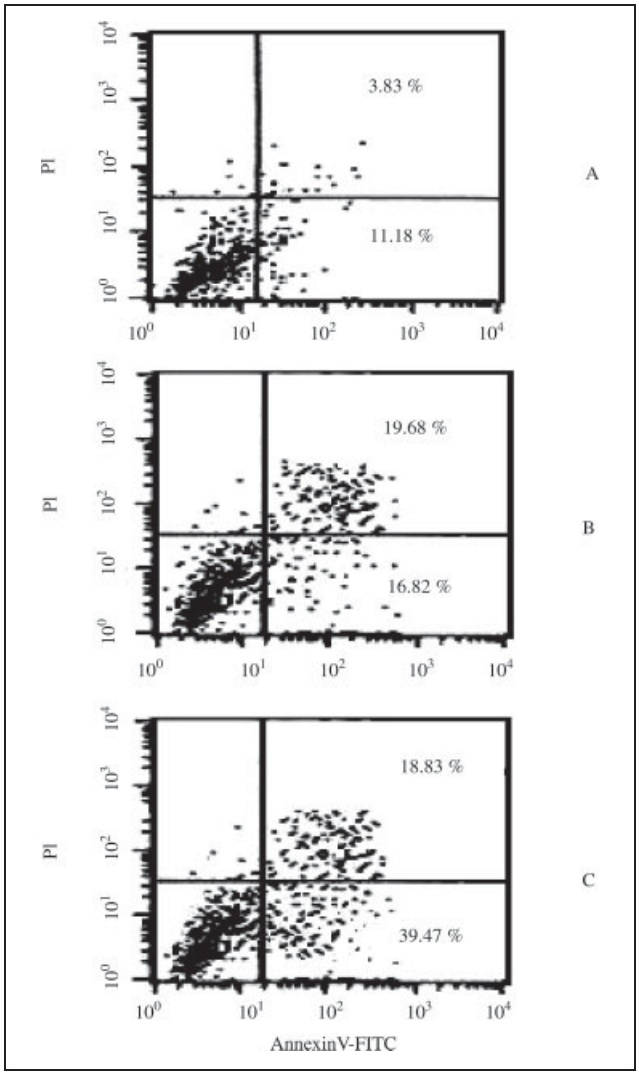


Fig. 3: Apoptosis was induced in 95-D cells under the FC-TEMPO treatment for 48 h: (A) 0 μM as control; (B) 260 μM; (C) 520 μM. Cells were double stained with annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (PI) and analyzed by flow cytometry as described in "Experimental". Each panel shows the relative distribution of viable cells (lower left quadrant), early apoptotic cells (lower right quadrant), and late-stage apoptotic/necrotic cells (upper right quadrant). Results shown are representative data of three individual studies

tion of 260, 520 μM for 48 h were about 2-fold and 3-fold respectively compared with controls. Treatment of 95-D cells with the compound resulted in a dose-dependent increase of early apoptosis.

In general, induced apoptosis is often associated with caspases activity, in paticular caspase-3 and caspase-8. Caspases as proteases play a key role in the downstream of the apoptosis pathway in mammalian cells. In this work, the activity of caspase-3 and caspase-8 was detected in 95-D cells treated by FC-TEMPO. The data are shown in Fig. 4A. FC-TEMPO remarkably stimulated the activity of caspase-3 in tumor cells in a dose dependent manner. The activity of caspase-3 increased about 2 fold under the concentration of 260 μM and 520 μM compared with the control. In contrast, the activation of caspase-8 let not to a significant difference between treated cells and the control (Fig. 4B). To identify which caspases are functionally important for FC-TEMPO induced apoptosis, selective inhibitors of caspases were applied in the investigation. As shown in Fig. 5, the caspase-3 inhibitor or pan-caspase inhibitor alone could significantly reduce the apoptosis mediated by FC-TEMPO, while the caspase-8 inhibitor did not suppress the apoptosis induction. The data suggest that FC-TEMPO could activate the activity of caspase-3 but not caspase-8 in the tumor cells.

Cell cycle arrest of tumor cells may also be responsible for the growth inhibition. In this experiment, the cell cycle arrest in tumor cells was observed as shown in the Table. The cell cycle block was dose-dependent. After cells were treated by FC-TEMPO for 48 h, the percent of cells at G_1 phase was increased from 49% to 70% at the drugs concentrations range from 130 μM to 520 μM, while the corresponding cell phase was decreased, such as S phase and G_2/M phase.

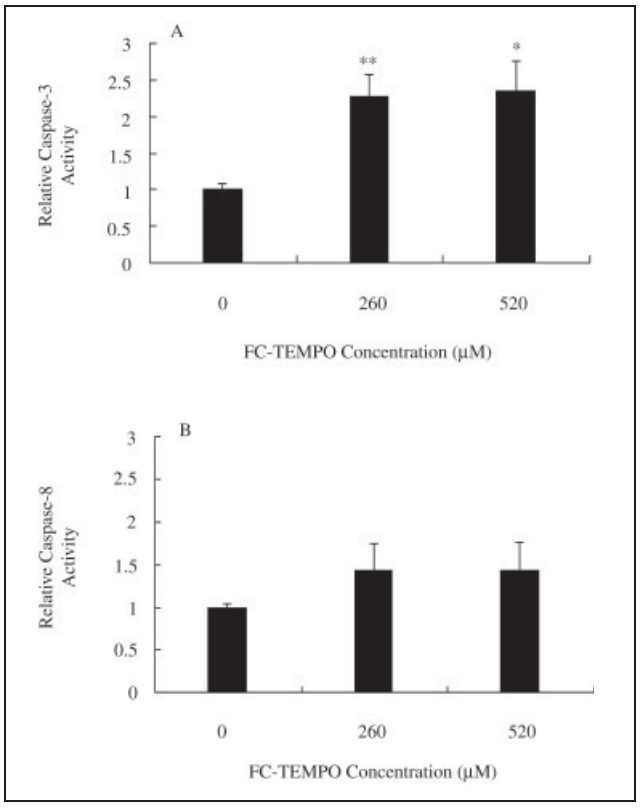


Fig. 4: Activities of caspase-3 and caspase-8 in tumor cells treated by FC-TEMPO. 95-D cells (1×10^6/ml) was treated with 260, 520 μM of FC-TEMPO and drug-free medium at 37 °C for 48 h, respectively. Caspase-3 (A) and caspase-8 (B) activities in the cytosolic extracts of cells were analyzed as described in "Experimental". The caspase-3 activity of cells treated by compound significantly increased from controls with the Student's t-test, (*) $p < 0.05$, (**) $p < 0.01$, (***) $p < 0.001$. However there was no statistical difference between caspase-8 activities in cells with compound and controls ($P < 0.05$). The results are shown as mean ± SD

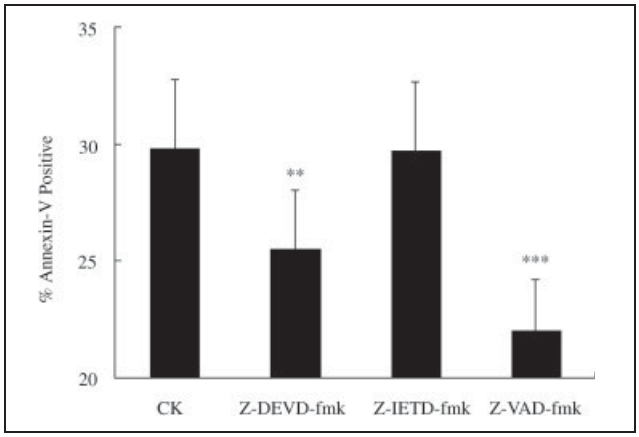


Fig. 5: The effects of caspase inhibitors on the apoptosis induction by FC-TEMPO. 95-D cells (1×10^6/ml) were pre-incubated for 60 min at 37 °C with Z-DEVD-fmk, 10 μM; Z-IETD-fmk, 10 μM; Z-VAD-fmk, 10 μM, before adding FC-TEMPO to the cultures. Cells were treated by 520 μM FC-TEMPO as described in "Experimental". Percentage of apoptosis was quantified by annexin-V staining, in the presence or absence of selected caspase-8, -3 and pan-caspases inhibitors. The results are shown as mean ± SD. Asterisks indicate significant difference from control (*) $p < 0.05$, (**) $p < 0.01$, (***) $p < 0.001$

Table: Effects of FC-TEMPO on cell cycle parameters of 95-D cells after 48 h exposure

FC-TEMPO (μM)	G_1 phase (%)	S phase (%)	G_2/M phase (%)
0	41.02	42.34	16.64
130	49.91	37.44	12.65
260	58.08	28.54	13.38
520	70.41	21.59	8.00

2.3. Increase of SOD and CAT in 95 cells

FC-TEMPO is a class of nitroxide derivative, the cytotoxicity of FC-TEMPO probably involved in intracellular oxidative stress. After 95-D cells were treated by FC-TEMPO for 48 h, the intracellular redox enzymes activities were altered. As shown in Fig. 6, FC-TEMPO increased the activities of SOD and CAT in 95-D cells. Moreover, the level of SOD was enhanced in a dose-dependant manner. The results indicated that FC-TEMPO could disturb the redox environment of 95-D tumor cells at 260 μM.

3. Discussion

In this work, TEMPOL and FCA did not markedly suppress the growth of 95-D cells in our experimental concentrations, but FC-TEMPO had significantly cytotoxicity under the some conditions. This toxic effect of FC-TEMPO could result from the presence of the ferrocenecarboxyl substituent at position 4 of the piperidine nitroxide radical. The cytotoxic effects caused by the ferrocenecarboxylic moiety can be explained in two ways: one is that the ferrocene derivative interacts with DNA (Vashisht et al. 2000); another aspect is that the addition of a ferrocenecarboxylic substituent possibly introduces the oxidizing/reducing genotoxic effects (Top et al. 2003).

FC-TEMPO inhibited the growth of tumor cells by apoptosis induction and cell cycle arrest at the same time.

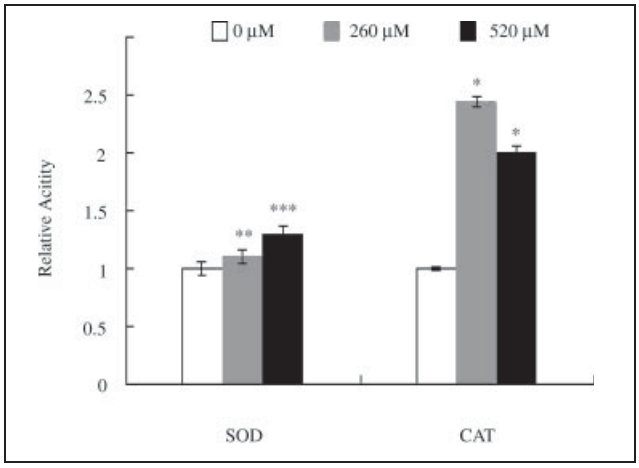


Fig. 6: The effect of FC-TEMPO on redox enzymes in 95-D cells after 48 h exposure. 2×10^6 cells were incubated with 260, 520 μM FC-TEMPO for 48 h and untreated cells were used as controls. The activities of CAT and SOD in cells were detected as described in "Experimental". Results are the mean ± SD of triplicates experiments. Asterisks indicate significant difference from control (*) $p < 0.05$, (**) $p < 0.01$, (***) $p < 0.001$

Caspase cascade plays a key role in apoptosis procedure. Caspase-3 typically functions at the downstream of other caspases and directly activates enzymes that are responsible for DNA fragmentation in intrinsic apoptosis pathway. Caspase-8 is considered a signaling and key caspase in the extrinsic pathway. Caspase-8 activation occurs either in association with the death receptor or via the stress-induced signaling pathway involving mitochondria. Our data suggest that activation of caspase-3 but not caspase-8 was involved in the tumor cells apoptosis induced by FC-TEMPO. The use of caspase inhibitors further proved that caspase-3 was associated with the induced apoptosis. Consistent with this conclusion is the finding that the caspase-8 inhibitor had no additive effect on preventing FC-TEMPO mediated apoptosis. Taken together, these results strongly suggest that apoptosis is induced by FC-TEMPO through the intrinsic pathway related to mitochondria.

Cell cycle assay showed the compound affected the cells progression through the accumulation of cells in the G_1 phase in a dose-dependent manner, with concomitant decrease in S and G_2/M cells. These results give hints at the damage mechanism induced by FC-TEMPO, which is associated both with the control of cell cycle progression and with the triggering of cells death. If the damage is regarded as repairable, cell progression is slowed down through the different phases in cell cycle and cells attempt to repair the damage. In contrast, when the damage is extensive and impossible to repair, cell death is triggered and cells are first killed in the G_1 phase of cell cycle. After cells 48 h exposure to high concentrations of FC-TEMPO, the triggering of an apoptotic mechanism may support the mode of the cell death induced by the compound.

Piperidine was nitroxides activated a program of apoptotic cell death possibly by inducing an oxidative stress dependent on free radical-mediated mechanisms (Gariboldi et al. 2000), so the FC-TEMPO cytotoxicity effects on tumor cells were possibly involved in the redox unbalance of intracellular environment. After 95-D cells were treated by FC-TEMPO, the activities of SOD and CAT in cells were increased. This result indicated that the intracellular redox status had been changed. Further investigation will be carried out since the metabolism of nitroxides in biological environment is very complex.

From the data mentioned above, we can draw the following conclusions: FC-TEMPO could exert cytotoxicity on tumor cells by cell cycle arrest and apoptosis induction. The apoptosis was possibly mediated by oxidative stress though activity of caspase-3. The anticancer efficacy displayed by FC-TEMPO is a potential benefit for the therapeutic use of this class of compounds.

4. Experimental

4.1. Reagents and chemistry

IR spectra (KBr) were recorded using a Nicolet Magna-IR 550 spectrometer. Mass spectra were recorded using a Micromass GCT spectrometer. Elemental analyses were obtained from Elementar Vario EL III. The EPR spectra were recorded on the Bruker EMX-8 series equipment under aerobic conditions. Melting points were determined on a SGWX-4 apparatus with a calibrated thermometer. All reagents were purchased from Sigma (St. Louis, USA) and Chinese medicine Co. Ltd (Shanghai, China) except additional illustration. All reagents were analytical grade.

4.2. Synthesis of FC-TEMPO

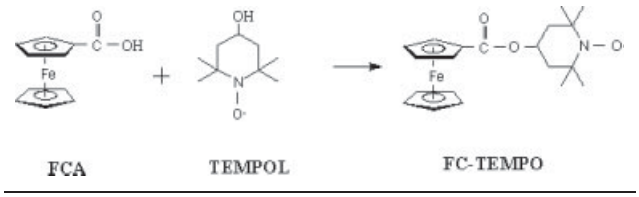
Freshly prepared ferrocenecarboxylic acid chloride 0.497 g (2 mmol) as described previously (Tomonori et al. 1977) was dissolved in dry petroleum ether (40 ml), and then added gradually to a stirred solution of 0.344 g (2 mmol) 4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPOL) in anhydrous ether (30 ml) and 0.3 ml of pyridine in an ice bath nitrogen atmosphere. The reaction was allowed to come to room temperature and stirring was continued for 36 h. The reaction mixture was filtered and the filtrate was evaporated under vacuum to dryness. The residue was purified using a silica gel chromatography column with a 15 : 1 (v/v) petroleum ether/ethyl acetate mixture as mobile phase (scheme). EPR spectrum of the product is shown in Fig. 7.

FC-TEMPO was orange crystal (m.p. 175–177 °C). MS (ESI, m/e): 384.1260 (M^+). IR(KBr, cm^{-1}): 3103.25, 2919.78, 2850.90, 1706.22, 1462.52, 1373.95, 1282.90, 1139.93. Elemental analyses: Calcd for $C_{20}H_{26}FeO_3N$: C, 62.56; H, 6.82; N: 3.65. Found: C, 62.62; H, 6.90; N: 3.39. EPR (toluene): $g = 2.0063$, $\alpha_N = 15.43G$.

4.3. Cell line and culture conditions

Human highly metastatic lung tumor cell line (95-D) and normal human lung fibroblast (HLF) cells were purchased from the Center of Cell Culture

Scheme



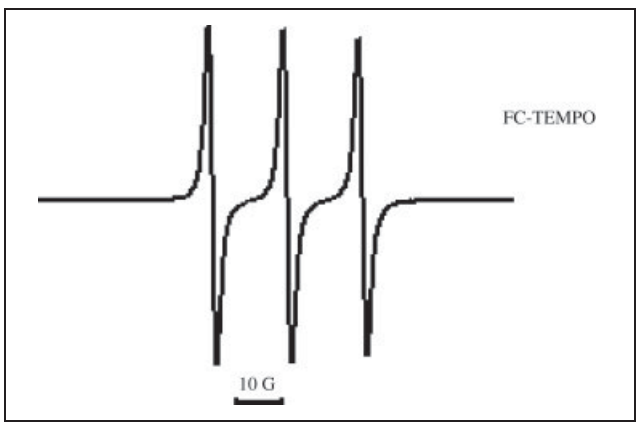


Fig. 7: The EPR spectra of FC-TEMPO under aerobic condition. The following parameters were used for the measurement: modulation frequency 100 kHz; microwave power 6.331 mW; receiver gain 6.32e + 002; modulation amplitude 0.1G; time constant 20.48 ms; scan range 100G.

Collection of Academica Sinica (Shanghai, China). The 95-D cell was cultured in RPMI 1640 medium, while the HLF cell line was cultured in DMEM medium, containing 10% heat-inactivated fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin in an atmosphere of 5% CO_2, 95% air at 37 °C. The cells (1×10^5/ml) at exponential growth phase were treated with compounds at various concentrations. Each experiment was repeated three times by using separate cell cultures each time.

4.4. Cell viability assay

Tumor cell viability was determined by the MTT reduction method, as described by Alley et al. (1988). The 1×10^5/ml cells were plated in 96 well tissue culture plates and allowed to grow for 4 h prior to treatment with different concentrations drugs. After 48 h incubation, the cells were stained with MTT for 4 h, the formazan crystal formed by mitochondrial reduction of MTT was dissolved in dimethyl sulfoxide (DMSO) and the absorbance was read at 570 nm for the measure of cell viability. Effects of the drugs on inhibition of cell growth were calculated and cells treated with DMSO at the same concentrations carried by the drugs were used as controls.

4.5. LDH release assay

The cytotoxicity assay was based on the measurement of lactate dehydrogenase (LDH) released from the cytosol of damaged cells (Cheung et al. 2000; Cardoso et al. 1998). In brief, 1×10^5/ml 95-D cells were plated into 24-well plates and allowed to incubate with either medium or medium containing 260, 520 μM FC-TEMPO for 48 h. LDH release from cells was measured by determining LDH activity (measured on a UNICO UV-2102PC spectrophotometer by NADH oxidation at 340 nm) in media. Results were normalized to 100% cells death caused by sonication.

4.6. Cell cycle parameter and apoptosis

95-D cells were seeded onto plates, and then were treated with different concentrations of FC-TEMPO (130, 260, 520 μM) and untreated cells were used as controls. After 48 h incubation, cells were harvested by trypsinization, washed in iced-cold phosphate-buffered saline (PBS), fixed in 70% ethanol. Cells were then stained with propidium iodide (PI). Cell cycle studies were performed using a flow cytometer (Becton Dickinson FACScan. USA) as described previously (Heerdt et al. 1998), the percentage of cells in the S phase, G_1, and G_2/M regions was determined. Apoptosis was detected by annexin V-FITC binding assay. Normal, apoptotic, and necrotic cells were distinguished by means of the annexin V-propidium iodide (PI) kit according to the manufacturer's instruction (Roche Diagnostics, Germany). After washing in PBS buffer, cells were resuspended for 10 min in the staining solution and analyzed by flow cytometry. The percentage of viable and dead cells was determined with 10000 cells per sample by using the FL1 channel for annexin V and the FL2 channel for PI.

4.7. Measurement of the activity of caspase-3 and caspase-8

The activity of caspase-3 was detected using a luminometer analyzer (BPCL, China) with caspase-Glo3 assay kit (Catalog G8090, Promege, USA) according to the manufacturer's protocol. The activity of caspase-8 was detected using a microplate reader at 405 nm according to the manufacturer's protocol (BD Biosciences, SanJose, CA). In some experiments, cells were pre-incubated for 60 min at 37 °C with selective caspase inhibitors, i.e., caspase-3 inhibitor, Z-DEVD-FMK, 10 μM; caspase-8 inhibitor, Z-IETD-FMK, 10 μM; pan-caspase inhibitor, Z-VAD-FMK, 10 μM, before addition of FC-TEMPO to the cultures.

4.8. Detection of intracellular catalase

The cells were washed in PBS and resuspended in a solution containing 10 mM Tris-HCl, pH 7.5, 0.25 M sucrose, 1 mM EDTA, 0.5 mM DL-dithiothreitol and 0.1 mM phenylmethylsulfonyl fluoride (PMSF). Triton X-100 was added to a final concentration of 1% and the samples were incubated for 30 min at 4 °C. After centrifugation at 3000 × g for 15 min, the supernatant fraction was assayed for enzyme activity. Catalase activity was measured as the decrease in the absorbance of H_2O_2 at 240 nm (Li et al. 2000). Protein content was measured according to Lowry et al. (1951).

4.9. Detection the activity of SOD

2×10^6 cells/ml were incubated with FC-TEMPO for 48 h and untreated cells were used as controls. Cells were washed with PBS and resuspended in the same buffer, then were sonicated and centrifuged at 12000 × g for 15 min at 4 °C. The SOD activity in supernatant was determined with the method of NADH-PMS-NBT on the UNICO spectrophotometer (Ewing and Janero 1995). In brief, the cell lysate was incubated with 50 mM phosphate buffer (pH 7.4), containing 0.1 mM EDTA, 50 μM NBT, and 78 μM NADH. The reaction was initiated by the addition of 3.3 μM PMS. Protein content was determined by the Lowry method.

All of the experiments were done at least three different times ($n = 3$) unless otherwise indicated. Data are expressed as means ± SD, and significance was assessed by Student's t test. Differences with $P < 0.05$ were considered significant.

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