Iron(III)-salen complexes with less DNA cleavage activity exhibit more efficient apoptosis in MCF7 cells†

Khairul I. Ansari,‡ James D. Grant,‡ Getachew A. Woldemariam,‡ Sahba Kasiri and Subhrangsu S. Mandal*

Received 25th September 2008, Accepted 26th November 2008
First published as an Advance Article on the web 15th January 2009
DOI: 10.1039/b816858j

To understand the relationship between DNA damage potential and biochemical activities, we synthesized nine different Fe(III)-salen derivatives with varying substituents, and analyzed their *in vitro* DNA cleavage properties and biochemical effects on cultured human cells. Our results demonstrated that Fe(III)-salen complexes affect cell viability, induce nuclear fragmentation, and activate caspases and apoptosis in cultured human cells. The nature and the position of the substituents in the Fe(III)-salen complexes play critical roles in determining their apoptotic efficiencies. Most importantly, our results demonstrated that the *in vitro* DNA cleavage activities of Fe(III)-salen complexes are not essential for their apoptotic activities in human cells. Instead, the lesser their DNA cleavage activity the greater is their apoptotic efficiency.

Introduction

Apoptosis is a process by which cells maintain a balance between proliferation and death and is often induced by external as well as internal stimuli.[1] Molecules that interact with nucleic acids, damage DNA and induce apoptosis in human cells find potential applications in medicinal chemistry and anti-tumour therapy. Notably, most of the anti-tumour agents irrespective of their mechanism of action induce efficient apoptosis in cancer cells and many of them directly interact with and/or damage DNA.[2–10] For example cis-diamminodichloro-platinum(ll) (cisplatin) and cis-diammine (cyclobutane-l, l-dicarboxylato) platinum(ll) (carboplatin), the widely used transition metal based anti-tumour drugs, induce crosslinking in DNA.[2–8] Similarly, Fe(III)-bleomycin, another well known naturally occurring anti-tumour antibiotic, produces free radicals that damage DNA.[11] As DNA interacting molecules find potential applications in novel anti-tumour therapy, intense research efforts are being invested towards developing novel DNA/RNA modifiers and in understanding their molecular mechanisms of action.[2,12,13]

In general redox active agents that damage DNA *in vitro* are assumed to exhibit apoptotic activities in live cells by inducing oxidative stress and/or DNA damage.[7,12,14–17] Metallo-salens with diverse structures and redox characteristics are shown to produce reactive oxygen species under oxidative/reducing environments and damage to DNA/RNA *in vitro*.[18–27] For example iron- and copper-salen derivatives produce hydroxyl radicals in the presence of dithiothreitol and damage DNA.[28,29] Water soluble cobalt-salen derivatives activate natural oxgen and induce DNA cleavage.[26] Nickel-salen derivatives induce crosslinking in DNA, RNA and proteins under oxidative environments.[29] Furthermore, various iron-salen derivatives and other metal-complexes have been implicated in efficient asymmetric catalysis and in catalyzing the hydrolytic cleavage of DNA and RNA.[30–34] Mn(III)-salens that damage DNA *in vitro* are shown to exhibit superoxide dismutase activities. Gust *et al.* showed that diaryl cobalt-salen derivatives possess novel anti-tumour activities.[35] Recently, we reported that Fe(III)-salen cleaves DNA *in vitro* and induces apoptosis in cultured human cells.[36]

Herein, to explore the relationship between the DNA cleavage activities of metallo-salens and their biochemical activities, we synthesized several Fe(III)-salen derivatives, and investigated their DNA cleavage properties and biochemical effects on cultured human cells (MCF7). Our results demonstrated that Fe(III)-salen complexes induce efficient apoptosis in MCF7 cells and interestingly, the DNA cleavage potentials of the Fe(III)-salen complexes are not essential for their apoptotic activities.

Results and discussion

In order to understand the relationship between the DNA damage potential of the redox active molecules and their biochemical activities in human cells, we synthesized nine different Fe(III)-salen derivatives (compds **1–9**) with different functionalities (hydroxy and methoxy) and diamino bridges (ethylene diamine, *o*-phenylene diamine and 2,3-diamino naphthalene) using a standard protocol (Fig. 1).[28,36] Each of the Fe(III)-salen complexes was characterized by mass spectrometry and elemental (CHN) analysis and the results are consistent with the proposed structures (Fig. 1).

Fe(III)-salen derivatives affect cell viability and induce nuclear fragmentation

Initially we examined the biochemical effects of the Fe(III)-salen derivatives (compds **1–9**) toward MCF7 cells (a human breast cancer cell line). We incubated cells with 20 μM of each of the Fe(III)-salen derivatives separately for 72 hrs and then

Department of Chemistry and Biochemistry, The University of Texas at Arlington, Arlington, Texas 76019, U. S. A. E-mail: smandal@uta.edu; Fax: +1 817-272-3808

† Electronic supplementary information (ESI) available: Results of TUNEL (terminal dUTP nicked end labeling) assay and cytotoxicity (IC_{50}) measurements for compds **1**, **5** and **6**. See DOI: 10.1039/b816858j

‡ These authors contributed equally

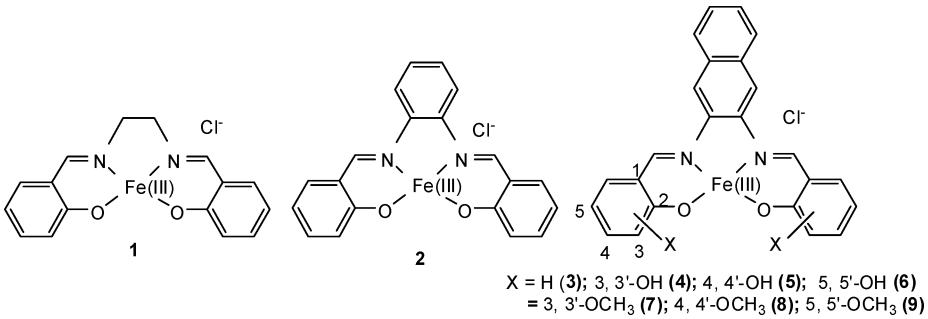

Fig. 1 Structures of Fe(III)-salen complexes (compds **1–9**).

visualized the cell morphology and viability under the differential interference contrast (DIC) settings of a microscope (Fig. 2a). Interestingly, in comparison to the control untreated cells (control panel, Fig. 2a), upon treatment with either of the Fe(III)-salen, Fe(III)-salphen and Fe(III)-salnaphen complexes (compds **1–3**), the cell population decreased dramatically and the remaining cells got clumped, rounded, and converted into debris, indicating signs of cell death (panels 1, 2, and 3, Fig. 2a). Based on the cell morphologies and the population densities, Fe(III)-salphen and -salnaphen appeared slightly more effective in inducing cell death (cytotoxic) than Fe(III)-salen (compare panels 1–3, Fig. 2a). The methoxy and hydroxy substituted salnaphen complexes showed different cytotoxicities towards MCF7 cells. In the case of hydroxy substituted Fe(III)-salnaphen derivatives, only 3,3′-dihydroxy Fe(III)-salnaphen (**4**) was highly effective in inducing cell death whereas 4,4′- and 5,5′-dihydroxy Fe(III)-salnaphen complexes (**5** and **6**) did not show any significant effect on cell viability (panels 4–6, Fig. 2a). However, all three corresponding methoxy substituted Fe(III)-salnaphen derivatives (compds **7–9**) showed effective cytotoxicity towards MCF7 cells (panels 7–9, Fig. 2a).

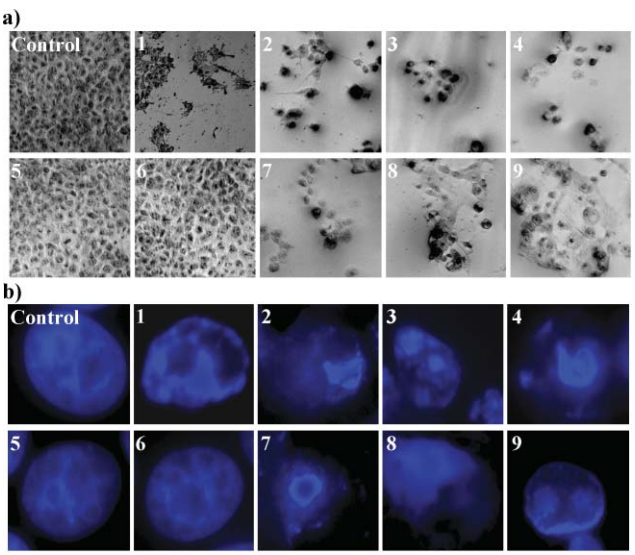

Fig. 2 Effect on cell viability: MCF7 cells were treated with 20 μM of the Fe(III)-salen complexes (in DMSO) for 72 hrs. (a) DIC (differential interference contrast) images of the cells under a microscope. (b) Cells stained with DAPI and visualized under a fluorescence microscope (Nikon TE200). Controls: cells treated with DMSO; panels 1–9: cells treated with compds **1–9** respectively.

To understand the nature of cell death, we performed the nuclear staining of the Fe(III)-salen treated cells with a DNA binding dye DAPI (4′,6-diamidino-2-phenylindole). Notably, upon DAPI staining, the cell nucleus appears blue under a fluorescence microscope and this technique has been routinely used to visualize nuclear morphologies.[8] Usually, more intensely DAPI stained regions indicate more condensed DNA (heterochromatin) while less intensely DAPI stained regions indicates less condensed DNA (euchromatins).[8] Herein, to visualize the effects on nuclear morphologies, Fe(III)-salen complex treated cells were stained with DAPI and visualized under a fluorescence microscope. Upon DAPI staining, the nucleus of the control untreated cell was visible with a distinct nuclear boundary and fairly uniform DNA distribution pattern (control panel, Fig. 2b). However, upon treatment with the biochemically active Fe(III)-salen complexes (compds **1–4**, **7**, **8** and **9**), the DAPI staining patterns of the cell nucleus was affected significantly (Fig. 2b). In most cases, the nucleus lost their well defined boundaries. DNA got heavily condensed (intensely DAPI stained regions), and fragmented (intensely DAPI stained speckles of nuclear fragments). Furthermore, some of the cell nuclei showed some ring-like DAPI stained structures around the nuclear boundaries (eg. in panels 1, 2, 4, and 9, Fig. 2b). Inactive compounds (**5** and **6**) showed no significant effect on the cell nucleus. Notably, the nuclear condensation and fragmentation as well as the ring-like DAPI staining patterns are hallmarks of apoptosis, indicating that Fe(III)-salen complexes induced apoptosis in MCF7 cells. The apoptotic activities of the Fe(III)-salen complexes were further confirmed by TUNEL (terminal dUTP nicked end labeling) assay (Fig. S1†).

Cytotoxicities of Fe(III)-salen derivatives (IC_{50} values)

To quantify the relative cytotoxicity we incubated MCF7 cells with varying concentrations of each of the Fe(III)-salen complexes for 96 hrs and subjected them to MTT assay as described previously.[36–38] The percent of viable cells relative to control were plotted against concentration of the Fe(III)-salen complexes to obtain IC_{50} values. IC_{50} values of the biochemically active compounds lie in the range 0.2 to 22.5 μM (Table 1, Fig. 3 and Fig S2†). The IC_{50} values for parent Fe(III)-salen (**1**), Fe(III)-salphen (**2**) and Fe(III)-salnaphen (**3**) are 22, 1.3 and 0.5 μM respectively which suggest that the increase in aromatic functionality in the diimmino bridge (from ethylene diamine to the more aromatic *ortho*-phenylenediamine and 2,3-diaminonaphthalene) in the

Table 1 IC_{50} values of Fe(III)-salen derivatives towards MCF7 and JAR cells

Compounds	IC_{50} (μM)[a]	
	MCF7	JAR
1	22 (± 0.7)	22 (± 0.8)
2	1.3 (± 0.1)	0.6 (± 0.03)
3	0.5 (± 0.02)	0.3 (± 0.01)
4	0.2 (± 0.06)	0.2 (± 0.05)
5	>100	>100
6	>100	>100
7	1.5 (± 0.2)	1.1 (± 0.03)
8	0.5 (± 0.03)	0.2 (± 0.01)
9	1.3 (± 0.07)	1.0 (± 0.3)

[a] The number in parenthesis indicates the standard error of the means (SEM).

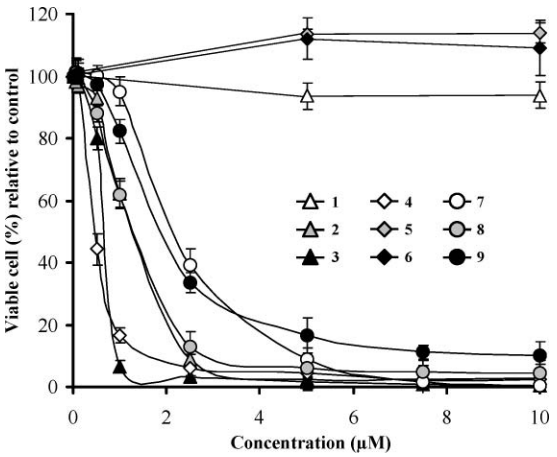

Fig. 3 Cytotoxicity (IC_{50}) measurements. Approximately 10 000 MCF7 cells (in 96 well microtiter plate) were treated with varying concentrations of Fe(III)-salen complexes for 96 hrs and the viable cells were quantified using MTT assay. The percent viable cells (relative to DMSO treated control) were plotted against concentrations for each compound. The concentration of the metal-complexes at which the conversion of MTT to formazan by viable cells is reduced by 50% compared to control cells is defined as the IC_{50}. Symbols (empty, shaded and filled triangles) represent the cytotoxicity curves for compds **1–3** respectively. Symbols (empty, shaded and filled diamonds) represent the cytotoxicity curves for compds **4–6** respectively. Symbols (empty, shaded and filled circles) represent the cytotoxicity curves for compds **7–9** respectively.

Fe(III)-salen complexes significantly increased their cytotoxicity. Notably, an increase in aromatic functionalities increases the lipophilicity of the molecules as well as maybe affecting their interactions with DNA. In the case of Fe(III)-salen complexes, both lipophilicity as well as DNA interaction factors may be contributing to the enhanced cytotoxicities of the more aromatic complexes.[26–28,35,39–42] In addition, 3,3′-dihydroxy and 4,4′-dimethoxy-Fe(III)-salnaphens (**4** and **8**) also have nanomolar IC_{50}s indicating that these Fe(III)-salen complexes are highly effective at inducing cell death in MCF7 cells (Table 1). Cytotoxicity curves, with an extended concentration range (0–60 μM), for the Fe(III)-salen (compd **1**) and the biochemically inactive compds (**5** and **6**) are shown in the supplementary Fig S2.† Taken together these studies demonstrated that the nature and the position of the substituents and the nature of the diimino bridge in the Fe(III)-salen complexes play critical roles in determining their cytotoxicity levels (IC_{50} values).

Fe(III)-salen complexes activate caspases-3/7

To confirm the apoptotic activities of Fe(III)-salen derivatives, we analyzed the activation of caspases-3/7 by using a commercially available kit. Notably, to assess the caspase 3/7 activity we used Jar cells (a placental cell line) instead of MCF7, as MCF7 cells lack the caspase-3 enzyme.[43] We treated cells with 100 μM Fe(III)-salens (compds **1–9**) for 0, 8, 16 and 24 hrs and then caspase-3/7 activities were analysed using a commercial caspase-3/7 assay kit. The caspase activities of the biochemically active Fe(III)-salen treated cells increased with time with maxima at 16 hrs and then declined at higher time points likely due to cell death (data not shown). The relative caspase-3/7 activities (at the 16 hr time point) of different Fe(III)-salen complexes were plotted in Fig. 4. For a reasonable comparison of caspase-3/7 activation with cytotoxicity, we measured the IC_{50} values for different Fe(III)-salen complexes in JAR cells (Table 1). Although there is difference in the IC_{50} values for the two different cells lines (MCF7 *vs* JAR, which is expected due to the difference in cell types), the caspase activities induced by different biochemically active Fe(III)-salen complexes in JAR cells correlated fairly well with their IC_{50} values in JAR cells (Table 1 and Fig. 4). More active (lower IC_{50}) compounds showed higher levels of caspase-3/7 activation. For example, Fe(III)-salen, salphen and salnaphen complexes (compds **1–3**) increased caspases-3/7 activities by 1.7, 9 and 14 fold compared to control untreated cells (JAR cells) and the IC_{50} values (JAR cells) for compds **1–3** are 22, 0.6 and 0.3 μM respectively (Fig. 4 and Table 1). Similarly, among the hydroxysalnaphen derivatives, the biochemically active 3,3′-dihydroxysalnaphen (compd **4**) induced an 18 fold increase (IC_{50}: 0.2 μM in JAR cells) in caspase activity, whereas the inactive compounds (**5** and **6**) induced no significant caspase activity (Fig. 4 and Table 1). The biochemically active methoxysalnaphen derivatives increased caspases-3/7 activities by 5 to 15 fold in comparison to untreated control cells (Fig. 4). These results demonstrated that Fe(III)-salen derivatives that affect cell viability, induce caspase 3/7 activation leading to apoptosis in JAR cells.

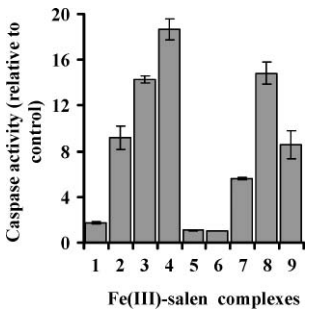

Fig. 4 Fe(III)-salens induced activation of caspase-3/7. JAR (human placental cell line) cells were treated with 100 μM Fe(III)-salens for 16 hrs and subjected to caspase 3/7 activation assay using a commercially available caspase-3/7 Assay Kit (AnaSpec Inc). The relative caspase-3/7 activities compared to the untreated control were plotted for different Fe(III)-salen derivatives. Bars indicate SEM.

Fe(III)-salen complexes with more efficient apoptosis activities induce less DNA cleavage

To correlate the DNA cleavage activities of Fe(III)-salen derivatives with their apoptotic activities, we performed DNA cleavage assays by incubating different Fe(III)-salen complexes with supercoiled plasmid DNA (pML20–47) with or without a reducing agent dithiothreitol (DTT). In agreement with our previous studies,[36,37] Fe(III)-salen (**1**) induced efficient DNA cleavage in the presence of DTT as evidenced by the conversion of supercoiled into nicked circular form DNA (lanes 3–4, Fig. 5a). Notably Fe(III)-salen itself has some DNA cleavage activity even in the absence of DTT (lane 5, Fig. 5a). The unmodified Fe(III)-salphen (**2**) and salnaphen (**3**) also cleaved DNA in presence of DTT. However, the cleavage efficiency decreased as the aromatic bridge increased from Fe(III)-salen to salphen to salnaphen (Fig. 5a). These results are in contrast to their apoptotic efficiencies where Fe(III)-salnaphen was the most active (IC_{50}: 0.5 μM) and Fe(III)-salen was the least active (IC_{50}: 22 μM).

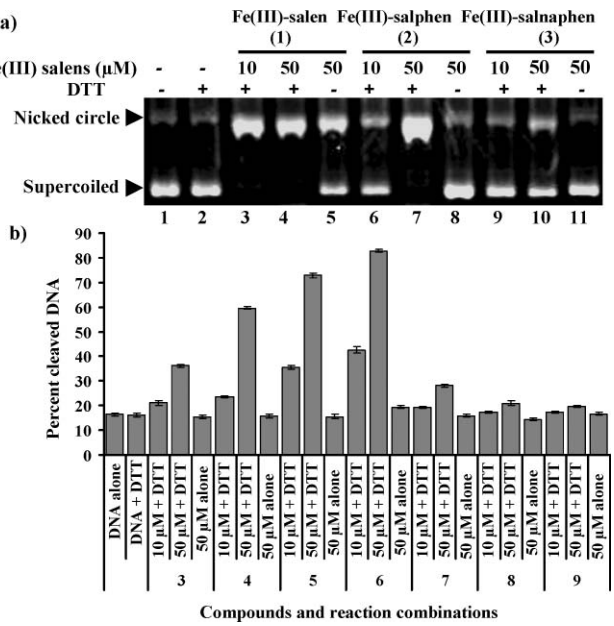

Fig. 5 DNA cleavage by Fe(III)-salen complexes. Supercoiled plasmid DNA pML20–47 (0.5 μg) was incubated with varying concentration of Fe(III)-salen complexes in the presence of 1 mM DTT at 37 °C for 1 hr, analyzed by agarose gel electrophoresis and quantified (ImageQuant). (a) Agarose gel picture showing the DNA cleavage analysis by compds **1**, **2** and **3**. (b) The percent DNA cleaved by compds **3–9** was quantified and plotted.

We also performed similar DNA cleavage experiments for different hydroxy and methoxy salnaphen derivatives and the amount of DNA cleaved was quantified and plotted in Fig. 5b. Interestingly, all the hydroxy-Fe(III)-salnaphen derivatives (**4–6**) showed efficient DNA cleavage activity (up to 85% DNA cleavage) (Fig. 5b). The DNA cleavage abilities of all the hydroxy derivatives were higher than the unmodified Fe(III)-salnaphen (Fig. 5b). These observations are again in contrast to their apoptotic activities (Fig. 5b and 2). The 3,3′-dihydroxy Fe(III)-salnaphen (**4**), which has the least DNA cleavage activity, induced the most efficient apoptosis. The remaining two hydroxy derivatives (**5–6**), which cleaved DNA efficiently, induced no apoptosis in MCF7 cells. Similarly, all three methoxy Fe(III)-salnaphens (**7–9**), which showed no significant DNA cleavage activities, induced efficient apoptosis in MCF7 cells (Fig. 5b and 2).

Conclusion

Understanding the correlation between the DNA damage potential of small molecules and their biochemical and apoptotic activities is important for designing novel anti-tumour agents. Herein, we synthesized several Fe(III)-salen complexes (compds **1–9**) with varying substituents and bridging spacers, and analyzed their DNA cleavage properties *in vitro* and apoptotic activities in cultured human cells. Cytotoxicity studies demonstrated that unmodified Fe(III)-salen, Fe(III)-salphen and Fe(III)-salnaphen complexes affect cell viability, activate caspases-3/7 and induce nuclear condensation/fragmentation leading to cell death. The activation of caspases-3/7, nuclear condensation and fragmentation indicated that Fe(III)-salen complexes induce apoptotic cell death in cultured human cells. Notably the level of caspase activation induced by different Fe(III)-salen complexes (compds **1–9**) correlated significantly with their cytotoxicity values (IC_{50} values) in JAR cells. These results further support that Fe(III)-salen complexes induce apoptosis in cultured human cells *via* caspase activation and the mitochondrial pathway.

The comparison of the IC_{50} values for the unmodified Fe(III)-salen, salphen and salnaphen complexes (IC_{50s}: 22, 1.3 and 0.5 μM for compds **1**, **2** and **3** respectively towards MCF7 cells), indicated that the increase in aromatic functionality in the diimino bridges of Fe(III)-salen complexes increases their cytotoxicity. Analysis of the cytotoxicities of the hydroxy and methoxy substituted Fe(III)-salnaphen complexes (compds **4–9**) demonstrated that the position and the nature of the substituents play critical roles in determining their cytotoxic efficiencies. For example, 3,3′-dihydroxy substituted Fe(III)-salnaphen (**4**) was highly effective in inducing cell death (IC_{50}.0.2 μM), whereas 4,4′- and 5,5′-dihydroxy Fe(III)-salnaphen complexes (**5** and **6**) were almost inactive toward MCF7 cells. However, all three corresponding methoxy substituted Fe(III)-salnaphen derivatives (compds **7–9**) showed effective cytotoxicity towards MCF7 cells (IC_{50s}: 1.5, 0.5 and 1.3 μM respectively). Notably, recent studies indicated that, in comparison to the hydroxy substituted derivatives, methoxy substituted cobalt-salen complexes are preferentially taken up by cells leading to a higher impact on cell growth.[35] This is likely because the methoxy compounds are more hydrophobic compared to the hydroxy compounds, which may increase their cellular permeabilities and uptake efficiencies.[35] A similar mechanism of action may be reflected in the cytotoxic behaviour of our methoxy-substituted Fe(III)-salnaphen complexes (compds **7–9**).

The comparison of the *in vitro* DNA cleavage activities of different Fe(III)-salen complexes (compds **1–9**) with their cytotoxicities was most intriguing. For example, DNA cleavage efficiencies were decreased from Fe(III)-salen to Fe(III)-salphen and Fe(III)-salnaphen complexes (compds **1–3**), while their cytotoxicities toward MCF7 cells were increased in a reciprocal manner (IC_{50} values decreased). Similar results were observed for various hydroxy and methoxy substituted Fe(III)-salnaphen complexes (compds **4–9**). The lower the DNA cleavage potentials of the Fe(III)-salen complexes, the higher are their cytotoxicities (lower

IC_{50} values). These results indicate that the *in vitro* DNA cleavage activities are not essential for the apoptotic efficiencies of Fe(III)-salen complexes in cultured human cells. Instead, less DNA cleavage active Fe(III)-salen complexes induced more efficient apoptosis.

Although, further studies are needed to address the detailed mechanism of the differential cytotoxicities, our studies demonstrated that Fe(III)-salen complexes induce apoptosis in cultured human cells with IC_{50}s in the nanomolar to micromolar ranges and that the cytotoxic activity does not necessarily depend on their *in vitro* DNA cleavage abilities.

Experimental

General

All reagents for organic synthesis and buffers were purchased from Sigma-Aldrich unless otherwise noted. Tissue culture medium DMEM (Dulbecco's Modified Eagle's Medium), FBS (Fetal Bovine Serum), penicillin and streptomycin were purchased from Sigma-Aldrich. Ferric chloride (anhydrous) was purchased from Spectrum Chemical Manufacturing Corporation. DAPI (4′,6-diamidino-2-phenylindole) was obtained from Chemicon. MTT (3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide) was obtained from Tokyo Chemical Industry Co. MCF7 cell line was purchased from ATCC (American Type Culture Collection). NMR spectra were recorded on JEOL Eclipse 500 (^{1}H, 500 MHz) spectrometer. FT-IR spectra were recorded by using a Bruker Vector 22 infrared spectrometer. Elemental analyses were performed using a Perkin Elmer Model 2400 CHN analyzer. Mass spectra were taken by using the ESI-MS method (positive mode, quadrupole ion trap-MS, MSLCQ Deca XP instrument from Thermo-Fisher Scientific).

Synthesis of Fe(III)-salen and their derivatives (compds 1–9)

Fe(III)-salen derivatives were synthesized and characterized following a general procedure as described previously.[28,36] In brief, ligands were synthesized by mixing the corresponding salicylaldehyde derivative and diamine in methanol which resulted in a dark colored (yellow, orange or red) precipitate of the ligands (yield 80–90%). The respective ligand was mixed with one equivalent of anhydrous ferric chloride in methanol, warmed at 60 °C for 20 min and then cooled down to room temperature. In most cases the metal-complexes were precipitated out upon cooling the reaction mixtures. In some cases the metal complexes were precipitated by adding diethyl ether into the cold reaction mixtures. The products were isolated, recrystallized from methanol and characterized by mass spectral analysis using ESI-MS.

[Fe(III)-salen] chloride (compd 1). The synthesis of this compound was reported by our lab previously.[36]

Fe(III)-salphen chloride (compd 2). Two equivalents of salicylaldehyde (2.2 g, 18.1 mmol) was mixed with one equivalent of o-phenylenediamine (920 mg, 8.4 mmol) in methanol which resulted in a light orange precipitate of salphen ligand. This ligand (500 mg, 1.6 mmol) was complexed with one equivalent of Fe(III) chloride (295 mg, 1.8 mmol) to obtain compd **2** (65% yield). FT-IR (KBr, cm^{-1}): 1600, 1590, 1530, 1450, 1430, 1380, 1320, 1200, 1140, 1110, 910, 880, 850, 800, 730, 600, 530. Observed M/Z value for compd. **2**: 370.18 (M^+, –Cl). CHN analysis: Calcd. (for $C_{20}H_{14}N_2O_2FeCl$. 2.5 H_2O) C: 53.37%, H 4.26%, N 6.22%; Observed: C: 53.32%, H 3.88%, N 6.00%.

Fe(III)-salnaphen chloride (compd 3). Two equivalents of salicylaldehyde (2.1 g, 17.2 mmol) was mixed with one equivalent of 2,3-diaminonaphthalene (1.3 g, 8.2 mmol) in methanol which resulted in a dark yellowish orange precipitate of salnaphen. This ligand (450 mg, 1.23 mmol) was complexed with one equivalent of Fe(III) chloride (226 mg, 1.4 mmol) to obtain **3** (60% yield). FT-IR (KBr, cm^{-1}): 1590, 1570, 1540, 1460, 1430, 1385, 1320, 1300, 1200, 1110, 1100, 900, 890, 810, 760, 620, 600. Observed M/Z value for compd **3**: 420.24 (M^+, –Cl); CHN analysis for salnaphen Fe(III) chloride (**3**) Calcd. (for $C_{24}H_{16}N_2O_2FeCl$. 1.5 H_2O) C: 59.80%, H 3.97%, N 5.81%; Observed: C: 60.05%, H 3.96%, N 5.78%.

3,3′-Dihydroxysalnaphen Fe(III) chloride (compd 4). Two equivalents of 2,3-dihydroxybenzaldehyde (2.1 g, 15.2 mmol) was mixed with one equivalent of 2,3-diaminonaphthalene (1.1 g, 6.9 mmol) in methanol which resulted in a red precipitate of 3,3′-dihydroxysalen. This ligand (300 mg, 0.75 mmol) was complexed with one equivalent of Fe(III) chloride (138 mg, 0.85 mmol) to obtain compd **4** (45% yield). FT-IR (KBr, cm^{-1}) 3400, 1590, 1500, 1430, 1390, 1300, 1280, 1250, 1129, 890, 802, 760, 500, 590, 530. Observed M/Z value for compd. **4**: 452.27 (M^+, –Cl). CHN analysis for **4**: Calcd. (for $C_{24}H_{16}N_2O_4FeCl$. 2 H_2O) C: 55.04%, H 3.85%, N 5.35%; Observed: C: 55.42%, H 3.59%, N 5.36%.

4,4′-Dihydroxysalnaphen Fe(III) chloride (compd 5). Two equivalents of 2,4-dihydroxybenzaldehyde (1.8 g, 13 mmol) was mixed with one equivalent of 2,3-diaminonaphthalene (1 g, 6.3 mmol) in methanol which resulted in a dark orange brown precipitate of 4,4′-dihydroxysalen. This ligand (450 mg, 1.3 mmol) was complexed with one equivalent of Fe(III) chloride (243 mg, 1.5 mmol) to obtain compd **5** (48% yield). FT-IR (KBr, cm^{-1}) 3395, 1577, 1538, 1435, 1369, 1251, 1213, 1174, 1129, 985, 850, 802, 760, 646, 602, 570. Observed M/Z value for compd **5**): 452.27 (M^+, –Cl). CHN analysis for **5**: Calcd. (for $C_{24}H_{16}N_2O_4FeCl$. 4 H_2O) C: 51.50%, H 4.32%, N 5.00%; Observed: C: 51.10%, H 4.04%, N 4.96%.

5,5′-Dihydroxysalnaphen Fe(III) chloride (compd 6). Two equivalents of 2,5-dihydroxybenzaldehyde (2 g, 14.5 mmol) was mixed with one equivalent of 2,3-diaminonaphthalene (1.1 g, 6.9 mmol) in methanol which resulted in a dark red precipitate of 5,5′-dihydroxysalen. This ligand (450 mg, 1.36 mmol) was complexed with one equivalent of Fe(III) chloride (235 mg, 1.4 mmol) to obtain compd **6** (52% yield). FT-IR (KBr, cm^{-1}) 3402, 1597, 1537, 1458, 1435, 1385, 1304, 1278, 1223, 1162, 1095, 874, 829, 750, 726, 554. Observed M/Z value for compd **6**: 452.27 (M^+, –Cl). CHN analysis for **6**: Calcd. (for $C_{24}H_{16}N_2O_4FeCl$. 3.5 H_2O) C: 52.35%, H 4.21%, N 5.09%; Observed: C: 52.70%, H 4.07%, N 5.01%.

3,3′-Dimethoxysalnaphen Fe(III) chloride (compd 7). Two equivalents of 2-hydroxy-3-methoxybenzaldehyde (2.7 g, 17.5 mmol) was mixed with one equivalent of 2,3-diaminonaphthalene (1.2 g, 7.6 mmol) in methanol which resulted in a bright orange precipitate of 3,3′-dimethoxysalen ligand. This ligand (410 mg, 0.96 mmol) was complexed with one equivalent of Fe(III) (186 mg, 1.15 mmol) chloride to obtain compd **7** (65%

yield). FT-IR (KBr, cm^{-1}): 1707, 1597, 1580, 1545, 1356, 1338, 1248, 1206, 982, 861, 779, 734, 604. Observed M/Z value for compd. **7**: 480.18 (M^+, –Cl). CHN analysis (compd **7**) Calcd. (for $C_{26}H_{20}N_2O_4FeCl$. 3.8 H_2O) C: 53.45%, H 4.76%, N 4.80%; Observed: C: 53.27%, H 3.95%, N 4.93%.

4,4′-Dimethoxysalnaphen Fe(III) chloride (compd 8). Two equivalents of 2-hydroxy-4-methoxybenzaldehyde (2.1 g, 13.8 mmol) was mixed with one equivalent of 2,3-diaminonaphthalene (1 g, 6.3 mmol) in methanol which resulted in an orange colored precipitate of 4,4′-dimethoxysalen ligand. This ligand (450 mg, 1.1 mmol) was complexed with one equivalent of Fe(III) chloride (194 mg, 1.2 mmol) to obtain compd **8** (58% yield). FT-IR (KBr, cm^{-1}): 1607, 1573, 1522, 1457, 1438, 1416, 1373, 1306, 1256, 1219, 1171, 1127, 1023, 976, 834, 751,648. Observed M/Z value for compd **8**: 480.25 (M^+, –Cl). CHN analysis for **8**: Calcd. (for $C_{26}H_{20}N_2O_4FeCl$. 1.0 H_2O) C: 58.51%, H 4.15%, N 5.25%; Observed: C: 58.43%, H 3.80%, N 5.27%.

5,5′-Dimethoxysalnaphen Fe(III) chloride (compd 9). Two equivalents of 2-hydroxy-5-methoxybenzaldehyde (2.89 g, 19 mmol) was mixed with one equivalent of 2,3-diaminonaphthalene (1.5 g, 9.5 mmol) in methanol which resulted in a dark reddish brown precipitate of 5,5′-dimethoxysalen. This ligand (400 mg, 0.94 mmol) was complexed with one equivalent of Fe(III) chloride (192 mg, 1.2 mmol) to obtain compd **9** (59% yield). FT-IR (KBr, cm^{-1}): 1621, 1580, 1536, 1461, 1365, 1295, 1264, 1224, 1167, 1036, 823, 744, 718. Observed M/Z value for compd **9**: 480.18 (M^+, –Cl). CHN analysis for **9**: Calcd. (for $C_{26}H_{20}N_2O_4FeCl$) C: 60.55%, H 3.91%, N 5.43%; Observed: C: 60.07%, H 3.73%, N 5.44%.

Cell culture

Human breast cancer (MCF7) and choriocarcinoma placental cells (JAR) were grown and maintained in Dulbecco's modified Eagle's media (DMEM) that was supplemented with heat inactivated fetal bovine serum (FBS, 10%), L-glutamine (1%) and Penicillin/streptomycin (0.1%).[8,36,44] Cells were cultured and maintained in humidified incubator with 5% CO_2 in air at 37 °C. Cells were grown on cover slips for microscopy experiments and in 96 well micro titer plates for cell viability and cytotoxicity assays.

Cell viability assay and DAPI staining

MCF7 cells were seeded onto a cover slip in a 60 mm plate and grown overnight under normal growth conditions followed by incubation with 20 μM Fe(III)-salen or its derivative for an additional 72 hrs. Control cells were treated with an equivalent amount of DMSO. Cells were fixed with 4% -formaldehyde in PBS for 15 minutes, washed twice with PBS, permeabilized with 0.2% Triton X-100 in PBS for 5 min. Cells were washed three times with cold PBS followed by incubation with DAPI (5 μg per slide) for 10 minutes at room temperature. DAPI stained cells were washed three times with cold PBS, mounted on a microscope slide with mounting media (Vectashield H-1000, Vector lab) and visualized under a fluorescence microscope.[8]

Cytotoxicity assay (IC_{50} measurement)

The cytotoxicities of the Fe(III)-salen and its derivatives were determined by using an MTT assay as described previously.[37,38] In brief, approximately 10 ,000 MCF7 (or JAR cells) cells were seeded into each well of a 96 well micro titer plate and incubated 24 hrs in presence of 150 μL DMEM. An additional 50 μL DMEM containing the required amount of each Fe(III)-salen to obtain a 0.05 to 60 μM final concentration (in 200 μL final volume) in 8 replicate wells was added. Control wells were treated with an equivalent amount of DMSO. After 96 hrs of incubation, 20 μL of MTT (5 mg/mL) was added into each well and cell viability was assayed by measuring the formazan absorption at 560 nm as described above. The absorbance (at 560 nm) values were plotted against concentration of the Fe(III)-salen complexes to determine the IC_{50}. The experiments were repeated twice with 8 replicates each time.

Caspase-3/7 activity assay

Caspase-3/7 activity was assessed using SensoLyte TM Homogenous AMC Caspase-3/7 Assay Kit (AnaSpec Inc). Briefly, JAR cells were treated with 100 μM Fe (III)-salens for 0, 8, 16, and 24 hrs, lysed, and centrifuged (2500 g for 10 min at 4 °C). The supernatant was diluted (to 1 μg/μL of protein) and 150 μL extract was mixed with 50 μL of assay buffer containing caspase-3/7 substrate, incubated for 15 min at 37 °C and then the fluorescence intensity ($350_{EX}/440_{EM}$) was measured every 10 min (up to 2 hrs) using FLOUstar Omega micro plate reader (BMG labTech). The concentration of activated caspase-3/7 was calculated by using a calibration curve with pure standard. The caspase-3/7 activity was finally expressed relative to untreated control cells.

DNA cleavage assay

DNA cleavage reactions were performed by incubating supercoiled plasmid DNA (pML20–47, 0.5 μg) with varying concentrations of Fe(III)-salen derivatives in the presence and absence of 1 mM dithiothreitol (DTT) for 1 hr at 37 °C in 20 mM Tris-HCl (pH 7.4) as described previously.[36] The products were analyzed by agarose gel electrophoresis. The gel was stained with ethidium bromide and visualized by using an Alpha Imager-2200 (Alpha Innotech corporation) instrument, and quantified by using Image Quant software.

Acknowledgements

We thank Saoni Mandal and other lab members for critical discussions. This work was supported by grants from Texas Advanced Research Program (00365–0009-2006) and American Heart Association (SM 0765160Y).

References

1 A. Basu and A. Miura, *Int. J. Mol. Med.*, 2002, **10**, 541.
2 K. R. Barnes and S. J. Lippard, *Met. Ions. Biol. Syst.*, 2004, **42**, 143.
3 C. X. Zhang and S. J. Lippard, *Curr. Opin. Chem. Biol.*, 2003, **7**, 481.
4 C. Brambilla, L. Ferrari, P. Passoni and G. Bonadonna, *Cancer. Treat. Rev.*, 1993, **19**, 3.

5 H. Robson, S. Meyer, S. M. Shalet, E. Anderson, S. Roberts and O. B. Eden, *Med. Pediatr. Oncol.*, 2002, **39**, 573.
6 L. Zhang, Y. Zhang, P. Y. Huang, F. Xu, P. J. Peng and Z. Z. Guan, *Cancer Chemother. Pharmacol.*, 2008, **61**, 33.
7 L. J. K. Boerner and J. M. Zaleski, *Curr. Opin. Chem. Biol.*, 2005, **9**, 135.
8 K. I. Ansari, B. P. Mishra and S. S. Mandal, *Biochim. Biophys. Acta*, 2008, **1779**, 66.
9 M. Pitie, C. J. Burrows and B. Meunier, *Nucleic Acids Res.*, 2000, **28**, 4856.
10 J. B. Biggins, J. R. Prudent, D. J. Marshall, M. Ruppen and J. S. Thorson, *Proc. Natl. Acad. Sci. U. S. A.*, 2000, **97**, 13537.
11 C. E. Holmes, R. J. Duff, G. A. van der Marel, J. van Boom and S. M. Hecht, *Bioorg. Med. Chem.*, 1997, **5**, 1235.
12 S. M. Cohen and S. J. Lippard, *Prog. Nucleic Acid Res. Mol. Biol.*, 2001, **67**, 93.
13 I. Ott and R. Gust, *Arch. Pharm. (Weinheim)*, 2007, **340**, 117.
14 J. Wang, *Cell Death Differ.*, 2001, **8**, 1047.
15 M. Valko, M. Izakovic, M. Mazur, C. J. Rhodes and J. Telser, *Mol. Cell. Biochem.*, 2004, **266**, 37.
16 D. M. Perrin, A. Mazumder and D. S. Sigman, *Prog. Nucleic Acid Res. Mol. Biol.*, 1996, **52**, 123.
17 A. J. Danford, D. Wang, Q. Wang, T. D. Tullius and S. J. Lippard, *Proc. Natl. Acad. Sci. U. S. A.*, 2005, **102**, 12311.
18 S. Bhattacharya and S. S. Mandal, *J. Chem. Soc., Chem. Commun.*, 1996, 1515.
19 C. J. Burrows, R. P. Hickerson, J. G. Muller, B. Felden and S. E. Rokita, *Biophysical J.*, 1999, **76**, A5.
20 J. L. Czlapinski and T. L. Sheppard, *J. Am. Chem. Soc.*, 2001, **123**, 8618.
21 J. G. Muller, S. J. Paikoff, S. E. Rokita and C. J. Burrows, *J. Inorg. Biochem.*, 1994, **54**, 199.
22 S. Routier, J. L. Bernier, M. J. Waring, P. Colson, C. Houssier and C. Bailly, *J. Org. Chem.*, 1996, **61**, 2326.
23 D. J. Gravert and J. H. Griffin, *J. Org. Chem.*, 1993, **58**, 820.
24 H. Y. Shrivastava, S. N. Devaraj and B. U. Nair, *J. Inorg. Biochem.*, 2004, **98**, 387.
25 S. R. Doctrow, K. Huffman, C. B. Marcus, G. Tocco, E. Malfroy, C. A. Adinolfi, H. Kruk, K. Baker, N. Lazarowych, J. Mascarenhas and B. Malfroy, *J. Med. Chem.*, 2002, **45**, 4549.
26 S. Bhattacharya and S. S. Mandal, *J. Chem. Soc., Chem. Commun.*, 1995, 2489.
27 S. S. Mandal, U. Varshney and S. Bhattacharya, *Bioconjug. Chem.*, 1997, **8**, 798.
28 S. Routier, H. Vezin, E. Lamour, J. L. Bernier, J. P. Catteau and C. Bailly, *Nucleic Acids Res.*, 1999, **27**, 4160.
29 S. E. Rokita and C. J. Burrows, *Met. Ions Biol. Syst.*, 2001, **38**, 289.
30 M. Komiyama and J. Sumaoka, *Curr. Opin. Chem. Biol.*, 1998, **2**, 751.
31 M. Irisawa, N. Takeda and M. Komiyama, *J. Chem. Soc., Chem. Commun.*, 1995, 1221.
32 M. Komiyama, N. Takeda and H. Shigekawa, *Chem. Commun.*, 1999, 1443.
33 D. Prema, A. V. Wiznycia, B. M. T. Scott, J. Hilborn, J. Desper and C. J. Levy, *Dalton Trans.*, 2007, 4788.
34 A. V. Wiznycia, J. Desper and C. J. Levy, *Chem. Commun.*, 2005, 4693.
35 R. Gust, I. Ott, D. Posselt and K. Sommer, *J. Med. Chem.*, 2004, **47**, 5837.
36 G. A. Woldemariam and S. S. Mandal, *J. Inorg. Biochem.*, 2008, **102**, 740.
37 S. Awasthi, S. S. Singhal, N. He, M. Chaubey, P. Zimniak, S. K. Srivastava, S. V. Singh and Y. C. Awasthi, *Int. J. Cancer*, 1996, **68**, 333.
38 T. Mosmann, *J. Immunol. Meth*, 1983, **65**, 55.
39 A. Silvestri, G. Barone, G. Ruisi, M. T. Lo Giudice and S. Tumminello, *J. Inorg. Biochem.*, 2004, **98**, 589.
40 W. Lu, D. A. Vicic and J. K. Barton, *Inorg. Chem.*, 2005, **44**, 7970.
41 W. D. Wilson, F. A. Tanious, H. J. Barton, R. L. Wydra, R. L. Jones, D. W. Boykin and L. Strekowski, *Anticancer Drug Des.*, 1990, **5**, 31.
42 G. D. Liu, J. P. Liao, Y. Z. Fang, S. S. Huang, G. L. Sheng and R. Q. Yu, *Anal. Sci.*, 2002, **18**, 391.
43 B. B. Wolf, M. Schuler, F. Echeverri and D. R. Green, *J. Biol. Chem.*, 1999, **274**, 30651.
44 R. Pavri, B. Zhu, G. Li, P. Trojer, S. Mandal, A. Shilatifard and D. Reinberg, *Cell*, 2006, **125**, 703.

