Characterization of Dequalinium as a XIAP antagonist that targets the BIR2 domain

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Abbreviations: BIRs, baculovirus inhibitor of apoptosis proteins repeats; Bz, benzethonium chloride; cIAP, cellular inhibitor of apoptosis proteins 1; Dq, dequalinium hydrochloride; Gfa, glafenine hydrochloride; HeLa, human epithelial carcinoma cell line; IAP, inhibitor of apoptosis proteins; MBz, methyl benzethonium; MTT, (3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide); NMR, nuclear magnetic resonance spectroscopy; Smac/DIABLO protein, second mitochondria-derived activator of caspases/direct IAP-binding protein with low PI; SOSA, selective optimization of side activities; STD, saturation transfer difference; water-LOGSY, water-ligand observed by gradient spectroscopy; TNF, tumor necrosis factor alpha; XIAP, X chromosome-encoded IAP.

Abstract

Inhibitor of apoptosis proteins (IAPs) regulate the activity of caspases in apoptosis. The human X chromosome-encoded IAP (XIAP) is one of the more potent members of the IAP family and it has been described as a central regulator of apoptosis. Thus, molecules that inhibit XIAP could offer therapeutic opportunities to treat unwanted apoptosis inhibition. In the present study we have applied the SOSA (selective optimization of side activities) approach to the discovery of XIAP inhibitors. In this sense, we have identified Dequalinium Hydrochloride (Dq) as an inhibitor of the XIAP/caspase-3 interaction both *in vitro* and in cellular assays.

Introduction

Inhibitor of apoptosis proteins (IAPs) regulate or control the activity of both effector and initiator caspases. IAPs were initially identified in baculovirus as proteins that enhance virus infectivity by inhibition of host cell apoptosis (1). The mammalian family of IAPs is comprised of 8 different members with different functions. Cellular IAP1 (cIAP1), cellular IAP2 (cIAP2) and human X chromosome-encoded IAP (XIAP) are implicated in regulation of apoptosis. XIAP is one of the more potent members of the IAP family and it has been described as a central regulator of apoptosis (2, 3). In addition to its role as caspase inhibitor, recent evidences suggest that XIAP directly participates in NF- κ B and MAP kinase activation (4). XIAP contains two types of conserved sequence motifs; the baculovirus IAP repeats (BIRs) and the zinc finger-like RING. It has been shown that recombinant XIAP is able to specifically block the activity of caspases-3, -7, and -9 (5, 6). The spacer region between BIR1 and BIR2 domains interacts with caspases-3 and -7 while BIR3 targets caspase-9 (7-9). As XIAP regulates caspase activation, the Smac/DIABLO protein (second mitochondria-derived activator of caspases/direct IAP-binding protein with low PI) inhibits XIAP when released from mitochondria upon activation of apoptosis.

When malignant cancer cells fail to activate apoptosis increase their survival factors. Thus, molecules that inhibit IAPs could offer therapeutic opportunities to treat unwanted apoptosis inhibition. As reviewed by Schimmer et al. (10) two approaches have been explored in developing XIAP inhibitors: antisense oligonucleotides and small molecule inhibitors. In the latter approach both rational design and screening of compounds have provided interesting XIAP inhibitors. The rational design approaches have been directed to mimic the Smac/DIABLO-XIAP interaction that is based in the targeting of both BIR2 and BIR3 domains in XIAP by the AVPI tetrapeptide motif found in Smac/DIABLO (11, 12). Small-molecule XIAP inhibitors were reported from successful screening campaigns of a 160,000 small-molecule library by Wu et al. (13) and of approximately 1 million, including peptidyl and non-peptidyl, compound library by Schimmer et al. (14). However, the SOSA (selective optimization of side activities) (15) approach has not been applied to the discovery of XIAP inhibitors. The SOSA approach consists of testing “old” drugs on new pharmacological targets. The aim is to subject to screening a limited number of drug molecules that are structurally and

therapeutically very diverse and that have known safety and bioavailability in humans. In this sense, we have screened the Prestwick Chemical Library® and identified Dequalinium Hydrochloride (Dq) as an inhibitor of the XIAP/caspase-3 interaction both *in vitro* and in cellular assays.

Materials and methods

Materials. The Prestwick Chemical Library® was purchased from Prestwick Chemical (www.prestwickchemical.com). A synthetic peptide including the first seven N-terminal residues of Smac (AVPIAQK - pSmac) was synthesized by Fmoc chemistry, characterized by mass spectrometry and purified by HPLC. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), Dequalinium Chloride, Glafenine Hydrochloride, Benzetonium Chloride and Methyl Benzethonium were purchased from Sigma. Ac-DEVD-afc and z-DEVD-CHO were from Biomol and Calbiochem, respectively. Tumour necrosis factor α (TNF) was from PeproTech. HeLa cell line was obtained from the American Type Culture Collection (ATCC). XIAP antibody (#2042) was from Cell Signalling.

Cell cultures and HeLa cell extracts. Cell cultures were grown at 37 °C in DMEM medium in the presence of 10% fetal bovine serum under atmosphere of 5% CO₂. Cells were seeded in 15 cm plates at 1-2 x 10⁶ cells per plate and harvested when confluent (approximately 10⁷ cells per plate). S-100 extracts from HeLa cells (16) were prepared from 2 x 10⁸ cells in 50 mM PIPES pH 7, 50 mM KCl, 5 mM EGTA, 2 mM MgCl₂, 1 mM DTT. Caspase-3 was activated by addition of 10 mM dATP and 10 μ M Cytochrome c to the cellular extract.

Caspase-3 de-repression assay. Caspase-3 de-repression assay was assessed as previously described (13). Briefly, the active form of caspase-3 was reconstituted from its constituent subunits (17). The assay was performed in 96-well plate format. Recombinant GST-XIAP (XIAP) in caspase buffer (50 mM HEPES, pH 7.4, 100 mM NaCl, 10% sucrose, 1 mM EDTA, 0.1% CHAPS) was preincubated for 15 minutes at room temperature with the library compounds at the indicated concentrations and then recombinant caspase-3 was added. As a control of de-repression the pSmac peptide was used. Caspase-3 activity was continuously monitored following the release of fluorescent afc from the caspase-3 substrate Ac-DEVD-afc, at 30 °C using a Victor 2 spectrofluorimeter (Perkin Elmer - λ_{exc} = 390 nm; λ_{em} = 510 nm).

Caspase-9 de-repression assay. Procaspace-9 in pET23b was transformed in *E. coli* BL21(DE3) and cells were grown to OD 0.6. Expression was induced with 0.2 mM de IPTG for 3 hours at 30 °C. Cells were then lysed by sonication and protein purified by

affinity in a His Trap HP column. The eluted protein was further purified by ionic exchange in a Hi Trap Q HP column in 20 mM Tris pH 8.5, 5% glycerol, 1 mM DTT buffer with a gradient of NaCl. For the de-repression assay 100 nM of procaspase-9 was previously pre-activated in SC buffer (50 mM Na₂HPO₄, 150 mM NaCl, 1.5% Sucrose, 0.05% CHAPS, 0.7 M Sodium Citrate pH 7.4) and then the complete reaction was developed in S buffer (50 mM Na₂HPO₄, 150 mM NaCl, 1.5% Sucrose, 10 mM DTT, 0.35 M Sodium Citrate, pH 7.4). Activated caspase-9 (100 nM) was then used in the de-repression assay following the procedure described for the de-repression assay of caspase-3 but in the presence of the caspase-9 specific substrate Ac-LEHD-afc at 25 °C.

Caspase-3 and caspase-9 activity assays. The enzymes were incubated in the presence (15 min) or absence of a defined concentration of the compounds to test in caspase buffer and then Ac-DEVD-afc or Ac-LEHD-afc, substrates for caspase-3 or caspase-9, respectively, were added. Enzyme activity was monitored following the release of fluorescent afc at 30 °C using a Victor 2 spectrofluorimeter (λ_{exc} = 390 nm; λ_{em} = 510 nm).

NMR experiments. All spectra were recorded at 298 K with a Bruker Ultrashield Plus Avance II 600 MHz spectrometer equipped with a 5 mm cryogenically cooled TCI probe. An NMR sample consisted of 1 μ M XIAP and 20 or 100 μ M Dq from a 50 mM stock in DMSO-d₆, at protein:ligand ratios of 1:20 and 1:100 for the water-LOGSY and STD experiments, respectively. The sample buffer was 50 mM phosphate pH 7.0 with 50 mM NaCl. 8 K points were used for a sweep width of 9,600 Hz and a total of 256 scans were accumulated for the water-LOGSY and STD experiments. In the water-LOGSY experiments, the large bulk water magnetization is partially transferred via the protein-ligand complex to the free ligand in a selective manner. A non interacting compound results in negative resonances, whereas protein-ligand interactions are characterized by positive signals or by a reduction in the negative signals obtained in the absence of the protein. In the STD experiment, a positive interaction is recognized by the presence of positive signals, a negative interaction yielding no signals.

Silencing of XIAP. HeLa cells were seeded in 24-well plates at a cell density of 40000 cells/mL and transfected with either 100 nM XIAP siRNA or random siRNA (Cell Signaling) using LipofectAMINE2000 (Invitrogen) according to manufacturer's

instructions. 24h post-transfection cells were treated with the indicated concentrations of Dq and left to grow for additional 24h. Cell viability was determined by MTT.

Caspase activation assays in cellular models. Cell extracts were prepared from HeLa cells seeded in 3.5 cm plates at 3×10^5 cells per plate. After treatments, cells were harvested, the pellets were resuspended in 30 μ L of extraction buffer (50 mM PIPES, 50 mM KCl, 5 mM EDTA, 2 mM $MgCl_2$, 2 mM DTT supplemented with protease inhibitor cocktail from Sigma) and kept on ice 5 min. After 3 rounds of freeze and thaw, cell lysates were centrifuged at 14000 rpm 5 min and supernatants were collected. Quantification of total protein concentration from these cell extracts was performed using the bicinchoninic acid method (Pierce). Aliquots with appropriate protein content were mixed with 200 μ L of caspases assay buffer (PBS, 10% glycerol, 0.1 mM EDTA, 2 mM DTT) containing 20 μ M of Ac-DEVD-afc. Caspase activity was continuously monitored following the release of fluorescent afc at 30 °C using a Victor 2 spectrofluorimeter (λ_{exc} = 390 nm; λ_{em} = 510 nm). DEVDase activity was expressed as either an increase of relative fluorescence units per min (Δ FU/min) or as percentage of the initial fluorescence signal value.

Results and Discussion

Identification of Dequalinium as inhibitor of XIAP

For the screening we employed an enzymatic de-repression assay (14) based on the XIAP-mediated inhibition on active caspase-3. The objective is to screen the library to identify compounds that relieve XIAP inhibition, allowing caspase-3 to cleave a fluorogenic substrate (see Materials and methods). From the screening and dose-response experiments we identified four compounds (Table I – Fig. 1A) as potential XIAP inhibitors with IC₅₀ (compound concentration that provides 50% inhibition of the XIAP/caspase complex) values similar to the polyphenylureas described by Schimmer et al. (14). Dequalinium Hydrochloride (Dq – chemical structure in top panel of Fig. 2) was the most promising hit with an IC₅₀ of 34 μ M against full-length XIAP (Fig. 1B). None of the hit-compounds identified had a direct activation effect on the enzymatic activity of caspase-3 (Fig. 1A) therefore the increase in activity obtained in the biological assay must be a consequence of a hit compound-mediated inhibition of the XIAP/caspase-3 complex. Furthermore, Dq and Glafenine Hydrochloride (Gfa) were not able to de-repress XIAP-inhibited caspase-9 (Fig. 1C, D) or to induce a direct inhibition on caspase-9 activity while we observed that Benzethonium Chloride (Bz) and Methyl Benzethonium (MBz) inhibited the enzymatic activity of caspase-9 (Fig. 1C).

Dequalinium binds to XIAP as characterized by nuclear magnetic resonance

The interaction between Dq and XIAP was further analyzed by nuclear magnetic resonance spectroscopy (NMR). We used two complementary ligand-based NMR techniques covering a wide range of binding affinities: water-LOGSY (water-Ligand Observed by Gradient Spectroscopy) (18); and STD (Saturation Transfer Difference) (19). Both experiments are ligand-based, i.e. the analysis is focused on the effects caused on the ligand NMR signals upon binding to the target. An excess of ligand is mixed with the target protein, and the exchange between the bound and free states of the ligand originates the effects observed on the latter. In the case of the STD experiment two spectra are recorded, one saturating all the protons of the protein and a second without saturation. Any ligand bound to the target protein will also be saturated in the first experiment, and this saturation will be transferred via exchange to the ligand in the free form. The difference between both spectra will yield only the signals from the

binding ligand, as a non-binding ligand will produce the same spectrum with and without saturation and therefore its signals will cancel out. The water-LOGSY experiment makes use of the water molecules bound at the target-ligand interface to transfer selectively the magnetization from the complex to the free ligand. A positive interaction between a ligand and a target protein is characterised by a positive signal in the 1D ^1H spectrum or by a reduction in the negative signal in the reference spectrum (experiment recorded in the absence of the target protein). In our experiments, 1 μM XIAP was incubated with Dq at a 1:20 (protein:ligand) excess in the water-LOGSY experiment and at a 1:100 excess in the STD experiment. Figure 2a shows the 1D ^1H spectrum of XIAP and Dq with the signals from the molecule identified. The unmarked resonances belong to other protonated chemicals present in the buffer. The water-LOGSY experiment (Fig. 2b) shows opposite sign for the resonances contributed by Dq (positive) and the non-binding chemicals present in the sample (negative), indicating that binding occurs between XIAP and Dq. A control water-LOGSY recorded in the absence of XIAP rendered negative signals for Dq (not shown). The XIAP-Dq binding was further confirmed by the STD experiment (Fig. 2c) which yielded positive signals for those resonances arising from Dq, while the non-binding compounds did not show any signals in the spectrum. As XIAP is expressed and purified using a GST protein fusion as a tag (see Materials and methods), the same experiments were repeated for GST and Dq (Fig. 2 d,e) and no interaction was found, thus confirming that Dq binds to XIAP.

Dequalinium inhibits XIAP in cells extracts and in cell cultures

The *in vitro* results suggested that Dq could be a potential hit as selective inhibitor of the XIAP/caspase-3 interaction. However in a cellular milieu, *in vitro* active molecules have to deal with a number of possible interactions that could compromise their activity on the molecular target. To further explore the efficacy of these hits in a cellular environment, their activity was initially evaluated in mammalian cellular extracts. Cytosolic extracts from human epithelial carcinoma cell line (HeLa) were activated to enter the apoptotic pathway by incubation with ATP and Cytochrome *c* (Cyt *c*) (16) that induces caspase-3 activity. We found that concentrations between 0.1 and 0.5 nM of exogenous recombinant XIAP were needed to induce close to 50% inhibition of cytosolic extract caspase-3 activity (Fig. 3A). In these experimental conditions Dq was able to de-repress XIAP-induced caspase-3 activity in the cell extracts (Fig. 3B).

Dq is a cation delocalized lipidic drug that has been described to accumulate in mitochondria due to the high mitochondrial membrane potential (20) inducing cell death in different cell lines. However, the molecular mechanism behind cell death induction is still unclear and different pathways have been postulated to be affected by Dq (20-22). In this study we have discovered that Dq, *in vitro* and in cell extracts, targets the complex XIAP/caspase-3 which seems to indicate that apoptosis induction could be at the basis of the molecular mechanism of action of Dq. In fact, when HeLa cells were treated with different concentrations of Dq we obtained cell death induction (Fig. 4) and caspase-3 activation (Inset Fig. 4). Western blot analysis demonstrates that Dq does not affect the cellular levels of XIAP (Fig. 4). Furthermore, siRNA-mediated depletion of XIAP drastically reduced HeLa cell sensitivity to Dq-induced cell death (Fig. 5A). It has been demonstrated that previously discovered small molecule inhibitors of XIAP synergize with tumor necrosis factor alpha (TNF) to induce both, caspase activation and apoptosis in human cancer cells (12) as well as tumor regression in xenograft mouse models (23). In the same line of evidence Dq-induced cell death was enhanced in the presence of sublethal concentrations of TNF, reinforcing the role of XIAP as cellular target of Dq (Fig. 5B).

Concluding remarks

In the present study we have shown that Dq binds to XIAP and inhibits the complex XIAP/caspase-3 (but not XIAP/caspase-9), induces apoptotic cell death and also is able to potentiate the pro-apoptotic activity of TNF. Dq is a classic bacteriostatic indicated for the use in mouth, skin and vaginal infections having also cytostatic activity in tumour cells (20, 22, 24). It was also shown that Dq was able to induce size reduction in rat colon tumour isografts (25). Dq accumulates preferentially in mitochondria of cancer cell lines (26). Protein kinase C has also been postulated as one of the potential targets (21) of Dq but the ultimate molecular mechanism of cell death induction has not been solved. Here we show that Dq is able to de-repress XIAP-mediated caspase-3 enzyme activity thereby re-establishing the progression of the apoptotic program in tumour cells. Taking into account the preferential accumulation of the drug into the mitochondria it can be postulated a mechanism of action for Dq similar to that of the natural inhibitors of XIAPs. Dq preferentially accumulates in the mitochondria of Dq-treated tumour cells, probably due to the different mitochondrial potential, and could be released after cell death induction. Accumulative interaction of Dq within

mitochondrial membranes could induce mitochondrial leakage which produces liberation of the drug to the cytosol and amplification of the cell death-signal by inhibition of XIAP-caspase-3 interaction. Recently, it has been shown that XIAP accumulates in the mitochondrial membrane shortly after cell death induction and promotes the formation of a complex that helps mitochondrial transition to permeability leading to Cyt *c* and Smac/DIABLO release (27). Therefore it is plausible an initial interaction between Dq and XIAP at the mitochondrial level that could compromise the anti-apoptotic activity of XIAP.

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Legends to Figures

Figure 1. The compounds identified as XIAP inhibitors hits (Dq, Bz, MBz and Gfa), de-repress caspase-3- but not caspase-9-XIAP mediated inhibition. Measure of caspase-3 (A, B) and caspase-9 (C, D) activity in the presence or absence of XIAP (100 nM and 400 nM, in panels A and C, respectively) and of Dq, Bz, MBz and Gfa at 50 μ M (A, C). The activity of the enzyme in the absence of XIAP or compounds (C+) was defined as 100%. In the presence of XIAP the enzyme activity is inhibited and, as a control, we used a synthetic peptide derived from the natural XIAP-inhibitor Smac/Diablo (pSmac at 1 mM - see Material and methods section) that de-repressed the XIAP-inhibited enzyme activity. Dq, Bz, MBz and Gfa were able to de-repress XIAP-inhibited caspase-3 (panel A) but not XIAP-inhibited caspase-9 (panel C). All four hits did not have any effect on caspase-3 activity in the absence of XIAP (panel A) but Bz, MBz and Gfa exerted an inhibitory effect on caspase-9 (panel C). Panels B and D are Dq full dose-response curves for de-repression of caspase-3 and -9 activities, respectively. In these panels the percentages of enzyme activity with respect to the value obtained in the XIAP-inhibited control are plotted. Data represent the means $\pm$ s.d. of three independent experiments.

Figure 2. Chemical structure of Dq and detail of the aromatic (left) and aliphatic (right) regions of the NMR binding experiments of XIAP with Dq. (a) 1D 1 H NMR of the mixture XIAP-Dq; the Dq resonances are labelled according to the chemical structure; the unmarked signals belong to other chemicals present in the protein buffer. XIAP-Dq water-LOGSY (b) and STD (c) experiments show positive signals arising from Dq, thus proving the interaction between the protein and the molecule. The same experiments were repeated for control GST and Dq (d-e; see main text). All spectra were recorded at 298 K.

Figure 3. Activity of the compounds identified as XIAP inhibitors hits (Dq, Bz, MBz and Gfa), in HeLa cellular extracts. (A) Measure of caspase-3 percentage of inhibition when ATP- and Cyt *c*-activated HeLa cellular extracts were incubated in the presence of increasing concentrations of XIAP. As control of the assay the same extracts were incubated in the presence (DEVD inh) or absence (Ctrl) of 100 nM of the irreversible caspase-3 inhibitor Ac-DEVD-CHO. (B) ATP- Cyt *c*-activated HeLa cellular extracts in the presence of 0.1 nM of XIAP were pre-incubated (15 min) in the presence or absence (-) of the different compounds (50 μ M) and the recovery of caspase-3 activity

respect the XIAP inhibited sample was evaluated. Data represent the means $\pm$ s.d. of three independent experiments.

Figure 4. Dq induces cell death by apoptosis in HeLa cell cultures without affecting the protein levels of XIAP. HeLa cells were incubated with increasing concentrations of Dq for a period of 24 hours. Cell viability was determined by MTT and XIAP protein expression by western blot. Caspase-3 activation in the presence of Dq 25 μ M was determined (Figure inset). Data represent the means $\pm$ s.d. of three experiments.

Figure 5. Dq activity is influenced by the presence of XIAP in the cell and synergizes with TNF. (A) Silencing of XIAP diminishes cell death induced by Dq. HeLa cells were transfected with either XIAP or a random siRNA (see Materials and methods) and incubated for 24h. Dq (5 μ M) was then added to the media and cells were incubated for an additional period of 24h. Cell viability was measured by MTT. (B) Treatment with Dq sensitizes cells to death induced by TNF. HeLa cells seeded in 96 well plates (4000 cell/well) were treated with 50 μ M Dq for 4 hours, then TNF 50 ng/mL was added to the media and cells were incubated for additional period of 20 hours. Cell viability was measured by MTT. Data represent the means $\pm$ s.d. of three experiments (* p <0.05).

Table I. Compound concentration that provides 50% inhibition (IC₅₀) for the XIAP/caspase-3 complex identified from the library screening.

Compound	Structure	IC ₅₀ (μM)
Dequalinium Chloride (Dq)		34
Methyl Benzethonium (MBz)		61
Benzetonium Chloride (Bz)		105
Glaufenine Hydrochloride (Gfa)		116

FIG 1

FIG 2

FIG 3

A

B

FIG 4

FIG 5