Redirecting Apoptosis to Aponecrosis Induces Selective Cytotoxicity to Pancreatic Cancer Cells through Increased ROS, Decline in ATP Levels, and VDAC

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Abstract

Pancreatic cancer cell lines with mutated ras underwent an alternative form of cell death (aponecrosis) when treated concomitantly with clinically achievable concentrations of arsenic trioxide, ascorbic acid, and disulfiram (Antabuse; AAA). AAA’s major effects are mediated through generation of intracellular reactive oxygen species (ROS) and more than 50% decline in intracellular ATP. N-acetyl cysteine and a superoxide dismutase mimetic prevented aponecrosis and restored intracellular ATP levels. DIDS (4,4’-diisothiocyanatostilbene-2,2’-disulfonic acid), the pan- Voltage-Dependent Anion Channel (VDAC), -1, 2, 3 inhibitor and short hairpin RNA (shRNA) to VDAC-1 blocked cell death and ROS accumulation. In vivo exposure of AAA led to a 62% reduction in mean tumor size and eliminated tumors in 30% of nude mice with PANC-1 xenografts. We concluded that early caspase-independent apoptosis was shifted to VDAC-mediated "targeted" aponecrosis by the addition of disulfiram to arsenic trioxide and ascorbic acid. Conceptually, this work represents a paradigm shift where switching from apoptosis to aponecrosis death pathways, also known as targeted aponecrosis, could be utilized to selectively kill pancreatic cancer cells resistant to apoptosis. Mol Cancer Ther; 12(12); 1–12. ©2013 AACR.

Introduction

The 5-year survival with pancreatic cancer is less than 5%, irrespective of the initial stage, and pancreatic cancer is responsible for approximately 41,000 deaths per year in the United States of America (1). Standard chemotherapeutic treatment, gemcitabine, for advanced pancreatic cancer, increases median overall survival in patients with metastatic disease by 5 to 7 weeks over no treatment or 5-fluorouracil (2). In the search for more effective therapies, metastatic disease by 5 to 7 weeks over no treatment or 5-fluorouracil (2). In the search for more effective therapies, we investigated shifting classical apoptosis toward alternate death pathways, such as necrosis, which are rarely activated by classic chemotherapy drugs and have the potential to overcome resistance to apoptosis (3). Although necrosis may invoke a nonspecific inflammatory response and nonspecific cell death, we hypothesized that specificity to cancer cells may be maintained and a nonspecific inflammatory response avoided, if cell death is initiated through apoptosis first then redirected into necrosis (also known as aponecrosis). The high levels of reactive oxygen species (ROS) in pancreatic cancer cells is partially due to mutated K-ras, found in 95% of human pancreatic cancer tumors (4), and the reduced levels of free radical scavengers in pancreatic cancer cells (5) may be an ideal milieu for regimens that exploit this difference to switch apoptosis to necrosis. Mitochondrial-generated ROS activate a variety of cell signaling and death pathways including apoptosis and necrosis (6).

We present a hypothesis-driven model of a switch from apoptosis to aponecrosis, or as we previously termed, targeted necrosis (7), which is dependent on increased generation of ROS in pancreatic cancer cells. This article utilizes a regimen (arsenic trioxide, ascorbic acid, and disulfiram; AAA) we have developed that has synergistic activity which kills pancreatic cancer cells in vitro and in vivo using the aponecrotic pathway and may be dependent on the Voltage-Dependent Anion Channel (VDAC). Major mitochondrial proteins, including VDAC, the Adenine Nucleotide Transporter, and cyclophilin D interact through critical cysteine residues (8) to form the permeability transition pore complex (PTPC). The PTPC provides a pathway for ATP/ADP exchange and is considered to be key for determining whether a cell enters...
mitochondrial-mediated apoptosis or necrosis. AAA was much less toxic to human marrow pluripotent stem cells and nonmalignant cells and had activity in mouse pancreatic tumor xenografts. We view AAA as a prototype regimen for the induction of aponecrosis.

Each component of AAA has been used in patients: arsenic trioxide (ATO; 2 μmol/L maximal serum concentrations) has been successfully and safely used for the treatment of acute promyelocytic leukemia (APL; ref. 9). However, 2 μmol/L ATO alone has not demonstrated antitumor activity against solid tumors (10), and more than 6 μmol/L ATO was required to kill solid tumor and pancreatic cancer cell lines (10). Six μmol/L ATO alone in vitro was toxic in humans, but required to induce in vitro apoptosis in pancreatic cancer cells (10, 11). Ascorbic acid (AA) works synergistically with ATO in non-APL myeloid leukemia cells (12), and 100 μmol/L AA and 2 μmol/L ATO serum concentrations are an effective in vitro/in vivo regimen for human multiple myeloma (13) and APL (9).

However, to have an antitumor effect in pancreatic cancer, which is highly resistant to apoptosis, we wanted to induce necrosis to bypass mechanisms of apoptotic resistance. Pathways for induction of necrosis include caspase inhibition and lowered ATP levels (14). Antabuse (disulfiram; DSF) has been clinically used as an alcohol deterrent, through inhibition of aldehyde dehydrogenase (15), but also inhibits caspase-3 and 7, at 5 μmol/L, which are vital to pathways of apoptosis (16). As shown later, DSF at 0.25 μmol/L provided maximal synergy for induction of aponecrosis when added to ATO/AA (AAA) by decreasing intracellular ATP levels by more than 50% and increasing ROS levels. Normally, DSF is generally used at a clinical dose of 500 mg/day, which achieved serum concentrations of more than 1.4 μmol/L. Thus, all components of AAA are easily achievable and safe in humans. The AAA therapy exploits intrinsically high levels of ROS generated by mutant Ras and intrinsically low levels of detoxification enzymes such as superoxide dismutase (SOD) and glutathione peroxidase in pancreatic cancer. This overwhelms the pancreatic cancer cell with O2− radicals along with specific inhibition of VDAC function, which lowers ATP levels, thereby forcing the pancreatic cancer cell into a completed aponecrotic cell death. AAA promotes in vitro and in vivo killing in human pancreatic cancer cells/tumors at nontoxic ATO concentrations (2 μmol/L), and promotes initial apoptosis, which gives specificity to pancreatic cancer cancer cells, with a delayed necrotic mechanism, thus completing cell death in intrinsically resistant pancreatic cancer cells.

Materials and Methods

Materials

Disulfiram (DSF), also known as tetraethylthiuram disulfide (DSF), sodium ascorbate, referred to in this article as ascorbic acid (AA), arsenic trioxide (ATO), 4,4’-diisothiocyanatostilbene-2,2’ disulfonic acid disodium salt hydrate (DIDS), dextran sulfate (DEX) and N-acetyl cysteine (NAC): Sigma-Aldrich Company; Paclitaxel (PAC) Mayne Pharma. Dihydroethidium (DHE) and dichlorodihydrofluorescein diacetate (DCFDA) were from Molecular Probes.

Cell culture

The human pancreatic cancer cell lines PANC-1, AsPC-1, BxPC-3, MIA PaCa-2, and nonmalignant cell lines MCF-10F (breast epithelial), and CCD-27sk (skin fibroblast) were from American Type Culture Collection. Cell lines were passaged no more than 10 times from liquid nitrogen before being discarded. Monolayer cultures, except MCF-10F, were maintained in RPMI-1640 medium (Life Technologies) supplemented with 10% heat-inactivated FBS (Atlanta Biologicals), 100 U/mL penicillin G, 100 μg/mL streptomycin, and 2 mmol/L glucose (Life Technologies). MCF-10F cells, a human breast nonmalignant cell line, were maintained in DMEM/F12 (1:1) supplemented with 5% horse serum, 2 mmol/L L-glutamine, 100 μg/mL penicillin/streptomycin, 20 mmol/L HEPS, 10 μg/mL insulin, 0.5 μg/mL hydrocortisone, 100 ng/mL cholora toxin, and 20 ng/mL EGF. For all experiments, cells were trypsinized, plated at 5 × 10^4 cells/cm² and allowed to adhere overnight and achieve exponential growth before drug treatments.

Stem cell toxicity assay for CFU-GEMM

Normal adult volunteers, who signed consent forms approved by our Institutional Review Board, underwent leukopheresis of peripheral blood for stem cell harvesting. Peripheral blood CD34+ stem cell progenitors for granulocytes, erythroid, monocytes, and megakaryocytes (CFU-GEMM) were exposed to various drug combinations for 48 hours. After exposure, the cells were washed in PBS, added to methylcellulose H4434 (Stem Cell Technology), with the necessary growth factors for CFU-GEMM. Cells were plated at 1 × 10^5 cells/well and incubated for 14 days at 37°C. The total number of colonies (>50 cells) were scored by a blinded observer.

TUNEL, Annexin V/ PI, Trypan blue, vybrant cytotoxicity assays, and microscopy

Cells were exposed to drug treatments as indicated, trypsinized and pooled with floating cells. TUNEL (terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling) was performed as previously described (7). For Anx V/PI analysis, cells were incubated with Alexa Fluor 488 (Molecular Probes) for 15 minutes followed by 0.33 μg/mL propidium iodide (PI) and analyzed immediately by fluorescence-activated cell sorting (channels FL-1 for Anx V and FL-3 for PI). For Trypan blue analysis, cells were resuspended in 100 mL PBS and Trypan blue (final concentration 0.04%), incubated at room temperature for 5 minutes and at least 200 cells scored/sample by a blinded reader. The Vybrant cytotoxicity assay (Invitrogen) was carried out according to the manufacturer’s instructions. Confocal and electron microscopy was performed as described in our previous experiments (7).
Intracellular glutathione, LDH, and ATP

After various treatments, cells were collected (including floating cells) and washed with PBS. Reduced glutathione (GSH) was analyzed using the ApoGSH Glutathione Colorimetric Kit (Biovision Research Products). Intracellular ATP levels and lactate dehydrogenase (LDH) were determined as described (7).

VDAC-1 shRNA adenovirus and stable cell line

Short hairpin RNA (shRNA) constructs pAd/U6/VDAC-1-shRNA and control pAd/U6/shuffled-VDAC-1-shRNA were synthesized in our laboratory using the human VDAC-1 target sequence 157-AAAATAGACGGGCAGTTCTGGGAA-177 and inserted into an E1/E3-deleted adenovirus vector 5 (18), and propagated in 293A cells according to the manufacturer’s instructions (Invitrogen). The virus particle titer after filtration-based concentration (Adeno-X Virus Purification Kit, Clonetech Laboratories, Inc.) was 1 × 10^9 pfu/mL as determined by plaque tituration assay in 293A cells. Pancreatic cancer cells in log phase were infected over a 24-hour period with pAd/U6/VDAC-1-shRNA or control pAd/U6/shuffled-VDAC-1-shRNA at 20 or 30 MOI (multiplicity of infection), and exposed to drug combinations and assayed.

Doxycycline-regulated expression pSingle-tTS-shRNA against hVDAC1 was stably expressed in the Panc-1 cell line. Specific silencing of the endogenous hVDAC-1 was achieved by using a shRNA-expressing vector. Nucleotides 159–177 of the hVDAC-1 coding sequence were chosen as the target for shRNA. This sequence is homologous to sequences of hVDAC-1. The hVDAC-1-shRNA encoding sequence was created by using the two complementary oligonucleotides indicated below, each containing the 19 nucleotide target sequence of hVDAC-1 (159–177), followed by a short spacer and an antisense sequence. The hVDAC-1-shRNA encoding sequence was cloned into the XhoI and HindIII sites of the pSUPERretro plasmid (Oligoengine), containing a G418 resistance gene. Transcription of this sequence and sequence was neutralized with HCl, and diluted in PBS. Fresh ascorbate and DSF were replenished in the drinking water at each ATO intraperitoneal injection, 3 times/week (calculated for mice weighing 25 g and drinking 5 mL water/day). An equal number of control mice received PBS intraperitoneal injections and normal drinking water. Tumor size was measured weekly for 4 weeks and volume was (mm^3) determined by the following formula: \( \frac{a \times b \times t}{6} \times L \times W \times \times H \).

ROS analysis

Following treatment, floating and adherent cells were harvested, and intracellular ROS were assessed using cytofluorometry with the following 7 probes added in the final 30 minutes of incubation at the following final concentrations: (i) 2', 7'-DCFDA (10 μmol/L; ref. 19), (ii) DHE (10 μmol/L), (iii) singlet oxygen sensor green (SOSG, 5 μmol/L), (iv) MitoSOX red mitochondrial superoxide indicator (MitoSOX, 5 μmol/L), (v) aminophenyl fluorescein (APF, 5 μmol/L; ref. 20), (vi) hydroxylphenyl fluorescein (HPF, 5 μmol/L), and (vii) Amplex Red, (AMP, 200 μmol/L; ref. 21). For ROS dyes #1–6, cells were collected, washed, centrifuged, resuspended in PBS, and fluorescent intensity of 5,000 cells was analyzed on a FACSCalibur flow cytometer (Becton Dickinson) for ROS and for #7, according to the manufacturer’s directions (Invitrogen). These probes mainly measured the following ROS according to the number above: (i) \( \mathrm{H}_2\mathrm{O}_2 \), (ii) \( \mathrm{COO}^- \), ONOO\(^-\); (ii) \( \mathrm{O}_2^- \); (iii) \( \mathrm{O}^- \); (iv) \( \mathrm{O}^- \), \( \mathrm{O}_2^- \); (v) \( \mathrm{OH}^- \), ONOO\(^-\), \( \mathrm{OCI}^- \); (vi) \( \mathrm{OH}^- \), ONOO\(^-\); (vii) \( \mathrm{H}_2\mathrm{O}_2 \). DHE detects general ROS, but especially \( \mathrm{O}_2^- \) (22). SOSG (23) and MitoSOX (24) are relatively selective for singlet oxygen radicals and superoxide radicals (\( \mathrm{O}_2^- \)), respectively. The names of these ROS abbreviations are listed in the Supplementary Methods.

Nude mouse xenografts

Five- to 6-week-old homozygous Nu/Nu (Crl:NU-Foxn1nu) male mice (Charles River Laboratories), were injected at two sites on the dorsal flank with \( 1 \times 10^6 \) Panc-1 cells in 1:1 Matrigel:RPMI media (final Matrigel concentration 5 mg/mL). After 7 days, visible tumors developed and treatment began three times per week when average tumor volume was 50 mm^3 at the following doses: ATO (6.0 mg/g, i.p.; ref. 25), ascorbate (0.75 mg/g/d; p.o.; ref. 26) and DSF (4.0 mg/g/d; p.o.; ref. 27). ATO was dissolved in 1.65 mol/L NaOH, neutralized with HCl, and diluted in PBS. Fresh ascorbate and DSF were replenished in the drinking water at each ATO intraperitoneal injection, 3 times/week (calculated for mice weighing 25 g and drinking 5 mL water/day). An equal number of control mice received PBS intraperitoneal injections and normal drinking water. Tumor size was measured weekly for 4 weeks and volume was (mm^3) determined by the following formula: \( \frac{a \times b \times t}{6} \times L \times W \times \times H \).

Results

Pancreatic cancer cell death induced by ATO + AA + DSF (AAA)

ATO alone at 2 μmol/L did not induce significant death of human pancreatic cancer cell lines in vitro (≤5%). However, when 2 μmol/L ATO was combined with 100 μmol/L AA, there was 22% apoptosis after 48 hours (Fig. 1A). Because DSF (Antabuse) is a potent oxidizer of GSH and a potent caspase-3 inhibitor (\( IC_{30} = 5 \mu\text{mol/L} \); ref. 16), we hypothesized that DSF would synergize with ATO + AA to induce cell death, and could preferentially shift early apoptotic cells to a necrotic cell death pathway. After 48-hour incubation of Panc-1 cells with ATO (2 μmol/L), AA (100 μmol/L) and DSF (0.25 μmol/L; AAA), the TUNEL+ cells (fractured DNA) were: 59% in AAA, 32% in AA + DSF, 22% in the ATO + AA, and 12% in ATO + DSF-treated groups (Fig. 1A). None of the drugs alone produced a significant number of TUNEL+ cells


Published OnlineFirst October 14, 2013; DOI: 10.1158/1535-7163.MCT-13-0234

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Addition of DSF to ATO + AA group synergistically increased cell death from 22% to 59%. Trypan blue analysis confirmed that AA + DSF substantially lowered the cell death IC50 of ATO in PANC-1 from 6 μmol/L to 2 μmol/L (Fig. 1B). At 2 μmol/L ATO, DSF and AA addition resulted in an increase in cell death from 10.0 ± 4.25 to 59.2 ± 4.6, as in the TUNEL results and in MIA PaCa-2 cells from 15.1 ± 2.1 to 74.5 ± 5.3%. Similar results were obtained with AsPC-1 and BxPC-3 cells lines (Supplementary Fig. S1A).

Figure 1. Effect of AAA on pancreatic cancer cell lines and normal cells. A, cells were treated ± ATO (2 μmol/L), AA (100 μmol/L), or DSF (0.25 μmol/L) and DNA fragmentation determined by TUNEL after 48 hours. %TdT+ cells are indicated (Cell Quest software). Veh, vehicle. B, % Trypan blue+ cells determined after 48 hours in PANC-1 or MIA PaCa-2 cells with increasing concentrations of ATO, ±AA, DSF, or (AA + DSF). For all experiments, the concentration of ATO, AA, and DSF was 2, 100, and 0.25 μmol/L, respectively, unless otherwise indicated (see Fig. 4D for determination of minimal effective DSF concentration). AAA = ATO + AA + DSF. For Trypan blue analysis, at least 200 cells were scored per sample and performed in triplicate. All controls contained vehicle additions: DMSO <0.1% and NaOH <66 μmol/L. Veh, vehicle control. P < 0.0124 for PANC-1 and P < 0.006 for MIA PaCa-2 over all ATO concentrations with addition of AA/DSF compared with all other drug combinations. Similar cytotoxicity results were obtained in human pancreatic cancer cell lines, BxPC-3 (83 ± 3.2%), and AsPC-1 (73 ± 5.6%). C, effect of AAA on normal human peripheral blood CD34+ stem cell progenitors for CFU-GEMM ± ATO, AA, or DSF for 48 hours. PAC, paclitaxel at 50 nmol/L, a common clinical concentration. Results represents percentage of control colonies ± SD, n = 3. ***, ***. P < 0.01, < 0.001, respectively, compared with AAA treatment group. D, electron microscopy of PANC-1 cell AAA. After 24-hour incubation with AAA, the cell morphology was primarily apoptotic with apoptotic bodies, condensed DNA, and a dense cytoplasm. By 36 hours, cells with perforated plasma membranes, dispersed hypodense cytoplasm (consistent with necrosis) appeared. By 48 hours, cells displayed a condensed apoptotic-like nucleus and a dispersed necrotic hypodense cytoplasm. Representative photomicrographs are shown for the indicated times, and the nuclei of each cell are circled. Bars = 2.5 microns. All experiments done three times in triplicate.
Effect of AAA on CFU-GEMM and nonmalignant cell lines

The effects of all combinations of AAA drugs were assessed for toxicity to rapidly proliferating normal human peripheral blood CD34+ stem cell progenitors for CFU-GEMM. The cytotoxicity to CFU-GEMM colonies were: ATO, 48% ± 4; AA, 42% ± 1.6; DSF, 38.6 ± 1.7%; AAA, 56 ± 3%; and paclitaxel, 72 ± 1.1% compared with untreated control. Paclitaxel at 50 nmol/L, an average clinically relevant serum concentration in humans, was nearly 2-fold more toxic than any combination of the AAA drugs (Fig. 1C). The cytotoxic effect of AAA on the human nonmalignant breast cell line MCF-10F and the fibroblast line 27 sk was also minimal compared with pancreatic cancer cell lines cytotoxicity: (59.2 ± 4.6 -PANC-1, 74.5 ± 5.3% -MIA PaCa-2, 83 ± 3.2% -BxPC-3, and 73 ± 5.6% -AsPC-1; Fig. 1B and Supplementary Fig. S1B).

Morphology of AAA-treated cells

PANC-1 cells were examined using phase contrast (Supplementary Fig. S1C) and electron microscopy (Fig. 1D). As early as 16 hours after AAA addition, approximately 50% of PANC-1 cells began to round up and detach from the plate and by 48 hours, more than 90% of PANC-1 cells were floating Trypan blue–positive (data not shown). The DNA was pyknotic on 4′, 6-diamidino-2-phenylindole staining. Similar to TUNEL analysis (Fig. 1A), minor death (<10%) was noted after each single drug treatment, and there was approximately 20% death in the AA+DSF-treated group and approximately 90% death from exposure to AAA as detected by phase contrast microscopy (Supplementary Fig. S1C). AAA-treated PANC-1 cells were examined by electron microscopy (Fig. 1D). These microscopic studies suggested that the cells initially entered apoptosis (nuclear features of apoptosis of chromatin along the nuclear membrane; crescent shapes and chromatin bodies), which transformed into aponecrosis (nucleus apoptotic and cytoplasm necrotic) between 24 and 36 hours. The cytoplasm had swelled and numerous breaks in the cell and nuclear membrane were observable, which is a characteristic of necrosis.

Characterization of AAA-induced death

AAA-induced cell death began by 4 hours, and similar to the death curve with pharmacologically high concentrations of paclitaxel (25 μmol/L; Fig. 2A), shown to induce necrosis in other cell types (28). After 4 hours, death from AAA followed a temporal course that was intermediate between apoptosis and necrosis. This was also confirmed in a glucose-6-phosphate dehydrogenase (G-6-PD) assay (Supplementary Fig. S2A). G-6-PD catalyzes
the first and rate-limiting step of the pentose phosphate pathway critical for maintaining NADPH levels, therefore it can be indicative of necrosis. AAA treatment resulted in a 4.25-fold decline in intracellular ATP at 4 hours in PANC-1 cells and a 50% decrease in MIA PaCa-2 (Supplementary Fig. S2B). The release of LDH, indicative of plasma membrane perforations, was not observed at this time (4 hours) suggesting that the loss of ATP by 4 hours preceded the formation of membrane perforations (occurred at >24 hours; Supplementary Fig. S2C). Paclitaxel, at 25 μmol/L, but not AAA, induced mainly necrosis at 4 and 8 hours. This is consistent with the electron microscopy data showing the appearance of necrotic cells after 24 hours.

A time course investigation of cells treated with AAA was demonstrated at various time points: 12 hours, 12% apoptotic and 8% necrotic cells; 24 hours, 10% apoptotic and 29% necrotic cells; 48 hours, 2% apoptotic cells and 38% necrotic cells (Fig. 2B). Therefore, cells that had been in apoptosis may have been shifted to necrosis. There was also extensive necrosis in AAA-treated MIA PaCa-2 cells similar to paclitaxel at 25 μmol/L after 24 hours (Supplementary Fig. S3A). Pancreatic cancer cells from the above time course studies demonstrated no PARP cleavage (Fig. 3A) or no increase in caspase-3 or 9 activity in AAA-treated cells as an explanation for the decline in ATP at the same time point (Fig. 3B). In addition, no increase in caspase-8 activity could be detected, and AAA did not result in cleavage of caspase-3, 8, or 9 until 24-hours unlike paclitaxel treatment, which resulted in caspase cleavage starting at 6 hours (Fig. 3C). The data suggested that AAA-induced cell death followed some of the features of the intrinsic pathway of apoptosis in the early stages such as cytochrome c release (Supplementary Fig. S3B), increased Bak, decreased Bcl-2 (Fig. 3D), but lacked some of the critical features of classical apoptosis such as caspase-3, 8, and 9 activation (Fig. 3B and C) and later demonstrated signs of necrosis such as increased G-6-PD release, and observations by phase contrast, and electron microscopy. These changes were accompanied by decreased ATP levels (>50%), which have been shown to shunt apoptosis into necrosis. Necrosis can occur with low levels of ATP, whereas apoptosis requires higher levels of ATP and apoptosis is shunted into necrosis when more than 50% of a cell’s ATP stores are lost (29).
Mechanism of AAA-induced cell death

NAC abrogated the AAA-induced cell death as measured by Trypan blue. NAC alone had no effect on cell death, but completely reversed AAA-induced cell death (Fig. 4A), and addition of NAC to AAA increased the intracellular ATP 1.8-fold over the AAA group (Fig. 4B). Because NAC may be a ROS scavenger (O_2^-, H_2O_2), and a critical component in the formation of GSH, we measured ROS and GSH levels after AAA treatment. Intracellular ATP and GSH decline after 4 hours correlated with the addition of DSF to ATO+AA in Panc-1 cells (Fig. 4C) and at 0.25 μmol/L DSF, declined by 56% as early as 4 hours after exposure to AAA in MIA PaCa-2 cell line (Supplementary Fig. S3C). Cell death maximized at 0.25 μmol/L DSF (Fig. 4D). Cell death as measured by Trypan blue increased approximately 4-fold over this concentration range of DSF (0.01–0.25 μmol/L; Fig. 4D). A 6.2-fold increase in the number of cells with low NADH correlated with the low mitochondrial membrane potential caused from AAA exposure (Supplementary Fig. S4A and S4B). To further study the relationship between DSF and lowered ATP levels, we assessed the drugs of AAA in various combinations. ATP concentrations fell by 70.7% in PANC-1 and 51% in MIA PaCa-2 cells from AAA exposure, and all other single and binary combinations of AAA had less effect on intracellular ATP concentrations (Fig. 4E and Supplementary Fig. S2B). Only when DSF was added, did ATP levels fall below 50%, a level that we showed earlier to cause apoptosis to transform into necrosis.

Figure 4. Relationship between ATP, GSH, and cell death in AAA-treated PANC-1. A, effect of 3.5 mmol/L NAC on AAA-induced death after 48 hours. "", P < 0.0001. B, effect of 3.5 mmol/L NAC on AAA-induced ATP decline after 4 hours. Intracellular ATP levels were determined by luminometer at 560 nm. Results expressed as % of untreated controls and represent mean luminosity ± SD, n = 3. **P < 0.01. C, intracellular ATP and GSH after 4 hours with ATP/AA and increasing concentrations of DSF. For ATP and GSH, P = < 0.006 for all points with disulfiram compared with 0 μmol/L DSF except GSH at 0.01 μmol/L. D, cell death with 2 μmol/L ATO and 100 μmol/L AA after 48 hours with increasing concentrations of DSF. E, intracellular ATP concentrations in PANC-1 exposed to ATO/AA/DSF/ATO+AA+ATP+DSF/AA+DSF or AAA for 4 hours (see details in Fig. 4B). All experiments done three times in triplicate. P values relative to AAA are indicated. ***, P < 0.001, ****, P < 0.0001, respectively. F, intracellular accumulation of O_2^- radical in PANC-1 cell lines ± ATO, AA, or DSF, or AAA ± NAC. Cells were analyzed by flow cytometry using Mitosox Red dye. All experiments done three times in triplicate.
ROS were analyzed by seven semispecific probes: MitoSOX Red and DHE (superoxide, $O_2^-$), SOSG (singlet oxygen), APF (hydroxyl radical), HPF (hypochlorite anion), Amplex Red ($H_2O_2$), and DCFDA (general ROS). Superoxide radicals appeared as early as 2.5 hours after the addition of AAA in PANC-1 as determined by DHE and MitoSOX Red. After 6 hours, MitoSOX Red showed 54% $O_2^-$ with ATO+AA treatment, and it further increased to 65% $O_2^-$ levels when PANC-1 cells were treated with AAA as compared with 6% baseline (Fig. 4F). NAC totally abrogated the AAA-induced cell death, ATP decline, and ROS ($O_2^-$) levels (Fig. 4A, B, and F). These findings were similarly demonstrated in MIA PaCa-2 with some of the above ROS probes (Supplementary Fig. S5). The $O_2^-$ scavenger, MnTMPyP completely abrogated the AAA-induced $O_2^-$, which was paralleled by a nearly total block of subsequent cell death (Supplementary Fig. S6A). Potassium cyanide (KCN), which blocks the electron transport chain, reduced the $O_2^-$ levels and cell death (Supplementary Fig. S6B). The ROS forms of singlet oxygen-free radical ($1O_2$), hydroxyl radical ($OH$), and hydrogen peroxide ($H_2O_2$) were also generated but to a

Figure 5. Effect of VDAC inhibitors and VDAC-1 shRNA on cell death by AAA. A, effect of 2.4 µg/mL DEX or 100 µmol/L DIDS on cell death as measured by Annexin V. Experiment done three times in triplicate. B, Western blot analysis of VDAC-1 in shRNA adenovirus-treated MIA PaCa-2 cells. C, effect of VDAC-1 shRNA-expressing adenovirus on AAA-induced cell death as assessed by Annexin V. D, Western blot analysis of VDAC-1 in a control PANC-1 cell line containing vector only, and a doxycycline (DOX)-inducible VDAC-1 shRNA-expressing, PANC-1 stable cell line/2 mg/mL doxycycline. E, cell death as assessed by Annexin V in PANC-1 control vector cells or in VDAC-1 shRNA-expressing PANC-1 stable cell line. F, effect of VDAC-1 overexpression by cytomegalovirus promoter-VDAC-1 adenovirus on AAA-induced cell death (AAA assessed at nontoxic, half of standard concentrations of AAA to maximize effect of VDAC-1 overexpression). Experiment was repeated three times. MOI, multiplicity of infection.
much lesser degree than O$_2^-$ free radicals from AAA exposure in these four pancreatic cancer cell lines.

The direct relationship between the action of AAA and its induction of apoptosis is positively correlated either casually or associatively to the mechanism(s) of ATP synthesis. Therefore, we studied the effects of specific inhibitors of the VDAC, an integral part of the mitochondrial PTPC. We found that specific inhibitors of VDAC 1, 2, 3, such as DIDS (4,4-diisothiocyanatostilbene-2,2′-disulfonic acid) and dextran sulfate (DEX), which alters the voltage dependence of VDAC (30) also potently inhibited cell death induced by AAA (Fig. 5A). Superoxide (O$_2^-$) accumulation was also inhibited by DIDS (Supplementary Fig. S6C). Because the VDAC inhibitors reversed cell death, we tested the involvement of VDAC-1 more directly by using shRNA adenovirus directed against VDAC-1.

Discussion

AAA induced an alternative cell death pathway with low, clinically achievable concentrations of readily available drugs that produced synergistic cytotoxicity in pancreatic cancer cells. Initial cell death displayed features of incomplete apoptosis, and later exhibited necrotic features, which suggested necrosis occurred within the same cell (aponecrosis). AAA did not demonstrate synergistic toxicity to normal human marrow CD34+ stem cells or nonmalignant breast epithelial or fibroblast cells, and reduced or eliminated human xenograft pancreatic cancer tumors in nude mice with no discernable toxic effects.

AAA cell death and ROS (mainly superoxide, O$_2^-$) were significantly decreased upon prior or concomitant treatment with NAC, MnTMPyP, (SOD mimetic), dextran, DIDS (VDAC inhibitor) and KCN, (inhibitor of mitochondrial-produced O$_2^-$). The primary ROS involved in AAA-induced cell death was O$_2^-$ and was necessary in the mechanism of cell death from AAA. However, other factors contributing to cell death are possible because ROS was also high in the ATO+AA-treated cells, which showed less cell death than AAA (O$_2^-$ in AAA-65% vs. ATO/AA-54%). The ROS formed, mostly O$_2^-$, is most likely involved in the rapid decline of intracellular ATP levels and reduced NADH levels, triggering a shift to aponecrosis. ATP levels fell rapidly (>50%) only when DSF (0.25 μmol/L) was added to ATO/AA (Fig. 4E). In a mechanism not yet understood, the addition of DSF to ATO/AA decreased ATP levels to initiate aponecrosis. Ample levels of ATP are needed to

**In vivo efficacy of AAA**

AAA was tested in homozygous Nu/Nu (Crl:NU-Foxn1nu) mice, after injection with PANC-1 cells, and tumor size monitored in AAA-treated and control mice (Fig. 6A and B). A significant difference in tumor size between control and AAA-treated mice was noted within 1 week (three AAA treatments) and continued over the next 2 weeks (Fig. 6A and Supplementary Table S1). There were rapid growth rates of control tumor by weeks 1 through 3 and potent cytotoxicity of AAA on PANC-1 tumors. By weeks 1 through 3, the control tumors had grown approximately 50% to 200% over the starting point. In the same time frame, AAA had shrunk the tumor by approximately 50% to 75% in comparison with the original tumor size. By week 3, the tumors in the control group compared with the AAA group was nearly 3-fold larger in size (P = 0.001). This implied that the AAA effect was cytotoxic and not just cytostatic upon the PANC-1 tumor xenografts. AAA treatment also increased the percentage of tumor-free mouse xenografts over the 3-week period (Fig. 6B). By week 3, 30% of AAA-treated mice were tumor free as compared with 5% of the control group by physical exam (P = 0.005; n = 42, 2 tumors/animal, 21 animals/group). There were no physically discernable changes or adverse effects from AAA treatment in the mice.
complete apoptosis, and if the ATP levels fall below a critical level, the cell diverts into a necrotic death pathway (29). However, although intracellular ATP declined early (<4 hours), electron microscopic signs of apoptosis were not observed until after 24 hours. First, cells showed an apoptotic-like nuclear chromatin condensation accompanied by TUNEL+ (Annexin V+/PI- cells) (apoptotic). After 24 hours, membrane perforations (Annexin V+/PI+ cells) appeared, indicative of necrosis. The time between the initial ATP decline and development of apoptotic features suggests that there may be a biochemical program leading to the development of apoptosis. In summary, evidence for early but incomplete apoptosis included: (i) Annexin V+/PI- cells, (ii) cytochrome C release by confocal microscopy, (iii) apoptotic nuclei by electron microscopy, (iv) increased Bak and decreased Bcl-2 expression, and (v) no LDH release. However, we did not observe caspase-3, 8, or 9 or PARP cleavage. Evidence for later necrosis included: (i) reduction of intracellular ATP by more than 50%, (ii) increased Annexin V+/PI+ cells, (iii) massive increase in ROS, (iv) markedly reduced GSH, and (v) a necrotic cytoplasmic morphology by electron microscopy.

VDAC inhibitors reversed AAA-induced O2− accumulation and Annexin V− cells suggesting that AAA may disrupt the PTPC, which is central to both ATP/ADP exchange between the outer mitochondrial membrane (OMM) and cytoplasm, as well as a key modulator of apoptotic/necrotic cell death (31). Other studies have observed a similar switch of apoptosis to necrosis eliciting a VDAC-dependent permeabilization of the OMM and release of cytochrome C (31). ATO has been shown to target VDAC possibly through cross-linking of transmembrane cysteine residues or homodimerization of VDAC molecules, which may disrupt its function (32). AAA exposure may oxidize critical thiol groups involved with the PTPC (8, 33). Importantly, DSF can also directly lower GSH (16), reducing the ability of cells to quench O2−. Decreased GSH could lead to an increase in intracellular arsenic concentrations because GSH is vital to the primary mechanism by which the cell eliminates heavy metals, including arsenic, via GSH metal linkage by GST-pi and efflux via MRP (34). Consistent with this model, we found an approximately 2-fold increase in intracellular arsenic by atomic absorption studies after AAA treatment in Panc-1 cells (data not shown).

AAA’s cancer cell specificity, particularly to mutant K-Ras cells, might be at least partially explained by three mechanisms: (i) pancreatic cancer cells with mutant K-Ras generate higher intracellular levels of ROS than cells without mutant K-Ras (4); (ii) pancreatic cancer cells have decreased levels of ROS-reducing proteins such as SOD and GSH peroxidase (5); and (iii) the possible role of VDAC in the Warburg effect, that may be targeted by AAA (32). For this latter mechanism, we plan to do additional experiments to examine the relationship between AAA and it’s potential activation of the Warburg effect to produce pancreatic cancer cell death.

An alternative and nonexclusive explanation may be that AAA-treated cells are redirected into the apoptotic pathway by blocking caspase activation and/or decreased ATP levels. Blocking activation of specific caspasors or inducing inactivation of active caspasors leads to uninhibited PARP activity, which can deplete ATP stores. PARP activity is modulated by caspases, specifically caspases-3 and 7. A seminal study by the laboratory of S. Snyder found that blocking caspase activity redirected apoptosis to necrosis causing a depletion of energy stores (ADP/ATP) through continual activation of PARP (14). However, we did not find PARP activation from AAA- or PARP-mediated loss of ATP (data not shown), nor membrane perforations (no LDH loss; Supplementary Fig. S2C), which could cause loss of ATP. However, activated caspase activity could also be inhibited from the high levels of O2− generated by AAA (35). In other studies, drastic falls in ATP stores immediately preceded and perhaps initiated the switch to necrosis (36).

There may be a therapeutic window for the clinical application of AAA therapy. First, the concentrations of all three drugs are clinically achievable as in the use of ATO/AA for APL and myeloma. In addition, there was only a minor additional cytotoxic effect of AAA to human pluripotent CD34+ stem cells as compared with ATO alone (8% increase) or to AA alone (14% increase), yet a large increase of cytotoxicity to pancreatic cancer lines (>83%). Our studies in nude mice have shown a promising reduction in tumor size with no observable toxic side effects. However, we plan to use an orthotopic imageable model of pancreatic cancer and a pancreatic cancer GEMM model to further strengthen the support for the effect of AAA in pancreatic cancer (37–40).

The complete abrogation of AAA’s apoptotic effect by NAC suggests a fail-safe to the clinical translation of this therapy where amelioration of AAA-induced clinical toxicity could be mediated by NAC, as it is used for acetaminophen overdose.

Conceptually, the AAA model represents a shift in therapeutic and mechanistic paradigms where alternate death pathways such as apoptosis, could be utilized to selectively kill mutant ras cancer cells resistant to apoptosis by classical chemotherapeutic agents. Targeted aponecrosis by AAA has an advantage over necrosis because it retains the specificity of apoptosis for mutant Ras pancreatic cancer cells but allows completion of cell death through the necrotic pathway while relatively sparing normal cells. We have previously termed this "targeted necrosis" (7). The AAA therapy exploits in pancreatic cancer cells their intrinsically high levels of ROS generated by mutant Ras and their intrinsically low levels of detoxification enzymes in pancreatic cancer and overwhelms the pancreatic cancer cell with O2− radicals along with inhibiting the VDAC machinery which lowers the ATP levels, thereby forcing the pancreatic cancer cell into a completed aponecrotic cell death.
Disclosure of Potential Conflicts of Interest

R.L. Fine has honoraria from speakers’ bureau from Celgene. No potential conflicts of interest were disclosed by the other authors.

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References


Molecular Cancer Therapeutics

Redirecting Apoptosis to Aponecrosis Induces Selective Cytotoxicity to Pancreatic Cancer Cells through Increased ROS, Decline in ATP Levels, and VDAC

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*Mol Cancer Ther* Published OnlineFirst October 14, 2013.

Updated version
Access the most recent version of this article at:
doi:10.1158/1535-7163.MCT-13-0234

Supplementary Material
Access the most recent supplemental material at:
http://mct.aacrjournals.org/content/suppl/2013/10/14/1535-7163.MCT-13-0234.DC1

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