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The Effect of 5-Aminolevulinic Acid and its Derivatives on Protoporphyrin IX Accumulation and Apoptotic Cell Death in Castrate-Resistant Prostate Cancer Cells

Ervin Teper^a, Peter Makhov^a, Konstantin Golovine^a, Daniel J. Canter^b, Cynthia B. Myers^a, Alexander Kutikov^a, Steven N. Sterious^a, Robert G. Uzzo^a, and Vladimir M. Kolenko^a

^aFox Chase Cancer Center, Philadelphia, PA 19111

^bWinship Cancer Institute of Emory University, Atlanta, GA 30322

Abstract

Objective—To examine whether pharmacologically relevant zinc-binding agents are capable of depleting XIAP in tumor cells. Our prior work reveals that treatment with zinc chelating agents induces selective down-regulation of the X-linked inhibitor of apoptosis protein (XIAP) in cancer cells of various origins. A precursor of the heme synthetic pathway, 5-aminolevulinic acid (5-ALA), is metabolized to protoporphyrin IX (PPIX), which is highly reactive with zinc. We assessed whether modified versions of 5-ALA with lipophilic side chains can enhance efficacy and selectivity with respect to PPIX accumulation, XIAP depletion, and TNF-related apoptosis-inducing ligand (TRAIL) – mediated apoptosis in human castration resistant prostate cancer (CRPC) cells.

Methods—Seven modified versions of 5-ALA (five esters and two amides) were synthesized. Levels of endogenous PPIX were examined by flow cytometry. XIAP expression was examined by Western blotting. TUNEL assay was used to assess cell apoptosis. Results were compared qualitatively.

Results—Accumulation of endogenous PPIX by CRPC cells was shown to be directly related to the carbon chain length of the esterified 5-ALA derivatives. In fact, treatment with ALA-HE was superior to that achieved by 5-ALA with respect to XIAP down-regulation. 5-ALA and ALA-HE in combination with TRAIL significantly enhanced apoptotic cell death in CRPC cell lines.

Conclusion—Esterified derivatives of 5-ALA alone or in combination with other agents may provide therapeutic opportunities in treatment of CRPC by harnessing apoptotic pathways that are triggered by cellular zinc imbalance.

Keywords

Prostate cancer; 5-Aminolevulinic acid; Protoporphyrin IX; XIAP; Apoptosis

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Correspondence to: Ervin Teper, MD, Fox Chase Cancer Center, 333 Cottman Avenue, Philadelphia, PA 19111, ervin.teper@fccc.edu, ervoski@yahoo.com.

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INTRODUCTION

Castration-resistant prostate cancer (CRPC) is the second leading cause of cancer death among American men.¹ Established agents, such as docetaxel, as well as newer agents such as Sipuleucel-T and abiraterone offer a modest survival advantage ranging from 1 to 4 months for patients with metastatic prostate cancer.^{2,3} As such, development of new therapeutic strategies that target CRPC represents an overwhelming need in today's clinical practice.

The transformation of normal prostatic epithelial cell into a cancerous one is associated with the loss of the cell's ability to sequester intracellular zinc (Zn).^{4,5} Zinc-deficient prostate cancer cells are markedly more sensitive to zinc depletion than are non-cancerous prostatic tissues. Of all the members of IAP family, X-linked inhibitor of apoptosis protein (XIAP) has received most interest and has been best characterized. Importantly, XIAP is the only member of IAP family able to directly inhibit both the initiation and execution phases of the caspase cascade. By inhibiting effector caspases, XIAP blocks the downstream portion of the apoptosis pathway and inhibits apoptosis from multiple stimuli including the mitochondrial and death receptor-mediated pathways of caspase activation.^{6,7} XIAP levels are elevated in many cancer cells, such as prostate, melanoma and mesothelioma,⁷⁻⁹ and inhibition or down-regulation of XIAP in cancer cells lowers the apoptotic threshold, thereby enhancing the cytotoxic action of anti-neoplastic agents.^{7,10,11} Our group has recently demonstrated that treatment of cells with zinc-chelating compounds, such as N,N,N',N'-tetrakis(2-pyridylmethyl) ethylenediamine (TPEN), results in a rapid depletion of XIAP, leading to accelerated apoptotic cell death in response to tumor necrosis factor-related apoptosis inducing ligand (TRAIL).¹² As such, zinc chelating agents and their derivatives may afford therapeutic opportunities for CRPC and lay the groundwork for future clinical trials.

In light of the established actions of TPEN, our study set forth to examine whether pharmacologically relevant zinc-binding agents are capable of depleting XIAP in tumor cells. Synthesis of the essential protein heme occurs as a multistep biochemical pathway within all mammalian cells. A precursor in the heme synthetic pathway, 5-aminolevulinic acid (5-ALA), is metabolized to protoporphyrin IX (PPIX). Porphyrins are naturally occurring chelating agents with an ability to sequester a variety of metal ions. Importantly, zinc is the second highest affinity metal after copper for binding to porphyrin.¹³

Our current study sought to determine whether PPIX reduces XIAP levels in prostate cancer cells. The assembly of 5-ALA into its biologically active compound, PPIX, preferentially occurs in tumor cells.^{14,15} Moreover, esterified 5-ALA derivatives with varied lipophilicity are processed differently within the malignant cell, potentially affording advantages over 5-ALA.^{16,17} As such, we investigated the biological properties of a number of 5-ALA derivatives.

MATERIAL AND METHODS

Synthesis of 5-ALA ester and amide derivatives

5-ALA esters were synthesized according to the method of Kloek et al.¹⁸ Evaporation of the solvent and crystallization from ether provided the esters in good yield. ALA hydrochloride (500 mg; 2.98 mM), 0.5 ml of freshly distilled thionyl chloride and 3 ml of the corresponding alcohol were heated to 70° C with stirring. The reaction was heated an additional 10 minutes after the ALA dissolved in the alcohol. The reaction mixture was evaporated under reduced pressure. Diethyl ether (10 ml) was added to the residue and the white solid was collected, 222 mg (38% yield). TLC analysis (methanol 10/acetone 15;

sprayed with Ninhydrin) $R_f=0.50$. Propyl ester 440 mg (66% yield) was obtained as a white powder. TLC analysis (methanol 10/acetone 15; sprayed with Ninhydrin) $R_f=0.57$. Butyl ester (380 mg; 57% yield) was obtained as a white powder. TLC analysis (methanol 10/acetone 15; sprayed with Ninhydrin) $R_f=0.54$. LC-MS and NMR data are available. Hexyl ester 559 mg (74% yield) was obtained as a white powder. TLC analysis (methanol 10/acetone 15; sprayed with Ninhydrin) $R_f=0.56$. LC-MS and NMR data are available. For tetrahydrofuranyl ester LC-MS and TLC indicated the presence of several components. The oil was purified by column chromatography over silica gel 60 (4×16cm ; 70–230mesh) and eluted with 10:15 methanol-acetone. Fractions 20–30 contained the THF ester, 155 mg (21% yield). LC-MS and NMR data are available for all. The amides were synthesized in a three step reaction sequence: conversion of 5-ALA to its benzyl ester using thionyl chloride in benzyl alcohol, reaction with the corresponding acid chloride in pyridine, followed by cleavage of the benzyl ester protecting group with hydrogenation using a palladium catalyst. The structures of these compounds were confirmed by LC-MS and ^1H NMR analysis.

The benzyl ester pentylamide (0.901 g; 2.95 mM), 200 mg of 10% Pd/C and 25 ml anhydrous ethanol were shaken under 35–40 psi of hydrogen at room temperature in a Parr apparatus for 16 hours. The catalyst was removed by filtration and the filtrate was evaporated under reduced pressure. The residue was triturated in 10 ml of ether and collected to give 635 mg of pentylamide ALA (50% yield). LC-MS and NMR data are available. The benzyl ester acetylamide (198 mg; 752 mM), 200 mg of 10% Pd/C and 25 ml anhydrous ethanol were shaken under 35–40 psi of hydrogen at room temperature in a Parr apparatus for 16 hours. The catalyst was removed by filtration and the filtrate was evaporated under reduced pressure to give 121 mg (93% yield) of acetylamide ALA. LC-MS and NMR data are available.

Cell lines and culture conditions

Castration-resistant prostate cancer cell lines (PC-3 and DU-145) and normal prostate epithelial cell line (PZ-HPV-7) were obtained from ATCC (Rockville, MD). PC-3 and DU-145 cells were maintained in complete cell culture Roswell Park Memorial Institute (RPMI) 1640 medium (Bio-Whittaker, Walkersville, MD) supplemented with 10% Fetal Calf Serum (Hyclone, Logan, UT), gentamicin (50 mg/L), sodium pyruvate (1mM) and non-essential amino acids (0.1mM). Cells derived from the peripheral zone of histologically normal prostate (PZ-HPV-7) were maintained in Keratinocyte-Serum Free medium (Invitrogen, Carlsbad, CA) supplemented with 5ng/ml of human recombinant epidermal growth factor and 0.05mg/ml of bovine pituitary extract. During the experiments, all cell lines were maintained in a complete cell culture medium. TNF-related apoptosis-inducing ligand (TRAIL) was obtained from Biomol (Plymouth Meeting, PA, USA).

Antibodies and reagents

Antibodies to XIAP and GAPDH were obtained from Cell Signaling Technology (Danvers, MA). 5-aminolevulinic acid and 5-aminolevulinic acid methyl ester were purchased from the Sigma–Aldrich Corporation (St Louis, MO). PPIX was obtained from Frontier Scientific, Inc. (Logan , UT).

Analysis of intracellular PPIX accumulation

Cells were cultured in medium alone or in the presence of 5-ALA or 5-ALA derivatives in triplicates at concentration of 200 μM for 1 hour. The intracellular synthesis of PPIX in various cell lines after exogenous administration of 5-ALA and its derivatives was determined by flow cytometry. PPIX emits a fluorescent signal, which falls within a 590+35 nm range, therefore FL3 channel was utilized to detect its intracellular accumulation.

Western Blot Analysis

Cells were lysed in a boiling sodium dodecyl sulphate (SDS) buffer (50mM Tris (pH 7.6), 150mM NaCl, 2% SDS) for 10 minutes. SDS-PAGE and Western blotting were performed as previously described.¹⁹

Measurement of apoptosis

DNA fragmentation was detected using Apoptosis-Bromodeoxyuridine (APO-BRDU) kit followed by flow cytometry analysis (The Phoenix Flow Systems, Inc., San Diego, CA).

Statistical analysis

Statistical analysis was performed using a two-sided Student's t-test (GraphPad InStat software).

RESULTS

Increasing linear carbon chain length of 5-ALA ester derivatives results in enhanced PPIX accumulation in malignant prostate epithelial cells *in vitro*

Treatment with PPIX significantly reduced XIAP expression in both normal and malignant prostate epithelial cells *in vitro* (Fig. 1A). Next, we investigated whether treatment of cells with a PPIX precursor, 5-ALA, is capable of producing PPIX levels sufficient to promote XIAP down-regulation. Incubation of cells in the presence of 5-ALA resulted in notable down-regulation of XIAP in malignant, but not in normal prostate cells (Fig. 1A). To assess the effect of increasing lipophilicity on biological activity of 5-ALA we synthesized several ester and amide 5-ALA derivatives (Fig. 1B).

A panel of malignant and normal prostate cell lines was incubated in triplicates with pharmacologically relevant concentrations of native 5-ALA and its derivatives (200μM)²⁰ for a period of 1 hour (Table 1). Intracellular PPIX accumulation was examined by flow cytometry analysis. Consistently reproducible results were obtained with PC-3 and DU-145 cell lines, indicating that accumulation of endogenous PPIX by castration-resistant prostate cancer cells is in direct proportion to the carbon chain length of the esterified 5-ALA derivatives. In both cell lines PPIX buildup showed a significant elevation when comparing 1-carbon (methyl) and 2-carbon (ethyl) ester side chain with the 3-carbon (propyl) side chain. A lesser, but still notable effect was observed in androgen-dependent LNCaP cells. Linear chain esters in ascending order of PPIX accumulation were as follows: ALA-EE < ALA-ME < ALA-PE < ALA-BE < ALA-HE. Of note, heterocyclic 5-ALA ester, 5-ALA-TE, as well as the two 5-ALA amide derivatives, Ac-ALA-Am and Pen-ALA-Am, did not induce any notable elevation of endogenous PPIX. 5-ALA itself demonstrated a marked rise of endogenous PPIX, effectively positioning it in the range of propyl and butyl ester side chains (Table 1).

Multiple studies demonstrate that production of PPIX induced by 5-ALA and its esterified derivatives occurs preferentially in tumor cells.^{16,21,22} Indeed, as demonstrated in Table 1, the levels of PPIX buildup in PC-3 and DU-145 prostate cancer cells treated with 5-ALA and its derivatives were substantially higher than in PZ-HPV-7 normal prostate epithelial cells.

Treatment of castration-resistant prostate cancer cell lines with ALA-HE is superior to treatment with the native 5-ALA with respect to XIAP down-regulation

Next, we compared the effect of 5-ALA and its most potent derivative, ALA-HE on the XIAP levels in prostate cells. Cells were incubated with increasing concentrations of 5-ALA

and ALA-HE. Our data indicate that while both compounds are successful at inducing XIAP depletion, ALA-HE consistently outperforms 5-ALA at each respective concentration (Fig. 2A). Consistent with data in Table 1, treatment of non-malignant, prostatic epithelial cell line, PZ-HPV-7, with 5-ALA or ALA-HE failed to induce depletion of XIAP (Fig. 2A).

Treatment with 5-ALA and ALA-HE sensitizes castration-resistant prostate cancer cells to TRAIL-mediated apoptosis

Our data demonstrate that TPEN-mediated depletion of XIAP sensitizes prostate cancer cells to TRAIL-mediated apoptosis *in vitro*.¹² Given that the administration of 5-ALA and ALA-HE promotes depletion of XIAP at the protein level, we set forth to evaluate their cytotoxic effect in combination with TRAIL. Our data show that none of the tested agents alone affect apoptosis in castration-resistant prostate cancer cells. When combined with TRAIL; however, both agents induced apoptosis in both cell lines (Fig. 2B). Importantly, identically treated normal prostate epithelial PZ-HPV-7 cells were shown to have negligible levels of apoptosis (Fig. 2B).

COMMENT

Our previous work indicates that treatment of prostate cancer cells with the zinc chelating agent, TPEN, induces selective down-regulation of a key anti-apoptotic protein, XIAP, and sensitizes cancer cells to death ligand-mediated apoptosis.¹² Our current study demonstrates a significant reduction of XIAP levels in cells treated with 5-ALA and its ester derivatives. Moreover, the intracellular accumulation of protoporphyrin IX after exogenous administration of its precursor 5-ALA also coincides with reduced XIAP expression in prostate cancer cells. A potential mechanism involved in XIAP depletion via 5-ALA/PPIX pathway is illustrated in Figure 3.

PPIX generates red fluorescence when excited by blue light. Because of these properties, 5-ALA and porphyrin-derived agents have been extensively used for photodetection of tumors. Moreover, PPIX and its analogs have a significant, tumor-concentrated photosensitizing capacity. Indeed, Kral et al. have demonstrated that PPIX and porphyrin analogs such as sapphyrins selectively accumulate in tumor tissue. The concentration ratios for PPIX and porphyrin analogs such as sapphyrins were as high as 280:1 in malignant xenograft pancreatic tissue versus surrounding skeletal muscle tissue²³. Extensive studies of 5-ALA and PPIX, as well as novel porphyrin-derived agents, porfimer sodium and temoporfin, show activity in photodynamic therapy for mouse model of ovarian cancer²⁴ as well as clinical trials on bladder cancer,¹⁴ pituitary tumors,²⁵ and glioblastoma multiforme.²⁶ In addition, under certain conditions porphyrins can also act as radiosensitizers. For example, hematoporphyrin dimethyl ether sensitizes an aggressive Ehrlich ascites carcinoma to low doses of ionizing radiation, inducing total tumor growth inhibition.²⁷ Recent clinical studies evaluated the feasibility of 5-ALA for the detection of the surgical margins during radical prostatectomy.¹⁵ This report clearly demonstrates that PPIX fluorescence was predominantly located in cancerous tissue, independent of tumor grade.

With that in mind, our present study set forth to examine whether modifications of 5-ALA via addition of more lipophilic side chains in the form of esters and amides can enhance efficacy and selectivity with respect to PPIX accumulation and XIAP down-regulation in castration resistant prostate cancer cells.

Our experiments show that increasing linear size of the ester side chain is directly related to intracellular PPIX accumulation and subsequent XIAP depletion in both PC-3 and DU-145 cell lines. Prior work by Casas et. al²¹ is consistent with our findings, offering evidence that

ALA-HE, which contains a 6-carbon side chain, is substantially more effective at inducing PPIX accumulation than its methyl ester counterpart, ALA-ME. Notably, intracellular accumulation of PPIX following treatment with 5-ALA and its derivatives is highly specific for malignant cell lines, PC-3 and DU-145, rather than normal prostatic cells, PZ-HPV-7. Interestingly, likely due to aberrant intracellular processing, neither of the two tested amide derivatives, Ac-ALA-Am and Pen-ALA-Am, nor the heterocyclic ester derivative, ALA-TE, produced any detectable intracellular PPIX buildup.

One would anticipate that esterified derivatives of 5-ALA will induce a higher PPIX formation rate in prostate tumors *in vivo* compared with 5-ALA given their lipophilic nature. As mentioned above, recent study by Zaak et al. demonstrates feasibility of oral administration of 5-ALA in a clinical setting. Oral drug delivery offers several advantages over injectable methods providing a more comfortable and convenient delivery route. However, the physicochemical properties of certain therapeutic agents have the potential to compromise their potency via oral administration. The intestinal esterase activities may condition the pharmacological availability of orally administered 5-ALA esters. A possibility exists that esterified derivatives might have superior *in vitro* tumor selectivity while being less selective *in vivo*. Thus, the next logical step would certainly involve the application of these agents to animal models. Such work could potentially include both xenograft and transgenic animal models in an attempt to move towards clinical efficacy of 5-ALA derivatives.

A vast majority of anti-neoplastic agents promote cell death via the initiation of the apoptotic cascade.²⁸ Given that the treatment with 5-ALA and ALA-HE promotes XIAP depletion, we were able to establish the existence of synergism between these compounds and TRAIL with regard to facilitating apoptosis in PC-3 and DU-145 cell lines *in vitro*. As expected, treatment with TRAIL alone had no significant effect on cell death in CRPC cell lines as they are generally resistant to it.²⁹ However, administration of either 5-ALA or ALA-HE aided in overcoming resistance to TRAIL-mediated apoptosis in prostate cancer cells. Furthermore, concomitant treatment with either 5-ALA or ALA-HE and TRAIL induced negligible level of apoptosis in normal prostate epithelial PZ-HPV-7 cells (Fig. 2B).

In summary, our data demonstrate for the first time that 5-ALA downregulates XIAP, a gatekeeper protein in the apoptotic cascade. Furthermore, we provide additional evidence that 5-ALA esters may be superior to the 5-ALA parent compound in terms of XIAP downregulation and sensitization of PC-3 cells to TRAIL-mediated apoptosis *in vitro*. These results are indeed promising, however further investigation is needed.

CONCLUSIONS

In the present report, we demonstrate that 5-ALA and its ester derivative compounds sensitize castration-resistant prostate cancer to TRAIL-mediated apoptosis *in vitro* via downregulation of the X-linked inhibitor of apoptosis protein (XIAP). In particular, ALA esters containing longer side chains are more effective than the parent 5-ALA at inducing these cellular changes. This may hold promise as an adjunctive therapeutic option for patients with CRPC.

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Effect of PPIX and 5-ALA on XIAP protein levels in normal and malignant prostate epithelial cells. (A) Effect of PPIX and 5-ALA treatment on the expression of XIAP in PC-3, DU-145 (malignant) and PZ-HPV-7 (normal) prostate epithelial cells. Cells were cultured in medium alone or in the presence of either PPIX (50μM) or 5-ALA (200μM) for 24 hours. Expression of XIAP and GAPDH was detected by immunoblotting with specific antibodies. Expression of GAPDH was used to document equal protein loading. (B) Structures of 5-ALA and the derivatives.

Figure 2.

Effect of 5-ALA and ALA-HE on the expression of XIAP and sensitization to TRAIL-mediated apoptosis in normal and malignant prostate cell lines. (A) PC-3, DU-145 prostate cancer cells and PZ-HPV-7 normal prostate epithelial cells were cultured in medium alone or in the presence of indicated concentrations of either 5-ALA or ALA-HE for 24 hours. Expression of XIAP and GAPDH was detected by immunoblotting with specific antibodies. Expression of GAPDH was used to document equal protein loading. (B) Cells were cultured in medium alone or in the presence of either 5-ALA or ALA-HE (200 μ M) with or without TRAIL (200ng/ml) overnight. The percentage of apoptotic cells was determined by TUNEL assay followed by flow cytometry analysis. Numbers represent percentage of apoptotic cells. Representative data provided from one of the three experiments.

Figure 3.
Proposed model for XIAP depletion by 5-ALA and its derivatives.

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Table 1

PPIX accumulation in malignant and normal prostate epithelial cells followed by administration of 5-ALA and derivatives at 200µM for 1 hour. Numerical values represent mean percentage PPIX positive cells ± SD (range).

CELL LINES	5-ALA AND DERIVATIVES									
	Med	5-ALA	ALA-ME	ALA-EE	ALA-PE	ALA-BE	ALA-HE	ALA-TE	Ac-ALA-Am	Pen-ALA-Am
PC-3	1.84±0.14	43.35±5.89	5.30±0.16	4.91±0.46	27.53±1.36	63.94±1.91	98.66±0.10	2.25±0.28	2.26±0.30	2.10±0.26
DU-145	2.85±0.11	26.79±1.09	7.92±8.88	3.23±0.51	22.02±0.74	57.09±4.11	90.89±1.48	2.63±0.20	2.59±0.18	2.55±0.18
LNCaP	1.04±0.33	9.01±0.54	2.29±0.54	49.49±1.43	81.75±0.79	77.87±1.46	79.74±1.69	2.14±0.98	2.54±0.32	2.57±0.71
PZ-HPV-7	2.00±0.15	7.36±0.49	1.70±0.14	9.32±0.32	23.17±3.89	24.99±0.76	26.93±3.86	1.34±0.05	1.69±0.08	1.55±0.16