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Anti-tumor activity and mechanism of action for a cyanoaziridine-derivative, AMP423

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Abstract

Purpose—Preclinical studies evaluated the anti-tumor activity and mechanism of action of AMP423, a naphthyl derivative of 2-cyanoaziridine-1-carboxamide with structural similarity to the pro-oxidant anti-tumor agent imexon.

Methods—The cytotoxic potency was evaluated in vitro against a variety of human cancer cell lines. Mechanism-of-action studies were performed in the human 8226/S myeloma cell line and its imexon-resistant variant, 8226/IM10. In vivo activity was evaluated against human myeloma and lymphoma xenografts in SCID mice. Pharmacokinetics and toxicology were investigated in non-tumor-bearing mice.

Results—The 72-h IC_{50} s for all cell types ranged from 2 to 36 μ M, across a wide variety of human cancer cell lines. AMP423 was active in SCID mice bearing 8226/S myeloma and SU-DHL-6 B-cell lymphoma tumors, with a median tumor growth delay (T–C) of 21 days ($P = 0.0002$) and 5 days ($P = 0.004$), respectively, and a median tumor growth inhibition (T/C) of 33.3% ($P = 0.03$) and 82% ($P = 0.01$), respectively. In non-tumor-bearing mice, AMP423 was not myelosuppressive. Mechanistic studies show that AMP423's mode of cell death is a mixture of necrosis and apoptosis, with generation of reactive oxygen species, inhibition of protein synthesis, and a decrease in reduced sulfhydryl levels, but no alkylation of nucleophiles. Unlike its structural

analog imexon, which causes cell cycle arrest in G₂/M, AMP423 induces the accumulation of cells in S-phase.

Conclusions—AMP423 has pro-oxidant effects similar to imexon, has greater cytotoxic potency in vitro, and has anti-tumor activity in hematologic tumors in vivo.

Keywords

AMP423; Imexon; Pro-oxidant; Cyanoaziridine; Preclinical

Introduction

A series of chemically novel derivatives of 2-cyanoaziridine-1-carboxamide were originally described in 1999 [1]. These agents comprised iminopyrrolidone ring-opened congeners of the anti-tumor agent imexon. Among these 2-cyanoaziridine-based analogs, the 1-naphthyl derivative (AMP423, Fig. 1) showed good potency against a battery of human cancer cell lines with IC₅₀ values <36 μM compared to up to several hundred μM for the parent compound, imexon (Fig. 1). Whereas imexon has very good water solubility, AMP423 is more lipophilic with a calculated octanol/water distribution coefficient (*clog P*) value of 3.32. This was important since the previous study showed a direct correlation between lipophilicity and cytotoxic potency for the cyanoaziridine series [1]. This relationship suggested that more lipophilic agents would have improved anti-tumor efficacy over imexon in vitro. However, no in vivo studies and no mechanism-of-action studies have been reported with any of these cyanoaziridine analogs.

The parent molecule imexon has been extensively studied preclinically and has shown activity in vitro in various transformed cell lines [2, 3], as well as in vivo in xenograft tumor models [4, 5]. Mechanism-of-action studies with imexon have shown that the agent binds to sulfur atoms in thiols but not to nucleophilic sites in DNA [6]. Importantly, sulfhydryl binding with imexon requires an intact aziridine moiety and can proceed by two molecular pathways: one involving classic aziridine ring-opening to yield a carbonium ion and the other involving a covalent interaction with the cyano group following opening of the iminopyrrolidone ring. In human multiple myeloma cells, imexon causes an accumulation of reactive oxygen species (ROS) [7, 8] followed by a loss of the mitochondrial membrane potential, mitochondrial swelling, and the release of cytochrome C from the mitochondrial inner membrane [9]. This is followed by activation of caspases 9 and 3 in the intrinsic apoptotic pathway, and the subsequent induction of apoptosis detected by classic morphology and by Annexin V exposure on the outer cytoplasmic cell membrane [10]. In an early study in 8226/S myeloma cells, cell cycle arrest was noted to occur in the proliferative phases of S, G₂, and M vs G₀/G₁ [3]. A later study showed that cell cycle arrest was actually occurring at the G₂/M interphase [8]. Similar mechanistic effects have been described in human pancreatic carcinoma cell lines treated with imexon in vitro [11].

Clinically, imexon has been studied in a variety of solid tumors, predominantly in combination with other agents in phase I clinical trials. These clinical studies include the initial single agent phase I trial of imexon in various refractory cancers [12] and combinations with the alkylating agent dacarbazine in malignant melanoma [13], with docetaxel in lung, breast, and prostate cancer [14] and with gemcitabine in pancreatic cancer [15]. The latter indication was chosen for a phase II randomized trial of imexon since there was synergy noted for the combination in vitro [16]. In the phase I trial of imexon, myelosuppression was not prominent [12] and radiolabeled drug uptake studies in animal models showed poor distribution into the bone marrow (unpublished data). This suggested that imexon would not be active in bone marrow-derived cancers. A more lipophilic analog,

AMP423, was therefore considered for further preclinical development for the treatment of hematologic malignancies.

Because AMP423 showed greater in vitro cytotoxic potency than imexon, anti-tumor efficacy and mechanism-of-action studies were performed to evaluate AMP423 as a potential second-generation analog of imexon with improved preclinical features that might yield a better agent for tumors involving the bone marrow. The studies reported herein show that AMP423 shares many but not all mechanistic features with imexon in human myeloma cell lines. It provides improved anti-tumor potency in vitro and is active in vivo as a single agent against the 8226/S myeloma and SU-DHL-6 B-cell lymphoma cell line.

Materials and methods

Cell lines

The following cell lines were purchased from the American Type Culture Collection (Manassas, VA): multiple myeloma RPMI 8226/S (CCL-155), U266 (TIB-196), and NIH-H929 (CRL-9068); follicular B-cell lymphoma SU-DHL-6 (CRL-2959); B myelomonocytic leukemia MV-4-11 (CRL-9591); pancreatic MiaPaCa-2 (CRL-1420), Panc-1 (CRL-1469), and BxPC-3 (CRL-1687); mammary adenocarcinoma MCF7 (HTB-22) and MDA-MB231 (HTB-26); colorectal HCT-116 (CCL-247); and prostate DU-145 (HTB-81) and PC3 (CRL-1435). An imexon-resistant clone of the 8226/S cell line was established by continuous exposure to increasing concentrations of imexon over 6 months [17]. After this time, these cells, designated 8226/IM10, possessed fivefold resistance to imexon and are continuously maintained in 90 μ M of imexon without significant cell death. The 8226/Dox 40 cell line, a gift from Dr. William Dalton (Moffitt Cancer Center, Tampa, FL), exhibits 120-fold resistance to doxorubicin and is continuously maintained in 40 μ M doxorubicin [18]. The acute myelogenous leukemia line, OCI-AML-3, and the mantle cell lymphoma line, Granta 519, were provided by the Experimental Mouse Shared Service (EMSS) of the University of Arizona Cancer Center, Tucson, AZ. The identity of all cell lines was confirmed by autosomal short tandem repeat (STR) analysis (Human Origins Genotyping Laboratory, University of Arizona) using the American Type Culture Collection database for comparison.

Reagents

Imexon (4-imino-1,3-diazabicyclo[3.1.0]-hexanone) was generously provided by the National Cancer Institute under a RAID grant (Bethesda, MD). AMP423 (2-cyanoaziridine-[N-(1-naphthyl)]-1-carboxamide) and AMP454 (Fig. 1) were synthesized by the University of Arizona Chemical Synthesis Facility as described by Iyengar et al. [1]. For AMP423, the route involved treating an ice-cooled mixture of 468 mg (5 mmol) of 2-cyanoaziridine and 2.5 ml of toluene with an ice-cooled solution of 887 mg (5.25 mmol) of 1-naphthyl isocyanate in 2.5 ml of toluene at a rate to keep the temperature below 5°C. The mixture was stirred for 1 h at ice-bath temperature and then placed in a refrigerator overnight. The precipitate that formed was collected, washed with toluene, and dried under vacuum. Purity of AMP423 was determined by combustion analysis, which showed the predicted values for C, H, and N. A proton NMR spectrum of AMP423 was consistent with the proposed structure. The chemical formula is $C_{14}H_{11}N_3O$, FW 287, with a melting point of 98–100°C. The stability of AMP423 was not studied extensively, but the solid can be stored indefinitely at 4°C. AMP454 was prepared by the same process using ethyleneimine and 1-naphthyl isocyanate.

Gemcitabine was purchased from ChemieTek (Indianapolis, IN). AnnexinV-Alexa488, JC-1, Cell Tracker Green CMFDA (5-chloromethylfluorescein diacetate), and

dihydroethidium (HE) were obtained from Invitrogen (Carlsbad, CA). The following reagents were purchased from Sigma Chemical Company (St. Louis, MO): 3-(4,5-dimethylthiazol-3-yl)-2,5-diphenyltetrazolium bromide (MTT), propidium iodide, RNase A, *N*-acetyl cysteine (NAC), dimethyl sulfoxide (DMSO), 4-(4'-nitrobenzyl)-pyridine (NBP), triethylamine, chlorambucil, and melphalan.

Growth inhibition assays

Growth inhibition assays were performed according to Mosmann [19]. Mitochondrial dehydrogenases reduce MTT in viable cells to produce a colored formazan product, which is solubilized in DMSO and read spectrophotometrically at 540 nm. The percent survival in drug treated wells is calculated compared to untreated cells. The IC₅₀ is defined as the drug concentration required to achieve 50% growth inhibition.

Caspase 3 assays

Caspase 3 cleavage and activity were measured in cell-free lysates using Western blot and a colorimetric activity assay, respectively (Biovision, Mountain View, CA). Cells were incubated with the indicated concentrations of AMP423 or imexon for 24 or 48 h. After drug treatment, cells were pelleted by centrifugation, washed once with ice cold PBS, and lysed for 20 min on ice. Lysates were cleared by centrifugation, and protein quantitated by BCA (Pierce, Rockford, IL). For identification of cleaved caspase 3, equal amounts of protein were separated by SDS-PAGE, transferred to PVDF membrane (Bio-Rad, Hercules CA), probed with caspase 3 antibody (Cell Signaling Technology, Inc. Danvers, MA) and developed using Pierce Supersignal[®] chemiluminescence substrate. For caspase 3 activity, equal protein lysates were incubated with the tetrapeptide substrate, DEVD-pNA. Absorbance of the released pNA is read at 405 nm, and the fold change in caspase 3 activity is calculated, compared to untreated control cell lysates.

Thiol modulation studies

RPMI 8226/S myeloma cells were pretreated overnight with 10 mM *N*-acetyl cysteine (NAC) to reduce free thiols. NAC was prepared as a 200 mM stock solution in PBS and pH balanced to 7.2 prior to using. NAC was either maintained at this same dose for an additional 72 h or removed and replaced with fresh media lacking NAC for the duration of the study. A range of imexon or AMP423 was added for a standard 72-h cytotoxicity assay or for 24 h in the protein synthesis assay.

Flow cytometric analysis

Viability, apoptosis, and necrosis were measured using concurrent AnnexinV-Alexa488 and propidium iodide staining. Mitochondrial membrane potential was measured using 10 µg/ml of JC-1 [20], superoxide radicals measured using 2 µM HE [21], and thiol binding measured using 5 µM CMFDA [22]. Propidium iodide staining of DNA was used for cell cycle analysis. Equi-toxic concentrations of AMP423 and imexon were used. The data acquisition and analysis was performed on a Becton Dickinson FACScan (San Jose, CA) using CellQuest Pro software (BD BioSciences, San Jose, CA) for Annexin V-488/PI, HE, and CMFDA, and Mod-Fit software (BD BioSciences) for cell cycle.

Alkylating activity assay

Alkylating activity was assayed using a modification of the Friedman and Boger NBP assay [23, 24]. Briefly, compounds to be tested were dissolved in their respective vehicle and brought to 200 µg/ml with water. An equivalent volume of 0.1 M sodium acetate buffer, pH 4.5, was then added. After the addition of 0.75 ml of 3.3% NBP prepared in acetone, the tubes were capped tightly, shaken vigorously and then placed in a boiling water bath for 20

min. The samples were allowed to cool to room temperature, and 2.5 ml of 50% triethylamine prepared in acetone was added. The samples were again shaken vigorously, and the purple color was immediately read spectrophotometrically at 575 nm.

Protein synthesis inhibition assay

Inhibition of protein synthesis was measured by ^{14}C -valine incorporation. Cells were plated in 96-well plates and exposed to drug for 24 h, with incorporation of ^{14}C -valine (Amersham Biosciences, Piscataway, NJ) for the final 6 h. Cells were harvested on a Packard FilterMate Harvester (Perkin Elmer, Waltham, MA). Data are expressed as percent of control, where the control is cells incubated with vehicle control (mean $\pm$ SEM, $n = 8$).

Pharmacokinetic (PK) analysis of AMP423

AMP423 PK was determined in 7–8-week-old SCID mice after a single 150 mg/kg dose administered intraperitoneally (IP). Blood was collected into heparinized tubes for up to 96 h after administration. Plasma was collected rapidly and frozen at -80°C . AMP423 concentrations were analyzed on a Thermo Finnigan Surveyor System with PDA detector with a BDS Hypersil C-18 column (150×2.1 mm, 5 μ particle size) and BDS Hypersil C-18 guard column (10×2.1 mm). UV detection is at 290 nm with a filter bandwidth of 9 nm and a sampling rate of 10 Hz. AMP423 elutes at approximately 12.2 min. Pharmacokinetic data are reported as $\mu\text{g/ml}$.

Growth inhibition of xenograft tumors by AMP423 in SCID mice

Subcutaneous xenograft tumors were established in SCID mice and treatment initiated at 150 mm^3 mean tumor volume. Mice with 8226/S, 8226/IM10, or SU-DHL-6 xenografts were treated with 3 doses of AMP423 (150 mg/kg) on days 1, 5, and 9. Mice with Granta 519 xenografts received two courses of AMP423 (150 mg/kg) with 5 days between courses. Body weight and tumor volume were measured at least twice per week and monitored for general health and drug effects. Imexon was given daily $\times 5$.

Tumor growth inhibition (T/C) and tumor growth delay (T–C) were calculated based on criteria established at the NCI [25]. Tumor growth inhibition was calculated at a median tumor volume of 1000 mm^3 , as follows.

$$\text{T/C (\%)} = \frac{\text{median tumor volume of treated mouse}}{\text{median tumor volume of control mouse}} (\times 100)$$

The tumor growth delay value (T–C) is the difference in the median number of days for the tumors in the groups being compared to reach $1,000 \text{ mm}^3$.

Toxicology

A single dose of 200 or 250 mg/kg of AMP423 was administered IP to 7-week-old male SCID mice. Exactly 24 h later, and on day 3 and day 5, blood was drawn from the retro-orbital eye socket. A complete blood count (CBC) and comprehensive chemistry profile was done by the University Animal Care Pathology Services Laboratory of the University of Arizona, Tucson, AZ. Samples were analyzed on an Endocheck Plus Chemistry Analyzer (Hemagen Diagnostics, Inc., Columbia, MD) and a Hemavet 850 (Drew Scientific, Oxford, CT).

Statistical considerations

ANOVA was used to analyze the differences in caspase 3 activity, protein synthesis inhibition, and growth inhibition of xenograft tumors in SCID mice. For cell cycle analysis, two-sample *t* test was used to determine the statistical significance. Alkylating activity was analyzed by one-way ANOVA, with the Bonferroni correction for multiple analyses.

Results

In vitro cytotoxicity of AMP423

To investigate the anti-tumor activity of AMP423, we examined growth inhibition in a broad range of tumor types. Initial studies using 24-, 48-, and 72-h exposure times showed no greater cytotoxic effect with longer exposures to AMP423 in vitro (data not shown). Using the 72-h exposure time, we saw an extremely narrow range of cytotoxic potency, from 2 to 36 μ M, for AMP423 among the various human tumor cell lines in vitro (Table 1). Tumor types represented include pancreatic, colorectal, mammary and prostate carcinomas, as well as leukemia, lymphoma, and multiple myeloma. By comparison, the range of cytotoxic potency for imexon was much greater and varied from 16 μ M to greater than 500 μ M (Table 1). It is interesting to note, however, that there is no direct correlation between drug sensitivity and cell type for either drug. Importantly, neither the imexon-resistant 8226/IM10 nor the *P*-glycoprotein-expressing 8226/Dox40 cells demonstrated cross-resistance to AMP423. Because we are directly comparing the mechanism of action of AMP423 to its parental compound, imexon, all subsequent studies are done using concentrations of each drug that conferred equivalent growth inhibition by MTT assay.

AMP423 induces apoptotic cell death

The MTT assay measures mitochondrial metabolic activity and does not directly measure cell death. We therefore used AnnexinV/PI staining to determine whether AMP423 is cytotoxic (induces apoptosis) or cytostatic (induces cell cycle arrest). Both 8226/S and 8226/IM10 cells demonstrate AnnexinV and/or PI positivity at 48 h (data not shown). Furthermore, to determine whether classic pathways of apoptosis were involved, we examined the cleavage and activation of caspase 3. Myeloma cells were incubated with increasing concentrations of AMP423 for 24 or 48 h, and caspase activity (Fig. 2a) and caspase cleavage (Fig. 2b) were analyzed. Additionally, we examined the loss of the mitochondrial membrane potential (MMP) in AMP423-treated or imexon-treated cells. No significant difference was seen (data not shown). Both assays demonstrated very similar effects by AMP423 and imexon and confirmed the activation of the classical caspase-3-dependent apoptotic pathway, although with slightly different kinetics.

Effects of AMP423 on cell cycle

Cells treated with AMP423 predominately showed an accumulation of cells in S-phase and a corresponding loss of cells in G₁-phase (Fig. 3). These results are in contrast to the action of imexon, which halts cell cycle progression in G₂-phase with a corresponding loss of cells in G₁-phase. These effects were observed in both 8226/S and 8226/IM10 cells.

Effects of AMP423 on cellular thiols

Previous studies have shown that cells treated concurrently with *N*-acetyl cysteine (NAC) and imexon were protected from imexon cytotoxicity [7]. Due to the structural similarity of AMP423 with imexon, we addressed whether NAC would antagonize AMP423 cytotoxicity as well. 8226/S cells were pretreated with 10 mM NAC for 16 h, then NAC was either completely removed or maintained for the duration of the experiment. AMP423 or imexon was added for an additional 72-h incubation and cytotoxicity measured by MTT. These data

demonstrate strong protection by continuous incubation with NAC (Fig. 4). In contrast, removal of NAC immediately prior to drug exposure negated its protective effect on both AMP423 and imexon.

We previously reported that imexon and the 2-cyanoaziridine-1-carboxamide analogs AMP404, AMP413, AMP415, and AMP416 bind to cellular thiols [6]. Due to the structural similarity of AMP423 to imexon, we hypothesized that AMP423 would also cause a loss of cellular thiols. We used concurrent CMFDA and PI staining to analyze intracellular thiol concentration. AMP423 treatment results in approximately 30 and 60% loss of reduced thiol content in the 8226/S and 8226/IM10 cell lines, respectively. This compares to a 20 and 30% loss of reduced thiol content with imexon in the 8226/S and IM10 cell lines, respectively (Supplemental Fig 1). AMP423 was particularly effective at decreasing intracellular thiols in the imexon-resistant 8226/IM10 cell line, which is known to have increased levels of reduced sulfhydryls compared to the parental cell line [17].

Because imexon is a known pro-oxidant, we evaluated whether AMP423 would also cause the accumulation of reactive oxygen species (ROS). Treatment with imexon and AMP423 at equi-toxic concentrations was performed in parallel to compare the effects. Both AMP423 and imexon generated similar amounts of reactive oxygen species at 24 and 48 h (Supplemental Fig 2).

Protein synthesis inhibition

Inhibition of protein synthesis has been identified as a salient feature of imexon cytotoxicity [26]. This is an early cellular response to oxidative stress that occurs prior to the growth inhibitory and cell death activity of the drug. To determine whether AMP423 similarly inhibits protein synthesis, ¹⁴C-valine incorporation was measured in 8226/S and 8226/IM10 cells treated with AMP423 or imexon for 24 h (Fig. 5). As with imexon, AMP423 inhibited protein synthesis at low concentrations prior to the appearance of any biochemical markers of cell death (See Figs. 2, 4). Additionally, co-treatment with 10 mM NAC abrogated the effects of AMP423 on protein synthesis to the same extent as imexon, suggesting this is an ROS-dependent activity (data not shown).

Analysis of alkylation by AMP423

Because AMP423 has chemical similarity to other aziridine containing molecules with alkylating activity, we used a colorimetric alkylation assay to assess reactivity toward the nucleophile γ -4-NBP. There was no evidence of alkylating activity by imexon or gemcitabine, which was used as a negative control (Fig. 6). In contrast, when the cyano moiety was removed from AMP423 resulting in the unsubstituted aziridine analog AMP454 (Fig. 1b), we saw significant alkylating activity ($P < 0.001$), comparable to melphalan and chlorambucil, the positive controls.

Pharmacokinetics of AMP423

Pharmacokinetic analysis (PK) of imexon in mice and in humans has been described in detail [5, 12]. To determine the pharmacokinetics of AMP423, we administered 150 mg/kg of AMP423 to mice intraperitoneally (IP) and analyzed plasma levels for up to 96 h. Results for up to 24 h are shown (Fig. 7). In contrast to plasma imexon levels, which reached a peak of almost 100 μ g/ml 15" after IV administration [12], plasma AMP423 levels never exceeded 6 μ g/ml (Fig. 7). However, similar to imexon,

Anti-tumor activity in vivo

Anti-tumor activity by AMP423 was investigated, in vivo, in 8226/S, 8226/IM10, SU-DHL-6, and Granta 519 xenograft models. Early experiments in SCID mice showed that

more drug could be delivered using a schedule of IP dosing on days 1, 5, and 9 of a 3-week cycle, compared to a daily $\times 5$ schedule, which produced some lethality on days 3–5 at the upper dose range. For that reason, the day 1, 5, and 9 schedule was adopted for subsequent anti-tumor efficacy studies. In SCID mice bearing 8226/S and SU-DHL-6 tumors, AMP423 delayed the median tumor growth by 21 ($P = 0.0002$) and 5 days ($P = 0.004$), respectively, compared to vehicle treated controls (Table 2). In these same tumors, AMP423 also inhibited median tumor growth to 33.3% of control ($P = 0.03$) and 82% of control ($P = 0.01$). We did not see a similar reduction in the imexon-resistant 8226/IM10 or Granta 519 xenograft tumors treated with AMP423. When directly compared to imexon, in SU-DHL-6 lymphoma xenografts, AMP423 appeared more active at limiting tumor growth, but the effect did not reach statistical significance. Similar results were seen in the 8226/S model, with better, but not statistically significantly greater activity by AMP423. Interestingly, in the Granta 519 model of mantle cell lymphoma, imexon was significantly more active than AMP423 ($P = 0.0054$). The molecular basis for this difference is not known.

Toxicology studies

To determine the maximum tolerated dose of AMP423, non-tumor-bearing SCID mice were administered 200 or 250 mg/kg AMP423, or an equivalent volume of DMSO. A single dose of 250 mg/kg was lethal at 24 h in 10/12 mice, but all animals tolerated a single IP dose of 200 mg/kg. Hematologic analysis after 200 mg/kg IP demonstrated negligible effects on red and white blood cell counts (data not shown). At lethal doses (250 mg/kg), there was an elevation of liver enzymes suggesting acute hepatic toxicity, but this was not seen at doses <200 mg/kg. There were also no apparent effects on renal function as evidenced by normal serum creatinine levels and blood urea nitrogen levels following AMP423 administration (data not shown).

Discussion

Aziridine-based anti-tumor agents such as mitomycin C have been well characterized for their anti-tumor activity based on aziridine ring-opening, leading to alkylation of DNA [27]. Many variants of these natural products have been isolated from marine and terrestrial sources [28], or synthesized from mitosene-like intermediates [29]. Mitomycin C is a classical DNA-binding agent that uses reductive activation of the aziridine moiety to form lethal DNA–DNA cross-links as well as more frequent mono-alkylations of DNA [30]. In contrast to these highly reactive aziridines, the cyanoaziridines do not bind to DNA, rather they may be selective for sulfur moieties in biological thiols as was shown for the related iminopyrrolidone compound imexon [6]. In the current report, we have shown that the cyano group mediates this selectivity since the molecule is identical to AMP423 but lacking the cyano group, and AMP454 was a potent alkylator in the nitro-benzyl pyridine reaction. These results suggest that the cyano group reduces the reactivity required for alkylation of DNA bases. This may also explain the lack of typical hematopoietic toxicities with AMP423. In this regard, AMP423 appears to be similar to imexon, which did not produce significant neutropenia or thrombocytopenia in humans [12]. Because of the marked lipophilicity of AMP423, we hypothesized that significant myelosuppression was possible, since the drug should distribute well into the fatty bone marrow compartment. However, we did not see any evidence of myelosuppression in animals given the drug at doses greater than we can expect to use therapeutically.

There were many mechanistic similarities between AMP423 and imexon but the two drugs are not identical. Both agents caused an accumulation of reaction oxygen species and induced a loss of the mitochondrial membrane potential, with decreases in reduced sulfhydryl content. Functionally, protein synthesis and cytotoxic activity of both drugs were inhibited by continuous exposure to NAC, suggesting a role for ROS in the activity of both

drugs. One significant difference we identified was in the cell cycle effects of AMP423 compared to imexon. AMP423 arrested cells in the S-phase of the cell cycle, while imexon caused a G₂/M-phase blockade.

In terms of growth inhibitory potency, AMP423 is approximately 20-fold more potent than imexon across a broad range of human tumor types. This may be due to better cellular uptake mediated by the naphthyl moiety's lipophilicity. The lack of cross-resistance with the doxorubicin-resistant 8226/Dox40 cell line suggests that AMP423 is not a substrate for *P*-glycoprotein mediated efflux. There was also no in vitro cross-resistance with AMP423 in the 8226/IM10 myeloma cell line, which has been selected for imexon resistance in vitro, approximately fivefold. However, there was a trend toward cross-resistance in vivo. We believe this effect may be related to the observation that the 8226/IM10 displays a higher growth rate in vivo compared to the parental 8226/S cell line (unpublished data). We have not specifically investigated the mechanistic basis of this phenomenon; however, there is no difference in the doubling time of the drug-resistant cells compared to the sensitive parental cell line in vitro, suggesting it may be the result of the absence of selection pressure.

We have not yet investigated the full pharmacokinetic profile of AMP423. Our current data demonstrate that although the mean plasma AMP423 concentration is approximately 10-fold lower than plasma imexon, the rapid clearance pattern is similar.

Because of the markedly greater lipophilicity and the higher potency seen in the hematopoietic cancer cell lines in vitro, and the anti-tumor efficacy in human myeloma and lymphoma tumors in vivo, we believe that AMP423 may be a good candidate for clinical development in hematopoietic malignancies such as leukemia, lymphoma, or multiple myeloma.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1.
Structures of AMP423 (**a**), AMP454 (**b**) and imexon (**c**)

Fig. 2.

Caspase 3 activation by AMP423 and imexon. 8226/S or 8226/IM10 cells were incubated with the indicated concentration of drug (AMP423/imexon) for 24 or 48 h. Cell lysates were analyzed for caspase 3 activity (**a**) and cleavage (**b**). Drug concentrations used induced equivalent growth inhibition by 72-h MTT assay. Data shown for the caspase activity assay are the mean $\pm$ SEM of three independent assays with duplicate samples in each. Statistical analysis compares AMP423 versus imexon treatment at equi-toxic doses. $*P \leq 0.05$

Fig. 3.

Analysis of cell cycle effects by AMP423 or imexon. Cell cycle effects in 8226/S or 8226/IM10 cells were analyzed by DNA content after treatment with either AMP423 or imexon. Drug concentrations used induced equivalent growth inhibition by 72-h MTT assay. Results shown are mean $\pm$ SEM ($n = 3$). Statistical analysis compares AMP423 versus imexon at equi-toxic doses. * $P \leq 0.05$

Fig. 4.

Effect of thiol modulation on AMP423 or Imexon cytotoxicity. 8226/S myeloma cells were preincubated with 10 mM NAC (16 h) and either washed out (Pre-tmt) or maintained (continuous) for an additional 72 h with AMP423 (**a**) or Imexon (**b**). Growth inhibition was measured by MTT assay and presented as % of control, where control is AMP423 or Imexon alone

Fig. 5. Effect of AMP423 on protein synthesis. The myeloma cell lines 8226/S (**a**) and 8226/IM10 (**b**) were incubated with AMP423 or imexon for 24 h, with ^{14}C -valine pulse for the last 6 h. Radiolabeled ^{14}C -valine incorporation was measured and calculated as percent of control. Data presented are the mean of 3 independent experiments. Statistical analysis compares the inhibition of protein synthesis by AMP423 or imexon against control. * $P \leq 0.05$

Fig. 6.

Alkylating Activity Assay. Alkylating activity was measured by colorimetric assay using the nucleophile γ -4-NBP as target. All compounds were prepared at 200 μ g/ml and compared to their respective vehicle (DMSO or water). Results shown are mean $\pm$ SEM ($n = 3$). * $P < 0.001$, ** $P < 0.002$

Fig. 7.

Pharmacokinetics of AMP423 in mice. Mouse plasma AMP423 levels after a single 150 mg/kg IP dose is shown. Plasma was analyzed for up to 96 h; results for up to 24 h is shown. Results shown are mean $\pm$ SEM ($n = 5$) plasma AMP423 also peaked shortly after administration and was rapidly cleared.

Table 1

AMP423 and imexon cytotoxicity in human tumor cell lines in vitro

Cell line	AMP423 (μ M)	Imexon (μ M)	Imexon:AMP423 Ratio
Multiple myeloma			
8226/S	3.0 $\pm$ 0.8	18 $\pm$ 2.0	6.0
8226/IM10	2.5 $\pm$ 0.2	100 $\pm$ 1.6	40.0
8226/Dox40	3.6 $\pm$ 0.1	30 $\pm$ 2.7	8.3
U266	5.0 $\pm$ 0.2	240 $\pm$ 12.0	48.0
NIH-H929	4.6 $\pm$ 0.2	48 $\pm$ 2.8	10.4
Lymphoma			
SU-DHL-6	2.7 $\pm$ 0.1	16 $\pm$ 0.8	5.9
Granta 519	6.0 $\pm$ 1.1	35 $\pm$ 1.09	5.8
Leukemia			
OCI-AML3	10.5 $\pm$ 0.3	52 $\pm$ 5.9	5.0
MV-4-11	6.2 $\pm$ 0.2	27 $\pm$ 0.8	4.4
Pancreatic			
MiaPaCa-2	6.3 $\pm$ 1.4	345.1 $\pm$ 28.2	54.7
Panc-1	23.3 $\pm$ 2.1	401.1 $\pm$ 42.3	17.2
Bx-PC3	33.4 $\pm$ 0.9	453.2 $\pm$ 93.2	13.5
Breast			
MCF7	36.0 $\pm$ 1.3	552 $\pm$ 6.2	15.3
MDA-MB231	12.0 $\pm$ 0.7	117 $\pm$ 2.8	9.8
Colorectal			
HCT-116	6.7 $\pm$ 0.1	146 $\pm$ 6.6	21.8
Prostate			
DU-145	10.0 $\pm$ 14.6	380 $\pm$ 7.3	38.0
PC3	17.0 $\pm$ 2.8	300 $\pm$ 12.7	17.6

Values are IC₅₀ (μ M) at 72 h, mean $\pm$ SEM

Table 2
Anti-tumor activity of AMP423 in human myeloma and lymphoma xenografts in SCID mice

Cell line	Cell type	Drug	Median T-C (days)	P-value	T/C (%)	P-value
8226/S	Myeloma	AMP423	21	0.0002	33.3	0.03
		Imexon	19	0.023	40	n.s.
8226/IM10	Myeloma	AMP423	-5	n.s.	135	n.s.
		Imexon	2	n.s.	89	n.s.
SU-DHL-6	Follicular B-cell lymphoma	AMP423	5	0.004	82	0.01
		Imexon	0	n.s.	106	n.s.
Granta 519	Mantle cell lymphoma	AMP423	1	n.s.	114	n.s.
		Imexon	10	0.01	20.5	0.006

One-way analysis of variance was used to test for the overall treatment effects on delay in tumor growth *n.s.* not significant