Abstract

Bcr-Abl activity in chronic myelogenous leukemia (CML) results in dysregulated cell proliferation and resistance against multiple cytotoxic agents due to the constitutive activation of proliferative signaling pathways. Currently, the most effective treatment of CML is the inhibition of Bcr-Abl activity by imatinib mesylate (Gleevec®). Imatinib efficacy is limited by development of resistance through either expression of Bcr-Abl variants that bind imatinib less avidly, increased expression of Bcr-Abl, or expression of multidrug transport proteins. N-Benzyladriamycin-14-valerate (AD 198) is a novel antitumor PKC activating agent that triggers rapid apoptosis through PKC-β2 activation and mitochondrial depolarization in a manner that is unaffected by Bcl-2 expression. We demonstrate that Bcr-Abl expression does not confer resistance to AD 198. Further, AD 198 rapidly induces Erk1/2 and STAT5 phosphorylation prior to cytochrome c release from mitochondria, indicating that proliferative pathways are active even as drug-treated cells undergo apoptosis. At sub-cytotoxic doses, AD 198 and its cellular metabolite, N-benzyladriamycin (AD 288) sensitize CML cells to imatinib through a supra-additive reduction in the level of Bcr-Abl protein expression. These results suggest that AD 198 is an effective treatment for CML both in combination with imatinib and alone against imatinib-resistant CML cells.

Keywords: AD 198; PKC; Imatinib; Bcr-Abl kinase; CML

1. Introduction

Chronic myelogenous leukemia (CML) is generated by the t(9;22)(q34;q11) reciprocal chromosomal translocation that results in the expression the fusion tyrosine kinase, p210 Bcr-Abl [1]. Unlike the normal c-abl kinase, which localizes predominantly in the nucleus and whose activity can lead to G1 arrest and apoptosis [2], Bcr-Abl localizes in the cytoplasm and constitutively activates multiple proliferative, anti-apoptotic, DNA repair, and differentiation pathways, including Ras/Raf/ERK, PI3-kinase/AKT, p38-MAPK, JNK, and JAK-STAT [3]. Consequently, CML cells are resistant to a wide variety of chemotherapeutic agents, including DNA damaging agents [4] and anti-microtubule agents [5]. Inhibition of cell cycle arrest and apoptosis by Bcr-Abl kinase activity following DNA damage is also associated with increased genomic instability [4]. CML progresses through three phases: (1) chronic phase, characterized by the hyperplastic growth of immature granulocytic cells that still retain the capability for terminal differentiation, (2) an accelerated phase in which the immature blast cells accumulate to higher levels in the circulation, and (3) blast crisis, in which Ph-positive blast cells flood the vasculature [6]. In this latter phase, the CML blast cells have lost the ability to differentiate, an event that is associated with Gab2-mediated reduction in Erk1/2 pathway activity [7].

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As a result of Bcr-Abl activity, standard treatment options for CML are limited to myeloablative chemotherapy followed by allogenic stem cell transplantation and α-interferon, alone or in combination with cytosine arabinoside [6]. Cytogenetic response (CR) rates to these treatments are limited; in one study, α-interferon treatment, alone, produced a 12-month CR of 24%, while α-interferon with cytarabine produced a CR of 41% [8]. Currently, the most promising treatment option for CML is imatinib mesylate (STI571; Gleevec®), which inhibits ATP binding to Bcr-Abl and blocks its activity [6–8]. Imatinib inhibits the growth of leukemia cells expressing both the p210 and p185 isoforms of Bcr-Abl, while exhibiting only marginal cytotoxic effects on normal hematopoietic progenitor cells [9]. In clinical studies, administration of high-dose imatinib is reported to produce high rates of both cytogenetic and molecular remission of CML [9–11]. However, incomplete cytogenetic remission is indicative of cellular resistance to imatinib through either Bcr-Abl-dependent or -independent mechanisms [12].

Clinical resistance, commonly observed in patients undergoing blast crisis, correlates principally with kinase mutations of Bcr-Abl, of which over 40 mutations have been identified from clinical samples [12]. These mutations occur either within the binding site of imatinib or elsewhere in the protein to produce allosteric changes in the kinase domain that abrogate imatinib binding [13]. In addition, approximately 10% of imatinib-resistant CML correlates with Bcr-Abl overproduction [14]. Imatinib resistance is also associated with the increased expression of P-glycoprotein (P-gp) and MRP1 multidrug transporters [15] and LYN kinase [16], which, in turn, is associated with elevated levels of Bcl-2 [17]. Resistance to imatinib is also attributed to the activation of Bcr-Abl-independent proliferative and anti-apoptotic pathways [18]. In response, alternate and/or adjuvant treatments have been developed, resulting in varied success. The imatinib structural analog, nilotinib (AMN107) and dasatinib, a modified carboxamide that is structurally distinct from imatinib, exhibit increased potency over imatinib and efficacy against most, but not all Bcr-Abl structural variants [13,19]. Like imatinib, dasatinib is largely ineffective against primitive quiescent CML cells [20], suggesting that alternative treatment strategies are required to eradicate CML cells that are refractory to treatment with current Bcr-Abl kinase inhibitors. The proteosome inhibitor Bortezomib in combination with histone deacetylase inhibitors has also been shown to be effective against imatinib-resistant cells lines and against CD34+ cells from a single patient with accelerated phase CML [21], suggesting that drug combinations may prove to be strategically effective against refractory CML.

We have previously demonstrated that N-benzyladriamycin-14-valerate (AD 198) and N-benzyladriamycin-14-pivalate (AD 445) bind to the C1b domain of conventional and novel isoforms of the protein kinase C (PKC) family of signaling enzymes, resulting in kinase activation [22]. As a result of activation of mitochondrial PKC-δ holoenzyme, AD 198 and AD 445 trigger rapid apoptosis in myeloid cells [23,24]. AD 198 and AD 445-induced apoptosis is not impeded by any of several mechanisms of drug resistance, including multidrug transporters, Bcl-2 and Bcl-XL expression, p53 dysfunction, and NF-κB expression [24–26]. Circumvention of multidrug transporters is largely due to the significant lipophilicity of AD 198 and AD 445, while circumvention of the anti-apoptotic effects of Bcl-2 and Bcl-XL correlate with the ability of these drugs to trigger apoptotic signaling in a manner that is independent of mitochondrial permeability transition pore complex activation and calcium flux [23]. Circumvention of p53 dysfunction correlates with the ability of AD 198 and AD 445 to induce rapid apoptosis in a manner that does not involve initial cell cycle arrest and also suggests that these compounds function by directly triggering apoptosis rather than inflicting cellular damage which would, in turn, cause growth arrest and apoptosis [24]. Based upon these cytotoxic characteristics, we assessed the cytotoxicity of AD 198 on leukemia cells expressing Bcr-Abl and found that Bcr-Abl does not confer resistance to AD 198 despite the proliferative signaling initiated by Bcr-Abl. Further, sub-cytotoxic doses of AD 198 and its principal cellular biotransformation product, N-benzyladriamycin (AD 288), enhance the sensitivity of CML cells to imatinib by accelerating the reduction of Bcr-Abl protein expression. These findings provide a strategy for the treatment of CML cells that are refractory to Bcr-Abl kinase-targeting agents.

2. Materials and methods

2.1. Cell culture and reagents

HL-60 acute myeloid leukemia cells transfected with Bcr-Abl (HL-60/Bcr-Abl), Bcl-XL (HL-60/Bcl-XL), or non-recombinant vector (HL-60/neo) were the generous gift of Dr. Kapil Bhalla (Univ. of Miami) and were maintained in RPMI-1640 medium with 1-glutamine (Atlanta Biological, Norcross, GA) supplemented with 10% fetal bovine serum (Atlanta Biological). K562 human chronic myelogenous leukemia cell lines were obtained from ATCC (Bethesda MD) and DOX-resistant K562 cells were the generous gift of Dr. Steven Grant (Virginia Commonwealth Univ., Richmond, VA). Both cell lines were maintained as described for HL-60 cells. Imatinib mesylate (Gleevec®) was the generous gift of Novartis Pharma AG (Basel, Switzerland). Phorbol 12-myristate 13-acetate (PMA) and doxorubicin (DOX) were obtained from Sigma Chemicals (St. Louis, MO), while AD 288, AD 445, and AD 198 hydrochloride salts were the generous gift of Dr. Mervyn Israel (Dept. of Pharmacology, University of Tennessee Health Science Center) and prepared according to previously described procedures [27]. Rottlerin, G66976, SP600125, and PD98059 were obtained from Calbiochem (La Jolla, CA). All drugs were dissolved in dimethylsulfoxide (DMSO).
2.2. Cell viability determinations

IC\textsubscript{90} concentrations (drug concentration required to kill 90\% of cells 24 h after 1 h drug treatment) of drugs for HL-60 and K562 cells were determined using the MTT assay [28]. Cells were exposed to drug at an initial cell density of 2 × 10\textsuperscript{5} ml\textsuperscript{-1} in complete medium for 1 h at 37 °C in a 5% CO\textsubscript{2} humidified environment. Control cells were treated with 1% DMSO for 1 h. In separate experiments, the rate of cell kill at a specific drug concentration was determined by the trypan blue dye exclusion assay described previously [24]. All data points are the mean of at least three independent determinations.

2.3. Cell fractionation, PKC translocation and immunoblot analyses

Isolation of total cell extract, cell fractionation, and analyses of PKC translocation and cytochrome c release were performed as described previously [24]. Antibodies used for immunoblot analyses are listed below. Antibodies purchased from Cell Signaling Technology (Beverly, MA) were: Bcr, 1:1000 rabbit polyclonal; phospho-STAT5, 1:1000 mouse monoclonal; STAT5, 1:1000 rabbit polyclonal; HSP70, 1:1000 rabbit polyclonal; Bcl-X\textsubscript{L}, 1:1000 rabbit polyclonal; ERK1/2, 1:1000 rabbit polyclonal. The following antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA): c-Jun, 1:500 mouse monoclonal; phospho-c-Jun, 1:500 mouse monoclonal; PKC-\(\delta\) (C-17), 1:1000 rabbit polyclonal. Other antibodies include: PKC-\(\beta\) (clone 36) 1:250 mouse monoclonal (BD Biosciences, San Diego, CA); phospho-MAPK, 1:500 goat polyclonal (Upstate Biotechnology, Lake Placid, NY); cytochrome c, 1:400 mouse monoclonal (BD Biosciences, San Diego, CA); \(\beta\)-tubulin (clone TUB2.1), 1:3000 mouse monoclonal.

2.4. RT-PCR

Relative mRNA quantitation of Bcr-Abl was performed using the MasterAmp\textsuperscript{TM} High Fidelity RT-PCR kit (Epicentre, Madison WI) as per the manufacturer’s instructions. \(\beta\)-Actin mRNA levels were used as quantitative controls. Bcr-Abl primers 5\textsuperscript{'}′-GGAGCTGCAGATGCTGACCAAC-3′ (sense) and 5\textsuperscript{'}′-TCAGACCCCTGAGGCTCAAAGTC-3′ (antisense) span the Bcr and Abl fusion site [29]. \(\beta\)-Actin primers were 5\textsuperscript{'}′-AGCGGGAAATCGTGCGTGACA-3′ and 5\textsuperscript{'}′-GTGGACTTGGAGAGGACTGG-3′.

3. Results

Bcr-Abl expression in HL-60 cells mediates anti-apoptotic signaling [3] and confers resistance DNA-damaging agents [4]. Predictably, Bcr-Abl confers resistance to DOX and the topoisomerase II catalytic inhibitor, AD 288 [30], as shown in Fig. 1. IC\textsubscript{90} drug concentrations (concentration producing 90\% cell kill 24 h following a 1 h exposure to drug) for DOX, AD 288, and AD 198 in HL-60/neo cells were determined by the MTT assay. HL-60/neo and HL-60/Bcr-Abl cells were then exposed to the IC\textsubscript{90} value for each drug in HL-60/neo cells and the rate of cell kill was compared. The rate of cell kill over 72 h was markedly slower in HL-60/Bcr-Abl cells for DOX and AD 288, with 90\% cell kill unachievable within 72 h. However, the rate of cell kill for AD 198 was the same for both HL-60/neo and HL-60/Bcr-Abl cells, indicating that Bcr-Abl expression does not confer resistance to AD 198.
As we have shown previously in murine myeloid cells, AD 198-induced apoptosis, unlike that of DOX and AD 288, requires the activation of mitochondrial PKC-δ holoenzyme [34–38]. Through this pathway, AD 198 is able to circumvent multiple mechanisms of drug resistance, including Bcl-2 and Bcl-XL expression [23,24]. Consequently, we examined the ability of AD 198 induce membrane translocation and activation of PKC-δ in human K562 CML cells that express endogenous Bcr-Abl kinase. Within 1 h of drug exposure, PKC-δ levels increase in the membrane fraction of K562 cells (Fig. 2A). In contrast, no significant membrane translocation is observed with PKCs-α and -β. The translocation/activation...
MEK inhibitor PD98059 (198-induced ERK1/2 phosphorylation was inhibited by the activation by AD 198, in turn, affects drug cytotoxicity, AD total ERK1/2 expression. To determine whether ERK1/2 acti-
nearly induces the phosphorylation of ERK1/2 within 30 min activated by Bcr-Abl kinase. As shown in proliferative/anti-apoptotic pathways in K562 cells that are fore, we determined whether AD 198 treatment modulates of apoptotic signaling despite Bcr-Abl activity. There-
reduced by 60% within the first hour of AD 198 treatment and then further reduced to control levels 2 h post-drug treat-
ment. Despite the inhibition of ERK1/2 phosphorylation, cell sensitivity to AD 198 remained unchanged (Fig. 3B), indic-
ating that AD 198-induced cell kill is not mediated through or impeded by ERK1/2 pathway activation.

Bcr-Abl expression results in constitutive STAT5B phos-
phorylation and activation [3,31]. Immunoblot analysis of K562 cells also shows increasing phosphorylation of STAT5 during and following exposure to the IC50 concentration of AD 198. Phosphorylation of STAT5 precedes AD 198-induced release of cytochrome c from mitochondria. During AD 198 exposure, transient fluctuations in STAT5 protein levels are observed, but do not correlate with the sustained increase in phosphorylation. Bcr-Abl induces sustained expression of Bel-XL [31], which is mediated by STAT5 activity [3]. AD 198-induced phosphorylation also corresponds with a modest, transient increase in Bcl-XL expression 1–3 h post-AD 198 exposure, which is insufficient to inhibit cytochrome c release. In fact, high level expression of Bel-XL in HL-60 cells prior to AD 198 exposure fails to inhibit AD 198-mediated cell kill (Fig. 4). HL-60 cells trans-
fected with a Bel-XL expression vector [32] were treated with increasing concentrations of DOX, AD 288 or AD 198 and cell survival assessed by the MTT assay. Bel-XL expression has no significant effect on both the IC50 and IC90 values of AD 198. In contrast, Bcl-XL expression inhibits both DOX and AD 288 cytotoxicity, with three- to five-fold increases in the IC50 values. IC90 values for DOX and AD 288 were not attained in HL-60/Bcl-XL cells at the drug concentration range that was used.

Bcr-Abl activates the JNK pathway leading to c-Jun phos-
phorylation and activation [33]. Likewise, AD 198 stimulates both c-Jun phosphorylation, initially observed 1 h after drug exposure and prior to cytochrome c release from mitochon-
dria, with markedly increased Jun expression 3 h after drug exposure. However, as observed with the chemical inhibition of ERK1/2, c-Jun activity does not appear to modulate AD 198 cytotoxicity, since combined treatment of cells with AD 198 and the JNK inhibitor SP600125 does not alter the rate of AD 198 cell kill (Fig. 5B).

Bcr-Abl also exerts its proliferative/anti-apoptotic effect, in part, through increased expression of hsp70, which in turn acts as a chaperone for nascent proteins to ensure proper folding [5]. Inhibition of hsp70 expression poten-
tiates drug-induced cytotoxicity in K562 cells [5,34]. AD 198 triggers apoptosis without inhibiting hsp70 expression in K562 cells, with hsp70 protein levels remaining con-
stant through the early events of AD 198-induced apoptosis (Fig. 5A).

The efficacy of AD 198 in the treatment of leukemia cells is not limited to its ability to circumvent resistance mediated by Bcr-Abl, but also in its ability to circumvent resistance against drugs that target Bcr-Abl. P-glycoprotein expression is reported to confer resistance to imatinib in K562 cells and likely to be a clinically relevant mechanism.
of imatinib resistance in CML cells [35]. However, P-gp expression in K562 cells selected for DOX resistance [36] has only a modest effect on the IC_{50} values of AD 198, but with no change in IC_{90} values (Fig. 6). In contrast, P-gp expression inhibits both DOX and AD 288 cytotoxicity. IC_{50} values of neither compound are attainable at 5–8 times IC_{50} concentrations of control levels. Other mechanisms of resistance to imatinib, such as increased Bcr-Abl expression and the expression of structural mutants of Bcr-Abl, will likely not inhibit AD 198 cytotoxicity, since our results show that AD 198-mediated apoptosis occurs independently of the proliferative effects of Bcr-Abl.

Our results thus far suggest that at cytotoxic doses, AD 198 circumvents multiple mechanisms of resistance against imatinib and other cytotoxic chemotherapeutic agents in CML cells and, consequently, show potential as alternative treatment for CML, alone or in combination with imatinib. Moreover, sub-cytotoxic levels of both AD 198 and its principal cellular biotransformation product, AD 288 also demonstrate therapeutic potential in combination with imatinib. Fig. 7A shows that 1 h exposure of K562 cells to 250 nM AD 198 or AD 288, alone, slows cell proliferation, but does

P-gp expression does not inhibit AD 198 cytotoxicity. K562 wild type and DOX-resistant cells were assayed for sensitivity to AD 198, AD 288, and DOX using the MTT assay, as described in Section 2.

Fig. 6. P-gp expression does not inhibit AD 198 cytotoxicity. K562 wild type and DOX-resistant cells were assayed for sensitivity to AD 198, AD 288, and DOX using the MTT assay, as described in Section 2.

Continuous exposure of cells to 10 μM imatinib exhibits no effect on cell proliferation in the initial 24 h with a 1.75-fold increase in cell number. However, in the following 48 h of imatinib exposure, cell viability decreases to 20% of peak cell number. Imatinib in combination with AD 198 or AD 288 shows a more rapid inhibition of cell proliferation, with less than 40% live cells at 48 h, compared with >100% following exposure to individual agents.

Increased sensitivity to imatinib in the presence of low dose AD 198 or AD 288 correlates with a rapid reduction in the expression of Bcr-Abl protein (Fig. 7B). Individual drug exposure does not significantly alter Bcr-Abl expression in the initial 48 h, but results in a complete or partial reduction.
of Bcr-Abl expression by 72 h for imatinib and AD 198/AD 288, respectively. However, combined treatment results in a greater than 90% reduction in Bcr-Abl protein expression by 24 h for combined imatinib/AD 198 and by 48 h for imatinib/AD 288. The supra-additive inhibition of Bcr-Abl expression does not occur at the transcriptional level, since RT-PCR of Bcr-Abl mRNA reveals no quantitative decrease following treatment of K562 cells with imatinib, AD 198, and AD 288 alone of in combination.

4. Discussion

The results of this study indicate that the cytotoxicity of the novel PKC-activator AD 198 is not blocked by Bcr-Abl kinase-mediated proliferative/anti-apoptotic activity in human leukemia cells. Further, mechanisms of resistance that impede the cytotoxic actions of the Bcr-Abl inhibitor, imatinib, have no effect on AD 198 cytotoxicity. In addition, sub-cytotoxic levels of AD 198 and its metabolite, AD 288, have a supra-additive effect in combination with imatinib to increase the rate of tumor cell kill through inhibition of Bcr-Abl protein expression. These drug actions provide an alternative strategy to the elimination of CML cells that are refractory to Bcr-Abl inhibitors. Current strategies to eradicate Bcr-Abl-expressing cells include direct inhibitors of Bcl-Abl protein expression, such as imatinib, dasatinib and nilotinib [19], or specific inhibitors of Bcr-Abl-mediated proliferative and anti-apoptotic pathways alone or in combination with cytotoxic agents [37–40]. In contrast, our results indicate that AD 198 does not require inhibition of Bcr-Abl-mediated signaling pathways in concert with the induction of apoptosis. Rather, AD 198 induces apoptosis through direct PKC-δ activation even as several proliferative pathways appear to be activated. AD 198 induces rapid ERK1/2 phosphorylation, yet its activation neither potentiates nor attenuates AD 198-mediated apoptosis, based upon the absence of either effect by the MEK inhibitor PD98059 on Bcr-Abl+ HL60 cells treated with AD 198. This may be due to the rapid induction of apoptosis by AD 198, which, consequently, does not afford sufficient time for ERK1/2 proliferative signaling to protect the cell. As shown in previous studies, MEK inhibition potentiates apoptosis in K562 cells induced by the PKC inhibitor UCN-01 [41]. However, under these conditions, inhibition of ERK1/2 precedes apoptosis by at least 24 h.

Similarly, in AD 198-treated K562 cells, STAT5 phosphorylation occurs rapidly and precedes cytochrome c release into the cytosol. The protective effects of STAT5 activation in K562 cells include increase Bcl-XL expression [3]. The stimulation of STAT5 activation and Bcl-XL expression in AD 198-treated cells likely occurs too late to impede apoptotic signaling. Further, even high levels of Bcl-XL expression prior to AD 198 treatment of HL-60 cells transfected with Bcl-XL expression vector does not inhibit apoptosis. The circumvention of Bcl-XL in HL-60 cells and, for that matter, other anti-apoptotic Bcl-2 isoforms in a variety of other cell types [24,26] is not due to enhanced expression of pro-apoptotic Bcl-2 isoforms (data not shown), but, rather, due to the novel ability of AD 198 to trigger mitochondrial-dependent apoptosis in a unique manner [23]. As such, elevated levels of Bcr-Abl activity, as observed in some imatinib-resistant CML variants [14], would predictably have little, if any, effect on AD 198 cytotoxicity, since Bcr-Abl-mediated proliferative signaling appears not to antagonize AD 198-induced apoptosis.

The signaling mechanism(s) by which AD 198 induces ERK1/2 and STAT5 phosphorylation is presently undetermined. PKC-α is instrumental in epoR-mediated erythrocyte differentiation through the phosphorylation of STAT5 and ERK1/2 [42], while PKCβII is has been reported to promote proliferative signaling, including STAT5 activation, in multipotent hematopoietic cells [43]. However, AD 198 does not appear to activate PKCs-α and -βII in K562 cells, based upon the absence of isoform translocation to cellular membranes. PKC-ε signaling crosstalk with ERK1/2 pathways has been demonstrated in K562 cells and is instrumental in phorbol ester-induced differentiation of K562 cells to megakaryocytes [44]. PKC-ε expression is only marginally detectable in proliferating K562 cells. Further, AD 198 does not induce PKC-ε expression within the time that ERK1/2, STAT5, and c-Jun phosphorylation is observed (data not shown). Alternatively, ERK1/2 activation could be achieved by direct stimulation of RasGRP by AD 198, which, like PKC, is a C1 domain-containing protein [45]. However, the ability of AD 198 to activate ERK1/2 via RasGRP stimulation remains to be determined.

Phosphorylation of c-Jun in K562 cells has been reported to contribute to the lethality of a number of agents, including flavopiridol [46], histone deacetylase inhibitors [39], and cisplatin [47]. AD 198 stimulates the phosphorylation of c-Jun prior the release of cytochrome c from mitochondria. The JNK inhibitor, SP600125, affords protection to leukemic cells against a wide variety of cytotoxic agents, including DOX [48]. However, inhibition of JNK activity by SP600125 fails to inhibit AD 198 cytotoxicity, suggesting that the JNK/c-Jun pathway is not essential for AD 198-induced apoptosis in K562. The JNK pathway has been implicated in promoting apoptosis through inactivation of Bcl-2 [49] and through Bax translocation to mitochondria [50]. Since AD 198-induced apoptosis is not altered by Bcl-2 protein family status [23,24,26], JNK/c-Jun-mediated signaling would not be expected to either delay or promote the effects of AD 198.

Bcr-Abl expression is associated with the constitutive activity of the proliferative PI3/Akt pathway in both clinical CML tumors as well as K562 cells [51]. While we have not examined the role, if any, of AD 198 on Akt signaling, we reason that Akt expression, which phosphorylates and inactives Bad in leukemic cells [3], would have little effect on AD 198 cytotoxicity, since the drug effects are independent of Bcl-2 family members. We do not rule out the possibility
that AD 198 might reduce Akt activity and potentiate the effects of Bad to work additively with AD 198.

The rapid rate at which apoptosis is triggered in HL-60 and K562 cells indicates that cell cycle arrest is not a pre-requisite event in AD 198-mediated apoptosis. This is consistent with our conclusion that cytotoxic levels of AD 198 do not inhibit Bcr-Abl activity, since reduced Bcr-Abl expression or activity results in G1 arrest in K562 cells [52]. The proximal downstream target of AD 198-mediated PKC-δ activation in mitochondria has not yet been identified in leukemia cells. We have demonstrated that in HeLa cells, the lipid shuttling protein phospholipid scramblase 3 (PLS3) is activated through phosphorylation by AD 198-mediated PKC-δ activity and induces mitochondrial membrane depolarization [53]. However, HL-60 and K562 cells express very low levels of PLS3 (Dr. Ray Lee, Medical Coll. of VA; personal communication), so alternative targets for AD 198-mediated PKC-δ activity remain to be determined.

In contrast to cytotoxic drug doses, we have shown that sub-cytotoxic levels of AD 198 and its principal cellular biotransformation product, AD 288, accelerate the loss of Bcr-Abl protein in combination with cytotoxic levels of imatinib. Unchanging levels of Bcr-Abl mRNA indicate that the loss of Bcr-Abl protein is at the translational or post-translational level. Since the effect is achieved with both AD 198 and AD 288, it is unlikely that the loss of Bcr-Abl protein correlates with direct PKC activation. Rather, this post-transcriptional down-regulation of Bcr-Abl has been observed with a variety of agents and through multiple pathways, such as proteosome-mediated turnover of Bcr-Abl protein by histone deacetylase inhibitors SAHA and LAQ824 [54,55] or disruption of Bcr-Abl interaction with hsp90 chaperone protein by curcumin [56].

The therapeutic potential of AD 198 against CML rests in its two-pronged efficacy: rapid apoptosis in a manner that circumvents Bcr-Abl activity and imatinib resistance, and in the ability of sub-cytotoxic levels of AD 198 and AD 288 further sensitive cells to imatinib. Consequently, AD 198 and other functionally related compounds may claim an effective role in the treatment of CML either as first line therapy in the treatment of blast phase disease or in combination with imatinib as a means of both potentiating anti-leukemic effects and targeting imatinib-resistant cells. The efficacy of AD 198 is not limited to leukemia cells, as we have previously demonstrated that AD 198 is also effective against solid tumor cells through PKC activation [24,53], as well as other proliferating non-tumor cells [23,26]. However, in in vivo pharmacology studies in rats, the dose limiting systemic toxicity of AD 198 is hematotoxicity at higher doses than DOX [57], suggesting that leukemia cells would be principal targets for AD 198. The lack of organ toxicity at myelosuppressive doses, combined with initial evidence of the cardioprotective effects of AD 198 through PKC-ε activation [58] further enhance the potential of AD 198 as an effective component of CML chemotherapy.

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References


