**Therapeutic Discovery**

**Adamantyl-Substituted Retinoid-Related Molecules Induce Apoptosis in Human Acute Myelogenous Leukemia Cells**

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**Abstract**

The adamantyl-substituted retinoid-related (ARR) compounds 3-Cl-AHPC and AHP3 induce apoptosis in vitro and in vivo in a newly established human acute myelogenous leukemia (AML) cell line, FFMA-AML, and in the established TF(v-SRC) AML cell line. FFMA-AML and TF(v-SRC) cells displayed resistance to apoptosis mediated by the standard retinoids (including trans-retinoic acid, 9-cis-retinoic acid, and the synthetic retinoid TTNPB) but showed sensitivity to apoptosis mediated by 3-Cl-AHPC and AHP3 in vitro and in vivo as documented by poly(ADP-ribose) polymerase (PARP) cleavage and apoptosis terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling assay. 3-Cl-AHPC or AHP3 exposure in vitro resulted in decreased expression of the antiapoptotic proteins (cellular inhibitor of apoptosis 1, X-linked inhibitor of apoptosis protein) and phospho-Bad and activated the NF-κB canonical pathway. A significant prolongation of survival was observed both in nonobese diabetic severe combined immunodeficient mice carrying FFMA-AML cells and treated with either 3-Cl-AHPC or AHP3 and in severe combined immunodeficient mice carrying TF(v-SRC) cells and treated with AHP3. We have previously shown that ARRs bind to the orphan nuclear receptor small heterodimer partner (SHP) and that the expression of SHP is required for ARR-mediated apoptosis. Induced loss of SHP in these AML cells blocked 3-Cl-AHPC- and AHP3-mediated induction of apoptosis. These results support the further development of 3-Cl-AHPC and AHP3 as potential therapeutic agents in the treatment of AML patients. *Mol Cancer Ther; 9(11); 2903–13. ©2010 AACR.*

**Introduction**

Numerous advances based on recent molecular observations have been made in both the classification and prognosis of acute myelogenous leukemia (AML). Whereas the inherent heterogeneous nature of AML was initially described using the French-American-British classification, the discovery of unique chromosomal translocations, gene amplification, and mutations and their effects on prognosis and response to therapy has resulted in new and more clinically relevant classification systems (1–3). Despite these advances, chemotherapy has remained the standard treatment for AML. Targeted therapy has played a role in the treatment of selective AML subtypes. Treatment of acute promyelocytic leukemia (APL) with pharmacologic concentrations of *trans*-retinoic acid (tRA) results in 90% of the patients achieving a complete remission (4). The dramatic response of APL cells to high concentrations of tRA is due to the presence of a unique 15;17 reciprocal translocation resulting in the generation of a promyelocytic leukemia (PML)-retinoic acid receptor α (PML-RARα) fusion product, which displays increased binding to corepressors in the presence of physiologic concentrations of tRA. This results in maturation arrest at the promyelocyte stage (5). Exposure of these cells to pharmacologic concentrations of tRA results in the disassociation of PML-RAR from the corepressors, enhancing its binding by coactivators with the subsequent initiation of gene transcription. Unfortunately, tRA efficacy is restricted to APL with no activity shown in the other AML subtypes. New targeted agents, including fms-related tyrosine kinase receptor (FLT-3) and farnesyltransferase inhibitors, are being evaluated as potential therapeutic modalities for the treatment of AML (6, 7).

Adamantyl-substituted retinoid-related (ARR) molecules are a unique class of compounds that have been found to induce apoptosis in a large number of tumor types, many of which display resistance to classic retinoids (8–10). The precise mechanism(s) by which ARRs induce cell death is not clear. Whereas 6-[3-(adamantyl)-4-hydroxyphenyl]-2-naphthaleneacrylic acid (CD437/AHPN)…

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was initially designed as a selective activator of RARs β and γ, it has been found to inhibit cell growth and induce apoptosis in a variety of malignant cell types using a RAR- and retinoid X receptor (RXR)-independent mechanism (11–13). In addition, we have found that 4-[3-(1-adamantyl)-4-hydroxyphenyl]-3-chlorocinnamic acid (3-CIAHPC), which binds to RARγ (but does not activate RARs or RXRs), is a potent inducer of apoptosis in AML cells in vitro (14). We have also reported that the novel nuclear receptor small heterodimer partner (SHP, NR0B2) is involved in the induction of apoptosis by the ARRs (15).

In this report, we show that 3-CIAHPC and its analogue (E)-3-[2-[3-(1-adamantyl)-4-hydroxyphenyl]-5-pyrimidinyl]-2-propenoic acid (AHP3) inhibit the growth and induce apoptosis of the AML cells both in vitro and in vivo. In these studies, we used the TF(v-SRC) AML cell line and the human AML cell line FFMA-AML, which we had previously established from primary AML cells; both of these AML cell lines grow in vitro and in vivo and are resistant to retinoid-mediated inhibition of cellular proliferation and induction of apoptosis, but are sensitive to the antiproliferative and apoptotic effects of the ARRs. In addition, 3-CIAHPC– and AHP3-mediated apoptosis was accompanied by activation of the canonical NF-κB pathway, decreased expression of a number of antiapoptotic proteins including the E3 ligase cellular inhibitor of apoptosis 1 (c-IAP1), and required the expression of orphan receptor protein SHP.

Materials and Methods

Adamantyl-substituted retinoid-related molecules

3-CIAHPC was synthesized as previously described (14). AHP3 was synthesized as described in Supplementary Data.

Retinoids and antibodies

The RAR-selective retinoid trans-RA, RAR, the RXR-selective 9-cis-RA, and the RAR-selective (E)-4-[2-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthyl)propenyl] benzoic acid (TTNPB) were synthesized in the laboratory. ARRs and retinoids were solubilized in DMSO before addition to cells. The maximum concentration of vehicle per culture was 0.1%. RPMI 1640; fetal bovine serum (FBS) and Trizol reagent were purchased from Invitrogen. X-linked inhibitor of apoptosis protein (XIAP), NF-κBp65, IκB kinase-α (IKKα)/IKKβ, and SHP antibodies were obtained from Santa Cruz Biotechnology. Phospho-NF-κBp65 (Ser276), phospho-IKKα (Ser180)/IKKβ (Ser181), phospho-Bad, caspase-3, and active cleaved caspase-3 antibodies were purchased from Cell Signaling, whereas c-IAP1 antibody and α-tubulin were obtained from R&D Systems, Inc., and Oncogene Research Products, respectively.

Acute myelogenous leukemia cells

The FFMA-AML cells were obtained from a patient diagnosed with AML as indicated by the immunophenotyping described in Supplementary Table S1. This patient was refractory to the chemotherapy regimens consisting of cytosine arabinoside administered with daunomycin as well as high-dose cytosine arabinoside. Peripheral blood samples were obtained from the patient under the guidelines of a Wayne State University Institutional Review Board–approved protocol. The leukemic blasts were isolated by Ficoll-Hypaque density gradient. The isolated leukemic cells (representing >99% of the cells) were subsequently cultured in RPMI 1640 supplemented with 10% heat-inactivated FBS and growth factors interleukin-3, granulocyte-colony stimulating factor, granulocyte/macrophage colony-stimulating factor, and stem cell factor as we have previously reported (14). After the cell line became established, cells were maintained in RPMI 1640 supplemented with 10% FBS and 500 μg/mL gentamycin (14). The establishment of the TF(v-SRC) cell line has been previously described (16). TF(v-SRC) cells were grown in RPMI 1640 supplemented with 10% heat-inactivated FBS.

Western blots and reverse transcription-PCR

Western blots, RNA preparation, and reverse transcription-PCR were done as we have previously described (17).

Apoptosis

Cell apoptosis was determined by (a) acridine orange and ethidium bromide staining to determine the percentage of cells with nuclear fragmentation and chromatin condensation as previously described (17), and (b) flow cytometry analysis of Annexin V-FITC binding together with propidium iodide (PI) staining (Annexin V-FITC apoptosis Detection kit 1, BD Biosciences). Data acquisition was done using a FACSCalibur flow cytometer (BD) and analyzed with CellQuest software (BD Biosciences).

shRNA SHP knockdown

shRNA SHP retroviral expression vectors were prepared as we have described (18). FFMA-AML and TF (v-SRC) cell lines were transiently transfected with retroviral shRNA-SHP plasmids for 48 or 72 hours. SHP protein expression was assessed using Western blots after 72 hours of infection with shRNA SHP retroviral expression vector in the FFMA-AML and TF(v-SRC) cells. Anti-SHP antibodies were obtained from MBL International Corporation and Santa Cruz Biotechnology. The effect of SHP knockdown on AML induction of apoptosis in cells was assessed 48 hours after infection with shRNA SHP expression vectors. Apoptosis was determined using an Annexin V-FITC apoptosis detection kit described above.

In vivo studies

All in vivo studies were conducted in accordance with Wayne State University–approved animal care and ethics committee guidelines and procedures. Nonobese diabetic...
severe combined immunodeficient (NOD-SCID) and ICR-SCID mice were obtained from The Jackson Laboratory and Taconic Farms, respectively.

**FFMA-AML and TF(v-SRC) systemic model.** NOD-SCID and ICR-SCID mice (4–5 weeks old) were injected i.v. with either FFMA-AML or TF(v-SRC) cells. Treatment with vehicle, 3-Cl-AHPC, or AHP3 was instituted the following day. If symptoms such as diarrhea, dehydration, weight loss, ascites, paralysis, or general weakness became evident, mice were euthanized.

**TF(v-SRC) subcutaneous mouse model.** ICR-SCID mice were bilaterally trocharred s.c. with TF(v-SRC) tumor fragments. Animals with equal tumor weights were assigned to three experimental groups as we have previously described (19): group 1, control (vehicle treated); group 2, s.c. injections of AHP3; and group 3, i.v. injections of AHP3. The percent increase in host life span of the FFMA-AML− and TF(v-SRC)-bearing mice was calculated by subtracting the median day of death of the drug-treated AML cell line−bearing mice from the median day of death of the vehicle-treated AML cell line−bearing mice divided by the median day of death of the vehicle-treated AML cell line−bearing mice.

To determine the efficacy of 3-Cl-AHPC and AHP3, the survival distributions of the 3-Cl-AHPC− or AHP3-treated (T) and vehicle (C) groups were compared using the log-rank test. Survival was characterized as the duration of the animal’s life span beginning 24 hours after the initiation of the xenograft until an observed event (euthanasia or death). P < 0.05 was considered statistically significant.

**In situ cell death detection and immunohistochemistry**

The terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) assay was done using the In Situ Cell Death Detection Kit, POD (Roche Applied Science) according to the manufacturer’s instructions. Frozen tumor samples were fixed for 24 hours in 10% formalin-buffered saline, dehydrated, embedded in paraffin, and cut into 4-μm-thick sections. The tissue sections were deparaffinized, rehydrated, and then incubated with proteinase K solution (10 μg/mL) for 30 minutes. Tissues were then rinsed twice in PBS and reacted with 50 μL of the TUNEL reaction mixture at room temperature for 60 minutes in a dark, humidified chamber. Sections were again rinsed in PBS and incubated for 30 minutes with 50 μL of the Converter-POD (Roche-Applied-Science) followed by 3-amino-9-ethylcarbazole. Sections were then counterstained with hematoxylin. As negative controls, corresponding sections were treated in the same way without terminal deoxynucleotidyl transferase. Under light microscopy, the number of TUNEL-positive cells was counted and expressed as a percentage of the total number of cells present in that field.

For immunohistochemistry, paraffin-embedded sections were deparaffinized and rehydrated, and antigen unmasking was done by immersing the slides in boiling 0.01 mol/L citrate buffer for 15 minutes. Endogenous peroxidase activity was blocked with 3.0% hydrogen peroxide for 30 minutes. Tissue sections were incubated overnight at 4°C with cleaved-PARP (Asp214) antibody (Cell signaling) at 1:25 dilution and then incubated with biotinylated secondary antibody. We used an avidin-biotinylated horseradish peroxidase complex (Vectastain ABC reagent, Vector Laboratories) with 3-amino-9-ethylcarbazole (BioGenex Laboratories, Inc.) as a chromogen for visualization of the immunoreaction. Slides were counterstained with hematoxylin. Primary antibody was omitted for negative control.

**Results**

**Structures of 3-Cl-AHPC and AHP3**

The chemical structures of the ARRs 3-Cl-AHPC and AHP3 are outlined in Fig. 1A. The synthesis and characterization of 3-Cl-AHPC has been previously described (14), whereas that of AHP3 is described in Materials and Methods.

**3-Cl-AHPC and AHP3 induce apoptosis and inhibit the proliferation of FFMA-AML cells**

The proliferation of TF(v-SRC) and FFMA-AML cells was inhibited by exposure to 3-Cl-AHPC and AHP3 (Supplementary Fig. S1A and B). 3-Cl-AHPC and AHP3 induction of apoptosis in FFMA-AML and TF (v-SRC) cells was examined by assessing the number of cells showing nuclear fragmentation and chromatin condensation (Fig. 1B–D). Cells were grown in the presence and absence of increasing concentrations of either 3-Cl-AHPC or AHP3 for 96 hours or were exposed to 1 μmol/L 3-Cl-AHPC or AHP3 from 0 to 96 hours. There was a progressive increase in 3-Cl-AHPC− and AHP3-mediated apoptosis in both FFMA-AML and TF (v-SRC) cells with increasing concentrations of the compounds and over time (Fig. 1B and C). Compared with FFMA-AML cells, TF(v-SRC) cells displayed less sensitivity to both 3-Cl-AHPC and AHP3 (ED₉₀ of 0.75 μmol/L), whereas FFMA-AML cells displayed ED₉₀s of 0.32 and 0.37 μmol/L for AHP3 and 3-Cl-AHPC, respectively (Fig. 1B and C). Previous studies have shown that APL cells undergo apoptosis in the presence of tRA, 9- cis-Ra, and the RARα-selective retinoid TTNPB through their ability to bind to the PML-RAR fusion protein (5). Therefore, we tested the sensitivity of FFMA-AML and TF(v-SRC) cells to apoptosis induced by tRA, 9-cis-Ra, and TTNPB (Fig. 1D). The addition of tRA, 9-cis-Ra, or TTNPB to FFMA-AML cells resulted in approximately 10% to 20% apoptosis (compared with those treated with the vehicle), whereas the addition of 3-Cl-AHPC induced apoptosis in 80% of the cells (Fig. 1D). TF(v-SRC) cells were resistant to apoptosis induction by these compounds, with 9-cis-Ra, tRA, or TTNPB exposure resulting in no real increase in apoptosis (Fig. 1D).
To further document 3-Cl-AHPC– and AHP3-mediated apoptosis in FFMA-AML and TF(v-SRC) cells, the percentage of cells undergoing apoptosis was assessed using flow cytometry. There was a progressive increase in the percentage of apoptotic cells (Fig. 2A and B). Previous studies have shown that (CD437/AHPN) and 3-Cl-AHPC induce apoptosis in variety of malignant cells through caspase-dependent processes (9, 18). Thus, we examined whether apoptosis was associated with the activation of caspase-3 in AML cells. 3-Cl-AHPC and AHP3 increased caspase-3 activity in the treated AML cells (Fig. 2C) and induced caspase-3 activation as indicated by the generation of the catalytically active 17-kDa cleaved caspase-3 protein (Fig. 2D).

**AHP3 inhibits the expression of the antiapoptotic proteins XIAP, c-IAP1, and phospho-Bad**

The proteins c-IAP1, c-IAP2, and XIAP bind caspases, resulting in inhibition of caspase activity (20). In addition, c-IAP1 and c-IAP2 possess E3 ligase activity, targeting protein destruction through the proteosome pathway, and play an important role in NF-κB activation (20). The Bcl-2 family member Bad enhances apoptosis and is inactivated through phosphorylation and the generation of phosphorylated Bad (21). To assess AHP3 modulation of antiapoptotic protein expression during the induction of apoptosis in AML cells, we assessed the expression of XIAP, c-IAP1, and phospho-Bad as well as the cleavage of the DNA-damage repair protein PARP.
following exposure of AML cells to AHP3 (Fig. 3A). Exposure of FFMA-AML and TF(v-SRC) cells to AHP3 in vitro resulted in a 70% to 80% decreased expression of the antiapoptotic proteins XIAP, c-IAP1, and phospho-Bad (Fig. 3A; Supplementary Fig. S1C). In addition, PARP cleavage was accompanied by a decrease in c-IAP1, XIAP, and phospho-Bad levels, further documenting initiation of apoptosis induced by AHP3 in AML cells (Figs. 3A; Supplementary Fig. S1D).

Activation of the NF-κB canonical pathway requires IKKβ activation, phosphorylation of IkBα, which sequesters the NF-κB p65 subunit in the cytoplasm, followed by destruction of phosphorylated IkBα through the proteosome pathway and the release and nuclear translocation of p65. Exposure of FFMA-AML and TF(v-SRC) cells to AHP3 or 3-Cl-AHPC resulted in a decrease in IkBα levels (Fig. 3B) and an increase in nuclear phospho-p65 (Ser276) levels (Fig. 3C), indicating activation of the NF-κB canonical pathway. Phosphorylation of the activation loops of IKKα and IKKβ has been associated with their conformational change and IKK kinase activation (22, 23). Thus, we assessed whether exposure of FFMA-AML and TF(v-SRC) to AHP3 resulted in IKKα and/or IKKβ phosphorylation and thus activation of the canonical and/or noncanonical NF-κB pathway. Exposure of FFMA-AML and TF(v-SRC) to AHP3 resulted in enhanced phosphorylation of both IKKα and IKKβ.

ARRs Induce Apoptosis in Human AML Cells

Figure 2. Induction of apoptosis and 3-Cl-AHPC- and AHP3-mediated caspase-3 cleavage and activation. FFMA-AML and TF(v-SRC) cells were exposed to 1 μmol/L 3-Cl-AHPC and AHP3. A, apoptosis in FFMA-AML and TF(v-SRC) cells after 24 and 48 h of ARR exposure, respectively. B, percentage of apoptotic cells for the indicated times. Induction of apoptosis and cell death was assessed using Annexin V-FITC labeling with PI staining. The percentage of apoptotic cells corresponds to the sum of percent noted on the top right (late apoptotic cells, Annexin V– and PI-positive cells) and bottom right (early apoptotic cells, Annexin V positive, PI negative) quadrants in A. C, activation of caspase-3 in FFMA-AML and TF(v-SRC) cells after exposure to ARR for varying time periods. D, generation of caspase-3 (17 kDa) fragments and caspase-3 protein levels. Caspase-3 activation was determined as described in Materials and Methods. Columns, mean of three separate determinations; bars, SD. *, P < 0.05; **, P < 0.01; ***, P < 0.001, in comparison with control cells (t test).
IKKα phosphorylation at 6 hours (1.6-fold) and 24 hours (1.2-fold) with no further increase at 48 hours (Fig. 3D; Supplementary Fig. S2), whereas IKKβ phosphorylation was increased at 6 hours (1.2-fold) with no further increase noted at 24 and 48 hours in these cells (Fig. 3D; Supplementary Fig. S2). Exposure of TF(v-SRC) cells to AHP3 resulted in a steady increase in IKKα phosphorylation at 6 hours (1.6-fold), 24 hours (1.6-fold), and 48 hours (1.9-fold) as well as a steady increase in IKKβ phosphorylation from 6 hours (1.2-fold) to 24 hours (1.8-fold) and 48 hours (2.1-fold; Fig. 3D; Supplementary Fig. S2). The compound JSH-23 selectively blocks nuclear translocation of the NF-κB p65/Rel A subunit and its heterodimerization with p50 and is associated transcriptional activation (24). We used JSH-23 to examine its effect on AHP3- and 3-Cl-AHPC-mediated apoptosis in TF(v-SRC) cells. Whereas JSH-23 induced apoptosis in approximately 20% of the cells alone, it inhibited 3-Cl-AHPC- and AHP3-mediated apoptosis in TF(v-SRC) cells, indicating that activation of the NF-κB canonical pathway is required for apoptosis induction by these compounds (Fig. 3D, right).

**Loss of SHP expression inhibits AHP3 inhibition of proliferation and the induction of apoptosis in TF(v-SRC) cells**

The orphan nuclear receptor SHP has been found to bind to numerous nuclear receptors resulting in the inhibition of their transcriptional activation (25, 26). SHP has been found to be expressed in a number of tissues including the human HL-60 AML cell line (26). We showed that SHP is expressed in the human FFMA-AML and TF(v-SRC) cells using reverse transcription-PCR (Supplementary Fig. S3A). Knockdown of SHP expression in TF(v-SRC) cells was achieved using shRNA directed at SHP (Supplementary Fig. S3B). Loss of SHP expression resulted in the inhibition of 3-Cl-AHPC-mediated apoptosis as indicated by Annexin V-FITC and PI staining.
(Fig. 4A and B). The loss of SHP expression also blocked 3-Cl-AHPC– and AHP3-mediated inhibition of TF(v-SRC) and FFMA-AML proliferation (Fig. 4C). Loss of SHP expression had no effect on the proliferation of MDA-MB-468, MEF, or KG-1 cells as reported previously (18).

**3-Cl-AHPC and AHP3 inhibit the growth of FFMA-AML cells in NOD-SCID mice**

The inhibition of FFMA-AML cell growth by 3-Cl-AHPC and AHP3 in NOD-SCID mice was determined. NOD-SCID mice were randomly assigned to two groups of eight mice and were injected with 1 million cells through the tail vein. The mice then received either vehicle or 3-Cl-AHPC (30 mg/kg) administered i.v. twice daily for 4 days. 3-Cl-AHPC treatment of the NOD-SCID mice resulted in a significant ($P = 0.0001$) increase in the length of survival (25%) compared with mice treated with vehicle only (Fig. 5A). Similar results were obtained when 3-Cl-AHPC was given through an i.p. route using the same dosage of 3-Cl-AHPC and treatment schedule (Fig. 5A, right). Treatment of the mice with 3-Cl-AHPC was associated with toxicity including weight loss (16.3% ± 17%) and decreased physical activity. Similarly, mice were treated with AHP3 at doses of either 5.0 or 7.5 mg/kg administered twice a day for 4 days. Although not resulting in a relevant antitumor effect, this treatment did result in a significant increase in survival time ($P = 0.0002$) compared with that in mice treated with vehicle alone (26% increase in host life span; Fig. 5B). In contrast to 3-Cl-AHPC, treatment with AHP3 therapy was extremely well tolerated, and no toxicity was evident (no weight loss and no lessening of physical activity).

**AHP3 inhibits the growth of TF(v-SRC) cells in SCID mice**

We next assessed the ability of AHP3 to inhibit the growth of TF(v-SRC) cells in SCID mice. SCID mice were injected with 10 million TF(v-SRC) cells and then treated...
i.v. with AHP3 (20 mg/kg) or vehicle every other day for a total of 15 doses. Treatment with AHP3 resulted in a marked increase in the length of survival with no evidence of leukemia in 87% of the treated mice (Fig. 5C). TF(v-SRC) cell growth in the SCID mice was documented by flow cytometry. Flow cytometric analysis of lymph node or tumor specimens obtained from the mice treated with vehicle revealed the presence of TF(v-SRC) cells exhibiting CD45, CD13, CD34, CD33, CD36, and CD40 expression in the lymph node and tumor specimens (Supplementary Table S2); no malignant cells were identified in the AHP3-treated surviving mice. Compared with vehicle-treated mice, AHP3-treated mice did not show evidence of toxicity such as weight loss (Fig. 5C, right) or evidence of diarrhea, dehydration, scruffy coat, or decreased physical activity.

**AHP3 inhibits the growth of palpable TF(v-SRC) tumors**

The ability of AHP3 to inhibit the growth of palpable TF(v-SRC) tumors was also examined. TF(v-SRC) cells were trocharred s.c. in SCID mice and allowed to form palpable tumors as described in Materials and Methods. When the palpable tumors reached a size of 100 mg, mice were divided into two groups of eight mice each and received either vehicle or AHP3 (Fig. 5D). AHP3 was administered either i.v. or s.c. (at a site distant from the palpable tumor) or i.v. at a dose of 20 mg/kg every other day for 4 doses. Vehicle was given i.v. to control mice. AHP3 treatment administered either i.v. or s.c. at a distant site from the tumor resulted in an approximately 50% reduction in TF(v-SRC) growth (Figs. 5D and 6A). In addition, AHP3 treatment of the mice resulted in the induction of apoptosis in the tumors as documented by immunohistochemical staining using the apoptosis TUNEL assay as well as PARP cleavage (Fig. 6B–D).

**Discussion**

We have previously shown that ARRs are potent inducers of apoptosis in leukemia cells (11). AHPN induced apoptosis in vitro in the HL-60 and HL-60R human
AML cells, with the latter expressing a deletion in the RARα and thus displaying resistance to retinoid-mediated differentiation and apoptosis (11). In addition, we have shown that exposure of AHPN to human primary chronic lymphocytic leukemia cells in culture resulted in apoptosis in chronic lymphocytic leukemia cells (19). Whereas tRA-induced apoptosis is restricted to APL cells, ARRs induce apoptosis in all categories of AML cells grown in culture (14). Human primary AML cells, which display resistance to tRA-mediated apoptosis, are exclusively sensitive to 3-Cl-AHPC-mediated apoptosis in vitro, with more than 80% of the cells undergoing apoptosis after 48 hours of incubation (14). In addition, incubation of these cells with 3-Cl-AHPC resulted in the activation of p38, extracellular signal-regulated kinase, and c-jun NH2-terminal kinase (14). Similar ARR activation of Jun, extracellular signal-regulated kinase, and p38 MAPK has been described by a number of investigators (27, 28). The role (s) of these kinases in ARR-mediated apoptosis is unclear.

In this study, we show that 3-Cl-AHPC and AHP3 induce apoptosis in human AML cells in vivo as well as in vitro. Exposure of FFMA-AML as well as T(v-SRC) cells to these compounds in vitro resulted in the induction of apoptosis; however, these cells were resistant to apoptosis induced by tRA, 9-cis-RA, and the RARα-selective TTNPB. Apoptosis was preceded by decreased expression of the antiapoptotic proteins XIAP, c-IAP1, and phospho-Bad. 3-Cl-AHPC- and AHP3-induced apoptosis in T(v-SRC) cells was dependent on the expression of the orphan nuclear receptor SHP and is similar to what we have shown in other malignant cell types (15).

AHP3- and 3-Cl-AHPC-induced apoptosis in the FFMA-AML and T(v-SRC) cells was also preceded by the activation of the NF-κB canonical pathway. NF-κB

Figure 6. AHP3 mediates apoptosis and PARP cleavage in treated tumor tissues. A, sizes of untreated and AHP3-treated paraffin-embedded tumor tissue sections (in millimeter). B, the DNA strand breaks in tumors obtained from mice treated with vehicle and AHP3 i.v. or s.c. were detected by TUNEL assay using the In Situ Cell Death Detection Kit, POD. C, TUNEL-positive cells in tumor tissues. D, immunohistochemical staining of paraffin-embedded tissue sections for cleaved PARP. Arrows, apoptotic cells. Details of slide preparation, visualization, and the antibodies used are described in Materials and Methods.
activation has been shown to inhibit apoptosis in a number of systems (17, 29). Exposure of breast or prostate carcinoma cells to CD437/AHPN or 3-Cl-AHPC resulted in NF-κB activation (17, 29). Inhibition of NF-κB signaling with a dominant negative IκB-α or knockout of p65 or helenalin, a specific inhibitor of p65 translocation to the nucleus, inhibited CD437/AHPN– and 3-Cl-AHPC–mediated NF-κB activation and apoptosis in these cells (17). Numerous studies have shown that NF-κB activation may also play a major role in the initiation apoptosis (17, 29, 30). The NF-κB-activated genes that play important roles in 3-Cl-AHPC/AHP3–mediated apoptosis are now under investigation.

3-Cl-AHPC or AHP3 treatment of NOD-SCID mice injected with FFMA-AML cells resulted in significantly prolonged survival. FFMA-AML–bearing mice treated with vehicle, 3-Cl-AHPC, or AHP3 eventually developed paralysis, requiring that they be euthanized as stipulated by the Wayne State University animal care guidelines. The explanation for the development of paralysis in the FFMA-AML–bearing mice is not clear but is under study. SCID mice injected with TF(v-SRC) cells showed 87% survival with AHP3 treatment; this was associated with minimal to no toxicity to the mice. Thus, AHP3 and 3-Cl-AHPC showed significant efficacy in inhibiting the growth of the AML cells both in vitro and in vivo.

The in vitro and in vivo mechanism(s) involved by which the ARRs induce apoptosis in the AML cells are still not clear. We and others have previously shown that RARs and RXRs are not involved despite reports that both AHPN and related ARRs can activate one or more RAR subtypes (8, 9). A number of potential mechanisms by which the ARRs may induce apoptosis have been suggested (31). Activation of the p38 and c-jun-NH2-terminal kinases, inactivation of the p38 MAP kinase, and mobilization of TR3 from its nuclear localization to mitochondria with its binding to Bcl-2 have all been implicated as important mechanisms by which the ARRs induce apoptosis in malignant cells (31). We have recently found that SHP expression as well as that of Sin3A is essential for ARR-mediated apoptosis in a variety of cell types (15); knockdown or knockout of either SHP or Sin3A resulted in the inhibition of ARR-mediated apoptosis (15). The importance of SHP in ARR-mediated apoptosis is further supported by our observation that the loss of SHP expression in TF(v-SRC) cells inhibited ARR-mediated apoptosis. In addition, we have found that the ARRs specifically bind to SHP (15). SHP functions primarily as a repressor of gene expression. This is mediated by a number of nuclear receptors including thyroid hormone receptor, RAR, RXR, estrogen receptor, glucocorticoid receptor, and hepatocyte nuclear receptor-4. SHP has been shown to be an activator of NF-κB– and peroxisome proliferator–activated receptor γ–mediated transcription and gene activation (32, 33). He et al. (34) have recently found that SHP functions as a tumor suppressor in the development of hepatocellular carcinoma. Furthermore, SHP seems to negatively regulate the proliferation of these cells through its ability to downregulate cyclin D1 expression (35). We have also found that ARR activation of NF-κB is essential for ARR-mediated apoptosis (17). Downregulation of SHP expression has been shown to block ARR-mediated activation of NF-κB as well as ARR-mediated apoptosis (18). Interestingly, Bruserud et al. (36) described high levels of NF-κBp65 in AML cells, which were suggested to play a role in the increased transcription of a variety of cytokines, resulting in enhanced cellular proliferation and perhaps resistance to therapy. The fact that ARR-induced apoptosis requires the activation of NF-κB in AML cells implies that it is not only NF-κB activation that is important but also the environment in which NF-κB activation takes place and which specific genes are activated.

The Sin3A protein serves as a scaffold protein, which, through its four highly conserved but imperfect repeats, the paired amphipathic helix domains, is capable of binding a variety of proteins (37–40). These include a number of important nuclear transcription factors such as p53 and MAD (37–40). 3-Cl-AHPC–treated KG-1 AML cells resulted in the binding of ARR to SHP and the subsequent binding of SHP/ARR to the Sin3A complex; this association between SHP/ARR and the Sin3A complex resulted in the modification of the Sin3A complex and the enhancement of its associated histone deacetylase inhibitor-1 and -2 activities (18).

In this report, we have shown that the ARRs displayed significant efficacy in the in vivo treatment of mice bearing AML and resulted in the prolongation of their survival. In addition, treatment of SCID mice bearing TF(v-SRC) cells with AHP3 resulted in 87% survival. We are now in the process of further delineating the role of SHP and ARR binding to SHP in ARR-mediated apoptosis as well as the synthesis of ARR analogues that will show greater efficacy, minimal toxicity, and better bioavailability.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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References


