Anacardic acid induces caspase-independent apoptosis and radiosensitizes pituitary adenoma cells

Laboratory investigation

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Object. Pituitary adenomas, which are common intracranial tumors, are associated with significant patient morbidity due to hormone secretion or mass effect or as a complication of therapy. Epigenetic regulation has emerged as an important component of malignant tumor pathogenesis, although the contribution in the progression of benign pituitary tumors remains largely unexplored. The present study evaluates the effect of anacardic acid (6-pentadecyl salicylic acid), a natural histone acetyltransferase inhibitor, on pituitary adenoma cells.

Methods. The concentration- and time-dependent effects of anacardic acid on the viability of GH3 and MMQ pituitary adenoma cells were determined by 3-(4,5-dimethylthiazoyl-2-yl)-2,5-diphenyltetrazolium bromide assay. Cell cycle phase distribution, protein expression, and percentage of apoptotic cells were assessed by flow cytometry and Western blotting. Colony forming assays were used to study the radiosensitizing effect of anacardic acid.

Results. The present study identifies a novel antiproliferative and cytotoxic effect of anacardic acid on pituitary adenoma cells. These effects were associated with an increase in poly([adenosine diphosphate]-ribose) polymerase cleavage, sub-G1 arrest, and annexin V staining, consistent with apoptotic cell death; however, the pancaspase inhibitor carbobenzoxy-valyl-alanyl-aspartyl-(O-methyl)-fluoromethylketone failed to reverse anacardic acid–induced cell death, suggesting a possible nonclassical apoptotic mechanism. Anacardic acid also reduced the expression of survivin and X-linked inhibitor of apoptosis protein, antiapoptotic proteins associated with cellular survival and radioresistance, and radiosensitized pituitary adenoma cells.

Conclusions. These findings warrant further exploration of anacardic acid as a single agent or as an adjunct to radiation therapy for the treatment of pituitary tumors. (DOI: 10.3171/2010.12.JNS10588)

Key Words • histone acetyltransferase • histone deacetylase • pituitary tumor • radiation • cell cycle • anacardic acid

PITUITARY adenoma, one of the most common primary brain tumors, accounts for as many as 20% of intracranial neoplasms. Although most are histologically benign, pituitary adenomas can present as significant mass lesions. In many macroadenomas, extensive infiltration of surrounding structures has already occurred at presentation, thus limiting surgical cure rates. Medical therapies, which can often adequately control the hormone hypersecretion and size of secretory pituitary tumors, may be associated with intolerable side effects and are often associated with poor patient compliance.

Radiation-based modalities are also frequently used in the treatment of pituitary tumors. Radiosurgery is widely used and can effectively control tumor growth, but application of this technology is limited both by the radiation tolerances of the parasellar structures and targeting issues relating to the infiltrative portion of the tumor. Radiation therapy remains an adjuvant option but is also associated with numerous complications, including decreased cognitive function and hypopituitarism. Although pituitary adenoma is manageable in most cases with existing modalities, there are limitations and exceptions that provide novel opportunities for additional therapeutic interventions. Toxicity related to radiation therapy and stereotactic radiosurgery is proportional to treatment dose; thus, the identification and development of novel radiosensitizing agents may allow more aggressive targeting using radiation with reduced collateral toxicity.

Epigenetic modifications represent a novel target for therapeutic drugs. Notably, aberrantly increased activity of HDACs, a class of enzymes that catalyzes the removal
of acetyl groups from histones, increases the expression of genes related to cellular proliferation and survival to promote tumor progression in a variety of malignancies.\(^5,35\) As such, HDAC inhibitors have emerged as a novel class of promising antineoplastic agents to facilitate cell cycle arrest and apoptosis. We first demonstrated an antiproliferative and proapoptotic role for the FDA-approved HDAC inhibitor suberoylanilide hydroxamic acid (vorinostat) in pituitary adenoma cells,\(^32\) suggesting posttranslational histone modification (Fig. 1) may represent a clinically viable therapeutic target in pituitary tumors.

Although HDAC inhibitors receive the most attention, HATs also regulate posttranslational histone modifications by catalyzing the transfer of acetyl groups from acetyl coenzyme A onto conserved lysine residues on histone proteins\(^24,37\) (Fig. 1). However, the contribution of HATs toward tumor progression remains largely unexplored. Anacardic acid (6-pentadecyl salicylic acid), a cell-permeable salicylic acid analog, was recently identified as a potent, noncompetitive inhibitor of distinct members of the HAT family, including p300, p300/CREB (cAMP-response element binding)-binding protein associated factor (PCAF), and Tip60,\(^2,26\) suggesting anacardic acid may globally reduce cellular HAT activity and attenuate histone acetylation. In the present study, we investigated the efficacy of anacardic acid in reducing the growth and proliferation of pituitary adenoma cells.

Methods

**Cell Culture**

All cell culture reagents, sera, and media were purchased from Hyclone Laboratories. Anacardic acid (95% pure; Fig. 2) and Z-VDAC-fmk were purchased from EMD Biosciences. Rat GH3 and mouse MMQ pituitary adenoma cells (American Type Tissue Collection) were cultured in Dulbecco modified Eagle medium supplemented with 5% fetal bovine serum, 5% bovine growth serum, and antibiotics in a 37°C humidified incubator at 5% CO\(_2\), as detailed previously by our group.\(^32\) The GH3 cells are a well-characterized model of secretory adenomas that oversecrete both prolactin and growth hormone and reflect the biology of human secretory tumors.\(^15,18,27,38\) The MMQ cells are a prolactin-secreting cell line that grows as a suspension culture.

**Cellular Viability Assays**

Cell viability was estimated by the addition of 5 mg/ml of MTT followed by incubation for 4 hours at 37°C, as described previously by our group.\(^20\) A yellow substrate, MTT is only cleaved by active mitochondria in living cells to yield a blue formazan product. Formazan crystals were then solubilized in 0.04 M hydrochloric acid in isopropanol, and absorbance was detected at 540 nm. Viability was normalized to the control, which was considered to represent 100%. To differentiate the mode of cell death, adherent and nonadherent cells were collected and washed. Cell suspensions were stained at room temperature with annexin V-fluorescein isothiocyanate conjugate (BD Pharmigen), an early apoptotic marker, and with 7-AAD, a fluorescent dead-cell marker. The percentage of apoptotic cells was quantified using a FACScan (Becton Dickinson) flow cytometer.

**Cell Cycle Analysis**

Exponentially growing cells were synchronized in serum-free Dulbecco modified Eagle medium overnight and then treated in complete culture media containing either vehicle (dimethyl sulfoxide) or anacardic acid. Cells were permeabilized with 70% methanol overnight at 20°C and then incubated with 50 μg/ml propidium iodide solution containing 200 μg/ml RNase A for 0.5 hours. Cells were immediately analyzed by flow cytometry using an FACSCalibur analyzer (BD Biosciences).

**Radiation and Colony-Forming Assay**

Cells were plated in 6-well dishes at 10\(^3\) cells/well and allowed to grow for 48 hours. Radiation was delivered to cells using a \(^{137}\)Cs γ-cell-40 Exactor (Nordion International, Inc.) at a dose rate of 1.14 Gy/minute, following 24 hours of pretreatment with anacardic acid. Media were then replaced, and cells were incubated for a further 10 days. Cell colonies were identified by incubation with 5 mg/ml of MTT for 2 hours at 37°C. Colonies were photographed with a digital imaging system and manually counted with the aid of a microscope, as detailed previously by our group.\(^3\)

**Histone Isolation**

Following treatments, cells were lysed in Triton extraction buffer (0.5% of Triton-X100 [v/v]), 2 μM phenylmethylsulfonyl fluoride, and 0.02% Na\(_2\)S in phosphate-buffered saline for 10 minutes on ice with gentle stirring. After a brief centrifugation (2000 rpm, 4°C), the pellet was washed in Triton extraction buffer, centrifuged, and resuspended in 0.2 M HCl for acid extraction overnight at 4°C. Samples were then centrifuged (2000 rpm, 10 minutes, 4°C). Protein content was determined following a buffer exchange with phosphate-buffered saline using a Centricon centrifugal filter (Millipore), as described by our laboratory.\(^20\)

**RNA Isolation and qRT-PCR**

Total RNA was isolated (SV RNA Isolation kit; Promega), and qRT-PCR was performed on a Cepheid SmartCycler II using a Superscript III Platinum SYBR Green One-Step qRT-PCR kit (Invitrogen), as described by our laboratory.\(^2,20,40\) Primers used were as follows: survivin forward 5ʹ-CCTACCGAATGAGCC TGA-3ʹ; reverse 5ʹ-ACGGTCATGTTCCACCTTG-3ʹ; XIAP forward 5ʹ-TGCTTTTGTTTGTTGG-3ʹ; reverse 5ʹ-CTGCCATGGGATTCTTCTT-3ʹ; and glderaldehyde-3-phosphate dehydrogenase forward 5ʹ-ATG GGAAGCTGTCATCAAC-3ʹ, reverse 5ʹ-GTTGGTC CACACCACACAA-3ʹ. Product specificity was confirmed by melting curve analysis and visualization of a single, appropriately sized band on a 2% agarose gel. Gene expression levels were quantified using a cDNA standard curve, and data were normalized to glderaldehyde-3-phosphate dehydrogenase, a housekeeping gene that was...
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Data are expressed as fold change versus vehicle treatment. Experiments were performed in triplicate, with 4 samples per trial.

**Western Blotting**

Whole cell lysates were collected in radioimmunoprecipitation buffer containing protease inhibitor cocktail, phosphatase inhibitor cocktail, and phenylmethylsulfonyl fluoride. Following sonication, cell lysates were centrifuged (5 minutes, 14,000 rpm, 4°C), and protein concentrations were quantified by BCA protein assay kit (Pierce). Thirty micrograms of protein was resolved (separated) on a 4%–20% sodium dodecyl sulfate-polyacrylamide gel and transferred onto a polyvinylidene difluoride membrane. Blots were incubated overnight at 4°C in primary antibody (survivin, Bcl-2, Bcl-xL, or Bax antibodies from Santa Cruz Biotechnology; XIAP, PARP, or cleaved caspase-3 from Cell Signaling Technology; and β-actin from Abcam) followed by a 2-hour incubation with an AlexaFluor 750 secondary antibody at room temperature. Blots were visualized using the Li-Cor Odyssey near-infrared imaging system, and densitometry analysis was performed using Quantity One software (Bio-Rad).

**Statistical Analysis**

Data were analyzed using the Student-Newman-Keuls post hoc test or 1-way ANOVA followed by the Dunnett post hoc test. Unless otherwise noted, the number of samples used was greater than 5, and experiments were performed in triplicate to verify results. Results are expressed as mean ± SEM. A p value < 0.05 was considered statistically significant.

**Results**

**Anacardic Acid and GH3 Cell Viability**

Anacardic acid significantly decreased the viability of GH3 and MMQ pituitary adenoma cells in a time- and concentration-dependent manner. A 48-hour treatment with anacardic acid (25, 50, or 100 μM) induced morphological changes and reduced cellular viability by 22% (p < 0.05 vs vehicle), 33% (p < 0.01), and 65% (p < 0.01), respectively, in GH3 cells, with a half maximal inhibitory concentration of 52.0 μM (Fig. 3A and B). Similarly, MMQ cellular viability was reduced by 39% (p < 0.05 vs vehicle), 47% (p < 0.01), and 85% (p < 0.001) after a 48-hour treatment with 25, 50, or 100 μM anacardic acid.
respectively, with a half maximal inhibitory concentration of 37.9 µM (Fig. 3A). A maximal effect was noted in both cell lines after a 72-hour treatment with 100 µM (p < 0.001 vs vehicle in both cell lines). Consistent with a proposed role as an HAT inhibitor, anacardic acid decreased lysine acetylation and specifically downregulated H3 histone acetylation at concentrations associated with reduced cellular viability in GH3 cells (Fig. 3C).

**Anacardic Acid–Induced Apoptosis and S-Phase Arrest**

Modulation of the cell cycle frequently precedes tumor cell death. As either growth arrest or cell death could account for the observed reduction in cellular viability using the MTT assay, the effect of treatment on cell cycle changes was analyzed. Anacardic acid (25 or 50 µM) significantly increased the percentage of cells in the S-phase following a 24-hour treatment (37.1% ± 1.7% and 44.9% ± 3.7%, respectively) compared with vehicle (24.7% ± 0.7%; p < 0.05), consistent with S-phase arrest, whereas 100 µM was not statistically different from controls (Fig. 4). Conversely, only 100 µM of anacardic acid significantly increased the percentage of cells within the sub-G1 phase (8.0% ± 0.9% vs 1.3% ± 0.4% in controls; p < 0.001; Fig. 4), suggestive of DNA fragmentation and apoptotic cell death.

To further clarify the form of cell death following treatment with 100 µM of anacardic acid, annexin V/7-AAD labeling was performed in conjunction with flow cytometry. Anacardic acid significantly increased the percentage of early apoptotic cells (annexin V/7-AAD−) from 2.6% in vehicle-treated cultures to 7.6% after 24 hours of treatment (p < 0.05 vs vehicle; data not shown). Consistent with an apoptotic mechanism of action, anacardic acid increased caspase-3 activation (Fig. 5A and C) and promoted the cleavage of PARP (Fig. 5A and B), confirming the functional activation of the caspase-3 path-
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way; however, despite this evidence implicating apoptotic cell death, the pancaspase inhibitor Z-VAD-fmk failed to reverse cell death, suggesting that caspase activation may be dispensable for anacardic acid–induced cytotoxicity (Fig. 5D).

**Anacardic Acid and Antiapoptotic Proteins Survivin and XIAP**

To elucidate potential mechanisms of anacardic acid–induced apoptosis, the expression profile of the established antiapoptotic proteins survivin, XIAP, Bcl-2, and Bcl-xL were studied. Notably, anacardic acid attenuated basal gene expression of the antiapoptotic IAP family members survivin and XIAP, as assessed by qRT-PCR (Fig. 6A and B). Survivin mRNA expression was reduced by 12.2% ± 3.6% and 87.9% ± 1.8% (p < 0.001 vs vehicle) after 24 hours of treatment with 50 and 100 μM of anacardic acid, respectively. Similarly, XIAP mRNA expression was reduced by 27.2% ± 6.2% (p < 0.05 vs vehicle) and 55.4% ± 2.9% (p < 0.01 vs vehicle) after 24 hours of treatment with 50 and 100 μM of anacardic acid, respectively. These changes in gene expression were similarly reflected at the protein level, as 100 μM of anacardic acid maximally reduced survivin and XIAP expression by 50.0% ± 1.0% (p < 0.01 vs vehicle) and 39.2% ± 5.3% (p < 0.05 vs vehicle; Fig. 6C). In contrast, anacardic acid did not significantly modulate the expression of Bcl-2 and Bcl-xL after a 24-hour treatment (Fig. 7). Likewise, the expression of the proapoptotic Bcl family member, Bax, was not significantly increased after exposure to anacardic acid (Fig. 7).
Anacardic Acid, GH3 Cells, and Radiation

Ionizing radiation remains a front-line treatment option for patients diagnosed with a pituitary tumor. Overexpression of survivin and XIAP frequently induces tumor radioresistance. Thus, we next tested whether anacardic acid may decrease the apoptotic threshold following exposure to radiation. Consistent with this hypothesis, a 24-hour pretreatment with anacardic acid significantly reduced the colony-forming ability of GH3 cells following exposure to a clinically relevant dose of radiation (2.5 Gy; Fig. 8). Specifically, 2.5 Gy alone reduced clonogenicity by 21.0% ± 1.1% (p < 0.05 vs control), whereas 100 μM anacardic acid alone reduced clonogenicity by 17.5% ± 1.5% (p < 0.05 vs control). In contrast, when exposure to 2.5 Gy was preceded by treatment with 100 μM anacardic acid, clonogenicity was reduced by 55.6% ± 4.4% (p < 0.01 vs control, 2.5 Gy alone). This effect exceeded an additive effect of the 2 treatments, suggesting that anacardic acid may be an effective radiosensitizing agent in pituitary tumor cells.

Discussion

Histone acetylation is increasingly recognized as an important regulator of tumor cell growth and proliferation. Despite the intense focus on HDAC inhibition, relatively few studies focus on the potential role of an HAT inhibitor in tumor progression. In this report, we demonstrate a novel proapoptotic and radiosensitizing effect of the natural, broad-spectrum HAT inhibitor anacardic acid in pituitary adenoma cells.

The effect of anacardic acid cellular viability was associated with a reduction in histone acetylation, consistent with its proposed role as an HAT inhibitor; although we cannot exclude the possibility that inhibition of prostaglandin synthase or lipoxygenase activity also contributed to the observed effects. The posttranslational acetylation of histones, particularly on the ε-amino groups on specific lysine chains in the N-terminal domains of core chromosomal histones, is crucial for the maintenance of chromatin structure, for the regulation of gene transcription, and for the control of DNA repair. As such, HDACs are an important therapeutic target in cancer biology. We recently demonstrated a proapoptotic role for suberoylanilide hydroxamic acid in pituitary adenoma cells. In the present study, we show for the first time that anacardic acid, a potent HAT inhibitor, time- and concentration-dependently reduced the viability of pituitary adenoma cells at concentrations below those used in previous studies. Together, these data suggest that an HAT inhibitor may represent a potentially novel and unexplored therapeutic agent for the treatment of pituitary adenomas.

At the mechanistic level, anacardic acid increased S-phase growth arrest and induced apoptotic cell death, as demonstrated by annexin V labeling, sub-G1 arrest, activation of caspase-3, and increased PARP cleavage. However, despite the appearance of classic hallmarks of...
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Apoptosis, caspase-3 activation may be dispensable for the induction of cell death, as the irreversible, cell-permeable pancaspase inhibitor Z-VAD-fmk failed to reverse anacardic acid-induced cell death. Notably, the potent HAT inhibitor 3 and curry spice, curcumin, also reduced cellular proliferation, induced apoptosis, and decreased hormone secretion in pituitary tumor cell lines and in a preclinical model of pituitary adenoma. Although the mechanism by which curcumin exerted these functions in pituitary tumors remains largely undefined, our laboratory reported that curcumin could radiosensitize and induce glioma cell death via a caspase-independent mechanism of action, supporting the observations made in this report.

Ionizing radiation (external beam radiation therapy) remains a treatment option for patients diagnosed with a pituitary tumor. However, radiotherapy is associated with delayed cognitive side effects and hypopituitarism. This limits its role to cases in which there is extensive infiltration of the tumor or where recurrence and refractory behavior have resulted after other options were exhausted. Furthermore, exposure of the optic apparatus (which is in close proximity to the anterior pituitary gland) to > 8 Gy of radiation increases the risk of visual complications such as loss of visual acuity and optic neuropathy. These comorbidities are directly proportional to the amount of radiation delivered to other areas of the brain outside of the pituitary gland. Thus, strategies to reduce the amount of radiation required (such as through an external beam radiation therapy or stereotactic radiosurgery protocol) to reduce tumor size can be clinically valuable. Survivin, an antiapoptotic protein that is associated with radioresistance in brain, pancreatic, and rectal cancer, is also highly overexpressed in invasive and secretory pituitary adenomas, compared with the normal pituitary gland. Notably, anacardic acid significantly downregulated the antiapoptotic IAP family members survivin and XIAP, but did not modulate the expression of either Bcl-2 or Bcl-xL, at concentrations that elicited cell death and radiosensitized pituitary adenoma cells to low doses of radiation. Coupled with reports showing low toxicity in nontransformed cells, these data suggest anacardic acid may be a useful adjunct to traditional ra-

Fig. 6. Anacardic acid attenuated survivin and XIAP expression. A: GH3 cells were treated for 24 hours with anacardic acid (25–100 μM), followed by quantification of survivin and XIAP using qRT-PCR. *p < 0.05, **p < 0.01, ***p < 0.001. B: Western blot analysis for survivin and XIAP following treatment with anacardic acid (ACA; 25–100 μM). Blots are representative of 3 independent experiments. β-actin was used as a loading control. C: Densitometry of survivin and XIAP Western blots (B). All data were obtained from 3 independent trials and were analyzed using 1-way ANOVA followed by the Dunnett post hoc test. *p < 0.05 versus vehicle-treated cultures.
Radiation therapy. Ongoing research in our laboratory will further characterize these effects at the cellular and molecular levels.

Anacardic acid, a cell-permeable salicylic acid analog, is obtained from a number of plant sources and is associated with many traditional medicinal uses worldwide. Anacardic acid is an active ingredient in the bark of *Amphipterygium adstringens*, which is used for the treatment of gastric ulcers, gastritis, and stomach cancers in Mexico. Anacardic acid is also present in *Ozoroa insignis*, an African shrub used to treat malaria; *Anacardium occidentale* (cashew nuts); and *Ginkgo biloba*, an Asian medicine under investigation for reducing dementia and improving cognitive outcomes in patients with Alzheimer disease. Although pharmacokinetic studies in preclinical animal models of pituitary tumors and carefully controlled clinical trials in humans remain to be performed, anacardic acid reduced the viability of primary human pituitary adenoma cells (unpublished observations). Coupled with data showing that anacardic acid did not reduce the viability of nontransformed human fibroblast cells, these findings validate the use of rodent pituitary tumor cell lines and further suggest a possible clinical application for anacardic acid (or structural analogs) in the medical management of pituitary adenomas.

**Conclusions**

These data show, for the first time, an antiproliferative and nonclassical apoptotic mechanism of action for anacardic acid in pituitary adenoma cells. Coupled with widespread utility and presumed clinical safety, anacardic acid may represent an adjuvant therapy in the treatment of pituitary adenoma.

**Disclosure**

The authors report no conflict of interest concerning the mate-
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tials or methods used in this study or the findings specified in this paper.
Author contributions to the study and manuscript preparation include the following. Conception and design: Vender, Sukumari-Ramesh, Jensen, Dhandapani. Acquisition of data: Sukumari-Ramesh, Singh, Dhandapani. Analysis and interpretation of data: Vender, Sukumari-Ramesh, Singh, Dhandapani. Drafting the article: Vender, Sukumari-Ramesh, Dhandapani. Critically revising the article: Vender, Sukumari-Ramesh, Singh, Dhandapani. Reviewed the final version of the manuscript and approved it for submission: all authors. Statistical analysis: Sukumari-Ramesh, Singh, Dhandapani. Administrative/technical/material support: Jensen. Study supervision: Vender, Dhandapani.

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