3-Phenylacetylamino-2,6-Piperidinedione Inhibition of Rat Nb2 Lymphoma Cell Mitogenesis (43274)

JOSEPH C. WOOD, JOHN A. COPLAND, THOMAS G. MULDOON,[1] AND LAWRENCE B. HENDRY[2]
Drug Design and Development Laboratory, Department of Physiology and Endocrinology, Medical College of Georgia, Augusta, Georgia 30912

Abstract. **3-Phenylacetylamino-2,6-piperidinedione (A10), an amino acid analog, has been reported to possess antineoplastic activity against certain neoplastic tissues. The antimitogenic properties of A10 were studied by determining its effect on prolactin (PRL)- and interleukin 2 (IL-2)-stimulated mitogenic responses in the rat Nb2 lymphoma cell line. The addition of A10 (1–12 m*M*) to PRL (0.4 ng/ml)-stimulated cells inhibited growth in a dose-dependent manner. DNA synthesis patterns studied by thymidine incorporation demonstrated that A10 was significantly inhibitory (25% at 20 hr; 50% at 40 hr, $P < 0.01$). IL-2 stimulation of mitogenesis was also sensitive to A10 inhibition. The inhibition of PRL stimulated mitogenesis was reversible when A10 was removed after 24 hr of culture and A10 showed no toxicity in a chromium release assay. These data suggest that A10 effects may be cytostatic, rather than cytotoxic.**

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The search for low-molecular weight amino-acid derivatives and modified peptides with antineoplastic activity led to the isolation of 3-phenylacetylamino-2,6-piperidinedione (A10) from freeze-dried human urine (1–3). A10 was identified by standard spectroscopic techniques; structural confirmation was obtained by total synthesis from the condensation of L-glutamine with phenylacetyl chloride to yield phenylacetylglutamine. Subsequent dehydration cyclized the glutamine moiety to a piperidinedione yielding A10 (Fig. 1) (3). A10 has been reported to lack toxicity (3–5); however, relatively little is known about A10 or its mechanism of action.

In this report, the rat Nb2 lymphoma cell line, a T cell-derived lactogen-dependent lymphoma, was chosen to examine the effects of A10 on hormonally stimulated mitogenesis. When Nb2 lymphoma cells are cultured in medium containing fetal calf serum (FCS) and horse serum, they demonstrate a doubling time of approximately 15 hr. If, however, the cells are cultured in medium devoid of FCS (a lactogen source), they enter a quiescent state of growth. Upon addition of exogenous prolactin (PRL), the cells resume proliferation in a dose-dependent manner (6, 7). In addition, interleukin 2 (IL-2) and phorbol esters have mitogenic effects in this cell line in the absence of PRL or FCS (8, 9).

The present analysis demonstrated a reproducible effect of A10 in the rat Nb2 lymphoma cell line. In this study: (i) A10 inhibited prolactin stimulation of cell proliferation in a dose-dependent manner; (ii) the inhibition correlated with a decrease in DNA synthesis; (iii) the inhibition was not selective for PRL stimulation of mitogenesis, since both PRL and IL-2 stimulated proliferation was inhibited; (iv) the inhibitory action of A10 was readily reversible; and (v) A10 was not cytotoxic in a chromium release assay.

Materials and Methods

Chemicals. A10 was obtained from the Burzynski Research Institute (Stafford, TX). A10 can also be obtained from Sigma Chemical Co. (St. Louis, MO). All A10 samples employed in this study were analyzed in our laboratories for chemical composition, purity, and stability. Analyses were routinely performed on

[1] Deceased.

[2] To whom correspondence and requests for reprints should be addressed at Department of Physiology and Endocrinology, CLW 334, Medical College of Georgia, Augusta, GA 30912.

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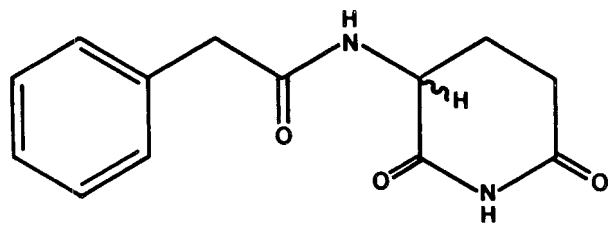
A10 solutions dissolved in dimethylformamide using a Finnigan 4023 computerized gas chromatograph-mass spectrometer under the following conditions: injector, 300°C; source, 300°C; oven, 265°C isothermal; DB5 30 *M* capillary column; electron energy, 70 ev; scan range, 35–450 amu. In each analysis, the results showed a single chromatographic peak with an electron impact mass spectrum consistent with A10 (*m/e* 246, M+; 155, M+ $-C_7H_7$; 127; 118; 110; 99; 91, C_7H_7+; 84; 65; 56). No discernible impurities were detected using reconstructed ion chromatograms; the ion chromatograms of the major diagnostic ions (*m/e* 246, 155, and 91) possessed single superimposable chromatographic peaks. The retention times and mass spectra obtained in all cases were consistent with those reported previously for A10, as well as those obtained from authentic samples (3, 10).

Interleukin 2 (IL-2), Nonidet P-40, and 2-mercaptoethanol were purchased from Sigma. Penicillin/streptomycin, fetal calf serum, and Fischer's medium were obtained from Gibco Laboratories (Grand Island, NY). [^{3}H]Thymidine (70–90 Ci/mmol) and sodium ^{51}Cr (100–400 mCi/mg Cr) were purchased from Amersham (Arlington Heights, IL). Horse serum was purchased from MA Bioproducts (Walkersville, MD). Rat prolactin (rPRL-RP3) was supplied by National Institute of Arthritis, Metabolism, and Digestive Diseases, National Institutes of Health. Tissue culture flasks (75 cm^2) and 24- and 96-well plates were obtained from Fisher (Pittsburgh, PA).

Cell Culture. Nb2 rat lymphoma cells were a gift from Dr. Peter W. Gout, Department of Cancer Endocrinology, Cancer Control Agency of British Columbia. The cells were maintained as suspension cultures in 75-cm^2 tissue culture flasks in Fischer's medium supplemented with 5% horse serum, 5% FCS, 0.1 m*M*-mercaptoethanol, 50 units/ml penicillin, and 50 μg/ml streptomycin, in an atmosphere of 5% CO_2 and 95% air at 37°C (8).

Measurement of Proliferation. Nb2 lymphoma cells were centrifuged (300*g*, 4 min), washed three times in medium containing only horse serum, and resuspended in the same medium. Cells were cultured in 75-cm^2 flasks for 24-hr prior to use. Cells were then aliquoted to 24-well plates and PRL, IL-2, or A10 was added. For cell counting, 100-μl aliquots of cells were added to 10 ml of Isoton (Coulter Counter Electronics); cell number was determined in a Coulter counter (model ZM). All experiments were performed in triplicate.

DNA Synthesis Studies. Incorporation of [^{3}H] thymidine (1 μCi/culture) into DNA was measured by pulsing cells for 1 hr at appropriate time intervals. Cells were then collected on glass fiber filters (Whatman, Clifton, NJ) with an automatic sample harvester



3(N-phenylacetylamino)-2,6-piperidinedione

Figure 1. Structure of A10.

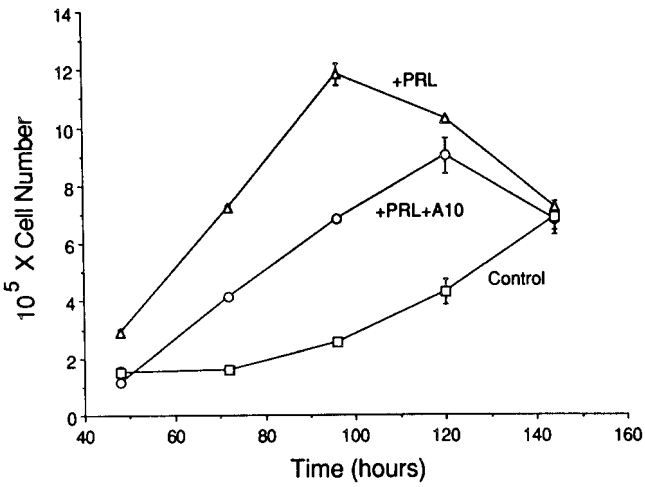


Figure 2. A10 time-course study in which 4 m*M* A10 (○) was added to PRL (0.4 ng/ml)-stimulated Nb2 lymphoma cells and compared to PRL alone (△). Control cells (□) received no A10 or PRL. The experiment was performed in triplicate. Cell counts were made at 48, 72, 96, 120, and 144 hr. Cell numbers are expressed as mean cells per ml ± SD.

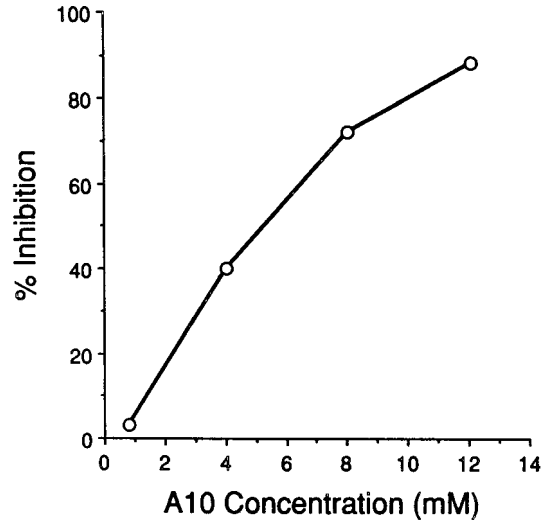


Figure 3. A10 dose-response inhibition. PRL (0.4 ng/ml)-stimulated Nb2 cells were treated with 1, 4, 8, and 12 m*M* A10. The experiment was performed in triplicate. Cell counts were made at 72 hr. The inhibition of growth is plotted as percentage of inhibition compared with PRL controls.

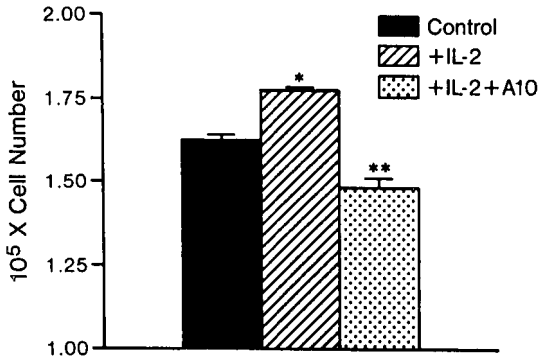


Figure 4. A10 inhibition of interleukin 2 (IL-2)-stimulated Nb2 cell growth. Quiescent Nb2 cells were stimulated with 10 units/ml of IL-2 (hatched bar). A10 (stippled bar) was added to IL-2-stimulated cells at a concentration of 4 m*M*. Control cells (solid bar) received no IL-2 or A10. Cell counts were made after 72 hr of culture. The experiment was performed in triplicate. Cell numbers are expressed as the mean cells per ml ± SD. $^*P < 0.05$ compared with control; $^{**}P < 0.05$ compared with +IL-2.

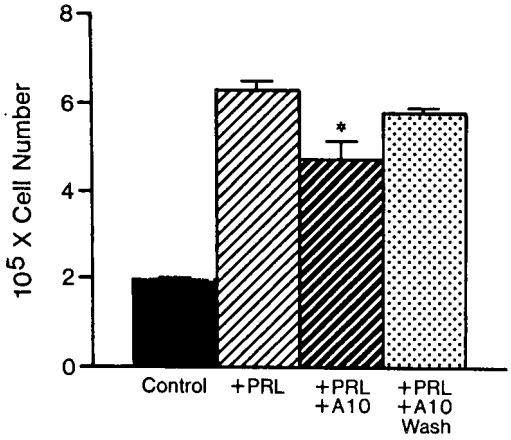


Figure 5. Reversibility of A10 inhibition. The supplemental A10 treatment group (+PRL+A10) received A10 (2 m*M*) for 48 hr, whereas the group that did not receive supplemental A10 treatment (+PRL+A10 Wash) had the A10 removed after 24 hr of culture. A PRL concentration of 0.4 ng/ml was used in +PRL, +PRL+A10, and +PRL+A10 Wash. Control cells received no A10 or PRL. The experiment was performed in triplicate. Cell numbers are expressed as the mean cells per ml ± SD. $^*P < 0.05$ compared with PRL control.

(Brandel, Rockville, MD), washed to remove unincorporated thymidine, and counted with a Beckman LS9000 beta counter. All culture experiments were performed in triplicate.

^{51}Chromium Release Assay for Cytotoxicity. This assay was performed as described previously (11). Briefly, aliquots containing 2×10^6 cells were labeled with 100 μCi of sodium ^{51}Cr for 2 hr in 1 ml of medium. After they were washed three times, 250,000 cells, in 0.1 ml of medium, were pipetted into 96-well plates. The various test substances were then added to triplicate wells and allowed to incubate for 4 hr. Two control groups were included: (i) no treatment, to measure spontaneous ^{51}Cr release; and (ii) cells treated with the detergent Nonidet P-40, to lyse all cells, thus representing maximum release. Following the incubation, 50 μl of supernatant were collected from each well and counted in a Beckman 4000 gamma counter. An increase in counts above spontaneous release would correspond to cell death.

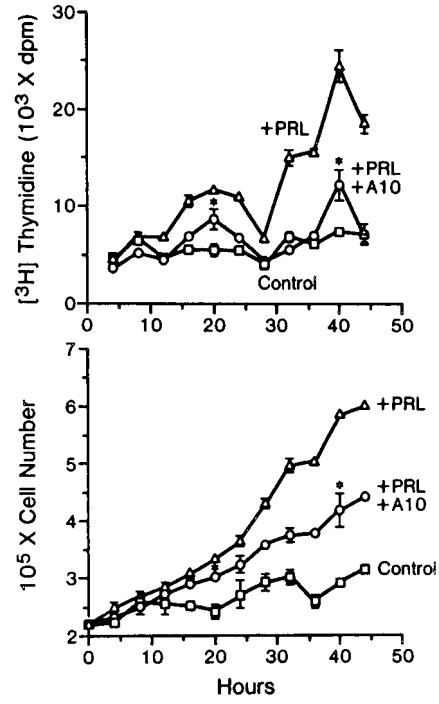


Figure 6. The effect of A10 on DNA synthesis in PRL-stimulated Nb2 lymphoma cells. (Top) Control (□), PRL-stimulated (Δ), and A10-treated cells (○) were pulsed with [^{3}H]thymidine every 4 hr. The PRL and A10 concentrations used were 0.4 ng/ml and 2 m*M*, respectively. DPM are plotted as the mean per well ± SD of three separate determinations. (Bottom) Cell numbers from the same experiment are plotted as the mean cells per ml ± SD. $^*P < 0.05$ compared with PRL control.

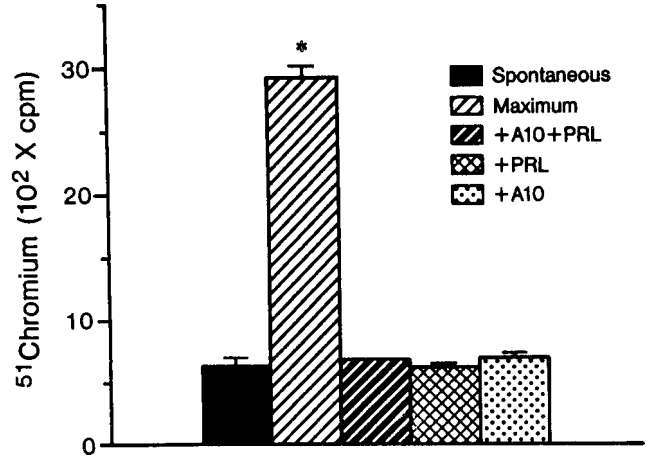


Figure 7. Chromium release assay for cytotoxicity. Quiescent Nb2 cells were loaded with ^{51}Cr as described in Materials and Methods, treated with PRL (0.4 ng/ml) and/or A10 (4 m*M*), and cultured for 4 hr. Spontaneous represents the amount of ^{51}Cr released in control cells (no A10 or PRL). Cells were treated with Nonidet P-40 for maximum release. CPM are plotted as mean per well ± SD of three separate determinations. $^*P < 0.05$ compared with spontaneous.

Statistics. Statistical analysis was performed by analysis of variance and Fisher PLSD.

Results

A10 Time Course and Dose-Response Inhibition of PRL-Stimulated Nb2 Cell Mitogenesis. A single concentration of A10 (4 m*M*) significantly inhibited PRL stimulation of Nb2 cell growth for as long as 120 hr (Fig. 2). To further characterize this inhibition, studies were performed to determine whether the inhibitory response to A10 was concentration dependent. A10 inhibited growth in a concentration-dependent manner, affording 90% inhibition compared with controls at the highest concentration used (12 m*M*) with an IC_{50} of 4–6 m*M* (Fig. 3).

Inhibition of IL-2 Stimulation of Cell Proliferation. IL-2 (10 units/ml) was used to stimulate mitogenesis in the cells in order to determine whether A10 inhibition was selective against PRL-stimulated growth. The stimulation of mitogenesis by IL-2 was completely blocked when A10 (4 m*M*) was included in the culture along with IL-2 (Fig. 4).

Reversibility of A10 Inhibition. In order to determine whether the inhibitory effects of A10 were reversible, two PRL-stimulated groups were initially cultured with A10 (2 m*M*). After 24 hr of culture, cells in both groups were centrifuged, washed three times with medium containing no A10, and plated again; one group received supplemental A10 treatment and the other did not. The cells were then cultured for an additional 24 hr and cell counts were made. A significant decrease in cell number was seen in the constant presence of A10 (i.e., the supplemental A10 treatment group). However, no statistical difference was noted in the magnitude of proliferative response to PRL when comparisons were made between PRL controls and the group that did not receive supplemental A10 treatment. The measurements demonstrated the reversibility of A10 inhibition (Fig. 5).

A10 Inhibition of DNA Synthesis. DNA synthesis in the Nb2 cells was studied by measuring [^{3}H]thymidine incorporation into DNA. Cells were pulsed with thymidine for 44 hr at 4-hr intervals. Following PRL stimulation, the quiescent cells that are predominantly in G_1 (12), began to enter S phase to resume cycling. PRL stimulation caused the cells to synthesize DNA in a synchronous manner, as seen by the wave of [^{3}H] thymidine incorporation, which peaks at 20 hr (Fig. 6, top). As cells proceeded through the cycle, there was a decrease in incorporation. Following mitosis, the cells began to synthesize DNA again, yielding a second and larger peak at 40 hr (Fig. 6, top). Control cells receiving no PRL or A10 showed no change in incorporation. The addition of A10 (2 m*M*) to PRL-stimulated cells in culture caused a significant decrease in [^{3}H]thymidine incorporation compared with PRL alone. A10 reduced [^{3}H]thymidine incorporation into DNA in the presence of PRL by 25% after 20 hr and by 50% after 40 hr. Corresponding cell numbers in the same experiment correlated well with the [^{3}H]thymidine incorporation data (Fig. 6, bottom).

Chromium Release Assay for Cytotoxicity. In order to determine whether the inhibitory actions of A10 are cytostatic rather than cytotoxic, a chromium release assay for cytotoxicity was employed. As revealed by the assay, addition of A10 to Nb2 lymphoma cells did not lead to a significant increase of ^{51}Cr release into the media above spontaneous control (Fig. 7). Therefore, A10 is not cytotoxic within the time interval of the assay.

Discussion

Our findings demonstrate that A10 can inhibit PRL-stimulated mitogenesis in a dose-dependent and readily reversible manner. Results from the 4-hr chromium-release assay for cytotoxicity suggest that the inhibition is probably through cytostatic actions. A10 may inhibit proliferation via postreceptor interactions, since both PRL and IL-2 stimulation of mitogenesis are inhibited by A10 treatment.

While the mode of action of A10 has not been established, the possibility that it may act at the genomic level has been raised. Stereochemical modeling studies demonstrated that A10 could insert into cavities between DNA base pairs (13). In addition, physicochemical studies (10) have suggested that A10 can interact weakly and reversibly with DNA. The possibility that A10 interacts with DNA is further supported by evidence that two structurally related experimental anticancer drugs, 1-(2-chloroethyl)-3-(2,6-dioxo-3-piperidyl)-1-nitrosurea and 5-cinnamoyl-6-aminouracil, interact with DNA (14, 15).

A10 has been undergoing clinical trials and animal studies as well as *in vitro* experiments (3–5). For example, in A/WySnJ mice, pulmonary adenoma formation induced by urethane was shown to be significantly reduced by feeding the mice a 1% A10-supplemented diet (16). Mean tumor number was shown to be reduced from 19.4 tumors per mouse in the urethane controls to 9.05 in the urethane + A10 group. Another chemopreventive study demonstrated a protective effect of orally administered A10 (1%) on aflatoxin B_1-induced hepatocarcinoma. The average number of lesions in the aflatoxin B_1-treated group was reduced from 5.84 to 0.67 with A10 treatment (17). Tumors in nude mice inoculated with human breast cancer cells (R-27) have also been shown to be inhibited by A10 treatment (18). We have obtained similar results in nude mice inoculated with Nb2 and MCF-7 cells (unpublished results). No changes in body weight, food intake, or in gross/microscopic anatomy were observed

in our experimental animals that were fed a diet in which 2% of their total food intake was A10. In all these cases, significant retardation in tumor growth was obtained with doses of A10 that showed no toxicity. Other cell lines in which mitogenesis was inhibited by A10 include: human leukemia (K562); human cervical carcinoma (HeLa); human medullary blastoma (T671); human bladder (T24); human breast carcinoma (MDA-MB-231; MCF-7); and mouse lymphoma (YAK) (4, 19, 20). While the levels of A10 required to establish a response in all studies including ours are high, no toxicity has been observed at those concentrations which inhibit mitogenesis (Fig. 7).

While we can now only speculate about the possible mechanism of A10 action, we believe the data presented provide us with the necessary information to direct further studies needed to understand the action of this novel compound. Experiments are currently being undertaken to explore the possibility that A10 perturbs mitogenic signaling pathways. Furthermore, work is in progress on the design, synthesis, and biological testing of A10 analogs with greater potency (20). This study will serve as a model system to evaluate these analogs.

This paper is dedicated to the memory of Dr. Thomas G. Muldoon.

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