Antitumor Activity and Murine Pharmacokinetics of Parenteral Acronycine

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INTRODUCTION

ACRO

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ACRO is an N-acridone alkaloid originally isolated from the bark of the scrub ash Achronychia bauen (1). The structure of acronycine (Fig. 1) includes a planar chromophoric ring system which lacks the ionizable side chain(s) that typify DNA intercalators such as doxorubicin (Adriamycin) or dactinomycin (actinomycin D). Mechanically, ACRO is reported (a) to act on cell surfaces or on membranous organelles (2), (b) to block cell division resulting in binucleation similar to cytochalasin B (3), and (c) to reduce nucleoside uptake (4, 5). Interestingly, ACRO is active in vitro and displays enhanced (colchicine).

MATERIALS AND METHODS

In Vitro Antitumor Assays

All in vitro assays for antitumor activity used ACRO dissolved in 1% VPD:99% D5W (% v/v). Solutions were made fresh using an ACRO stock solution (1 mg/ml in 100% VPD). Final plating concentrations of VPD were ≤0.1%, a concentration which was noncytotoxic in control experiments. Antitumor studies were performed using colony formation in soft agar in a standard two layer system (13). An underlayer of 1.0 ml culture medium in molten 0.3% agar was poured into 35-mm plastic Petri dishes. A single cell suspension of tumor cells was then plated over the underlayer. Drug exposures were for 1 h (prior to plating) or continuous (drug solution added to the final plating medium). The plates (3/exposure) were then incubated at 37°C in 5–10% CO2/air at high humidity for 7–14 days. Tumor cell colonies ≥60 μm were counted using an automated image analysis instrument (FAS-II; Omnicron; Bausch and Lomb, Rochester, NY) (14). Logarithmic ranges of ACRO concentrations were tested using 1 h and/or continuous drug exposure methods. Each experiment was repeated and mean values used. Fresh human tumors were disaggregated mechanically and enzymatically prior to drug testing (15). Sensitivity was inferred at a percentage survival ≤50% of concurrent, untreated control plates.

Because of the suggestion of collateral sensitivity to ACRO in MDR phenotype CHO cells (7), ACRO activity in other P-glycoprotein-positive MDR cell lines was evaluated using colony-forming assays. These results were compared to the sensitive parental strains of each cell line carried in concurrent passage. The MDR cell lines included: (a) L1210 murine leukemia cells selected for resistance to mitomycin C (16); (b) 8226 human multiple myeloma cells selected for resistance to doxorubicin (17); (c) CCRF-CEM human lymphoblasts selected for resistance to vinblastine (18); and (d) the original CHO cells of Ling and Thompson (19) which were selected for resistance to colchicine.

In Vivo Antitumor Studies

ACRO Solutions. ACRO powder was dissolved initially in 100% sterile VPD cosolvent system (11). Briefly, each ml of this cosolvent of ACRO suspensions in the non-ionic surfactant Emulphor which contains polyethoxylated castor oil (9). In a Phase I-II clinical trial in refractory multiple myeloma, oral ACRO capsules produced 1 partial response in 16 patients. This remission was maintained for 72 weeks using a daily dose of 300 mg/m2 (10). Clinical toxicities in this study included dose-limiting nausea, vomiting and anorexia, and cumulatively, neurotoxicity which was manifested by ataxia.

These findings suggested that ACRO is an active, novel anticancer agent. A serious limitation to further testing was the lack of a parenteral formulation of the drug. Recently, this problem was solved using a commercial lipophilic cosolvent system (11). This vehicle originated with the epipodophyllotoxin derivative, etoposide (12). Using this etoposide diluent (VPD) we were able to give mice i.p. ACRO injections without causing severe acute toxicity. In the current paper, we extend the parenteral use of ACRO in VPD to the successful treatment of several murine tumors in vivo. In addition, ACRO was further evaluated in vitro against several fresh human tumor cells and four established MDR cell lines to see whether the drug might be useful in MDR clinical malignancies.

ABSTRACT

The lipophilic antitumor alkaloid acronycine (ACRO) was solubilized in the cosolvent system used for etoposide. ACRO in this etoposide diluent (VPD) was found to be cytotoxic (70% colony formation in soft agar) in fresh human tumors from patients with renal cell cancer, ovarian cancer, uterine cancer, and metastatic tumors of unknown primary. In P-glycoprotein-positive, multidrug-resistant (MDR) cell lines, ACRO in VPD was active in MDR Chinese hamster ovary cells but not against MDR L1210 murine leukemia cells, 8226 human myeloma cells, or human CCRF-CEM lymphoblasts. In mice, ACRO in VPD was not active in two solid tumor models and an i.p. MOPC-315 plasmacytoma model. ACRO i.p. in 10% VPD (v/v%) produced significant tumor growth delays in nude mice bearing human MCF-7 breast cancer xenografts and CS7BL mice bearing colon 38 tumor. In MOPC-315-bearing mice, a single i.p. ACRO dose of 25 mg/kg was as effective as melphalan (15 mg/kg) at prolonging life span. Finally, ACRO pharmacokinetics was evaluated in mice given single 25–mg/kg doses i.p. or p.o. The oral bioavailability of an ACRO solution in VPD was only 50% but both i.p. and p.o. regimens achieved plasma levels >1.0 μg/ml. The plasma half-life was just under 2 h. These results show that parenteral ACRO in VPD comprises a cytotoxic antitumor agent with improved bioavailability over p.o. administration. ACRO is active in vitro against several human solid tumors but is cross-resistant in 3 of 4 MDR cell line. The prior clinical activity of i.p. ACRO in myeloma and the new results in MOPC-315 plasmacytomas in mice suggest that ACRO in VPD could have activity against human multiple myeloma.

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the MCF-7 model. An i.p. treatment of 10% VPD in D5W (0.1 ml/10 control treatment was also studied in the Colon 38 model. This involved MOPC-315 plasmacytoma study (20). No positive control was used in to comprise a consistently active regimen in this model (21). For similar a daily for 9 days regimen of floxuridine, 50-mg/kg dose given i.p. on of 750 mg size by >7 days compared to control animals (21). A positive

where a and b are the longest and shortest diameters in mm, respectively

includes polysorbate 80 (80 mg), polyethylene glycol 300 MW (650 mg), benzyl alcohol (30 mg), and absolute ethanol (sufficient to yield 1.0 ml). Subsequent dilutions were made using D5W prewarmed to 37°C. All drug injections were performed i.p. using 0.1 mg/10 g of up to a 3.0-mg/ml ACRO solution (10% VPD in 90% D5W, v/v). The stability of ACRO in VPD dissolved into human plasma was tested in vitro at 37°C.

Antitumor Studies in Mice. Three established murine tumor models were used to evaluate the antitumor activity of parenteral ACRO in vivo. Since ACRO was known to be inactive in the standard leukemia models (8), other solid tumor models were chosen for the current trial. These included (a) the mineral oil-induced plasmacytoma tumor MOPC-315, growing i.p. in BALB/c mice (20), (b) Colon 38 grown in the flank of C57BL mice (21), and (c) MCF-7 human breast cancer grown in the flank of nude mice (22). Tumors were passed and treated according to published methods (Table 1). The MCF-7 cells and Colon 38 tumor fragments were implanted in the forelimb muscle of anesthetized mice using 22- and 14-gauge needles, respectively. MOPC-315 tumor cells were injected into the peritoneum of mice pretreated 7 days earlier with i.p. pristane (2,6,10,14-tetramethylpentadecane) to induce a local granulomatous reaction (20). Each experimental group included 10 adult mice. All animals were obtained from The Jackson Laboratory, Bar Harbor, ME, and were treated at body weights of 25-32 g. They were housed 5/cage on wood chip bedding with 12-h light-dark cycles. Food and slightly acidified water (to reduce enteric pathogens) was provided ad libitum.

The end points for significant antitumor activity included survival in MOPC-315-bearing mice, tumor volume change in MCF-7-bearing nude mice, and tumor weight change in Colon 38-bearing mice. For the latter two solid tumor models, the widest perpendicular diameters were measured using micrometers. MCF-7 tumor volume was calculated according to published criteria (22) and Colon 38 tumor weight was calculated according to the formula

\[ \text{Tumor wt (mg)} = \frac{a \times b^2}{2} \]

where a and b are the longest and shortest diameters in mm, respectively (21).

Significant antitumor effects were inferred at time points wherein the ACRO treatments (a) delayed MCF-7 tumor volume change by ≥50% of control, and (b) delayed the attainment of Colon 38 tumors of 750 mg size by >7 days compared to control animals (21). A positive control treatment was also studied in the Colon 38 model. This involved a daily for 9 days regimen of flouxuridine, 50-mg/kg dose given i.p. on Days 3-12 after tumor implantation. Previous studies have shown this to comprise a consistently active regimen in this model (21). For similar reasons, an active melphalan treatment (15 mg/kg) was included in the MOPC-315 plasmacytoma study (20). No positive control was used in the MCF-7 model. An i.p. treatment of 10% VPD in D5W (0.1 ml/10 g body weight) was added to evaluate whether the diluent alone had any antitumor efficacy. For the MOPC-315 ascites tumors, mice were observed twice daily and survival differences were statistically compared using the log rank method (23).

Pharmacokinetic Studies. Adult male CD-1 mice (5-6/time point) were given i.p. or p.o. ACRO in 10% VPD:90% D5W at a dose of 25 mg/kg. Treatments were administered p.o. by gavage tube. At serial time points, 5 mice in each group were anesthetized under diethyl ether and 1-2 ml of blood were removed by cardiac puncture. The mice were then killed by rapid cervical dislocation. The blood was separated by centrifugation. The plasma fraction was then deproteinized with 5% perchloric acid and extracted with ethyl acetate. The concentration of ACRO was then analyzed by reverse phase HPLC (Varian 5020 chromatograph; Valco 10-μl sample loop; Beckman UV detector). The chromatographic conditions involved an isocratic elution with a mobile phase of 75% acetonitrile-25% water, a flow rate of 1 ml/min, and a solid phase of octadecylsilane (C18 ODS; Altex Corp). ACRO detection was by UV spectroscopy at 280 nm (11). Quantitation was by comparison to peak heights from a series of sequentially run external standards. No internal standard was used since prior studies documented >99% ACRO recovery using the nonpolar solvent ethyl acetate (11).

Pharmacokinetic parameters were determined by computer using NONLIN (24). Several models for disposition of oral and parenteral ACRO were evaluated. The final model was chosen to minimize the sum of squared residuals for observed and model-calculated ACRO concentrations. For both i.p. and p.o. data sets, a lag time for systemic absorption was used.

RESULTS

In Vitro Cytotoxicity against Fresh Human Tumor Cells. Table 2 summarizes the in vitro activity of ACRO in VPD against fresh human tumor cells. These results show that ACRO has activity against a variety of solid human tumors including malignant mesothelioma, uterine cancer, ovarian cancer, kidney cancer, and metastatic tumors with an unknown primary. The aggregate response rate in these sensitive tumors was approximately 38% using ACRO concentrations of 0.1 to 1.0 μg/ml by 1 h and continuous exposures, respectively. Other fresh human tumors which were not sensitive to ACRO by either exposure method included colon cancer, head and neck cancer, lung cancer, melanoma, and stomach cancer (at least 5 specimens tested in each category).

In Vitro Cytotoxicity against MDR Cell Lines. Colony-forming assays in soft agar showed that ACRO in VPD was not consistently more active against MDR tumor cell lines (Table 3). We were, however, able to reproduce the enhanced sensitivity of MDR CHO cells to ACRO seen in earlier studies (19). This represented a 300-fold enhancement in ACRO sensitivity in the colchicine-resistant MDR CHO cells. In the other 3 F-glycoprotein-positive MDR lines (16-18), ACRO was not more active than in the sensitive parental lines.

In Vivo Antitumor Activity. ACRO in VPD was dissolved in human plasma to test for compatibility of drug in a physiological medium. At 37°C the drug remained in solution for over 8 h at a concentration of 0.3 mg/ml (10% VPD cosolvent concentration). This simulates the peak plasma concentration obtainable after administering a dose of 30 mg/kg i.p. to a mouse.

In a previous murine toxico logical study, ACRO in 10% VPD was highly toxic by the i.v. route (50% lethal dose, 32 mg/kg). In contrast, it was well tolerated using 5 daily i.p. doses of up to 30 mg/kg/dose (11). This formed the upper limit of doses studied in the tumor-bearing mice. Fig. 2 shows the significant antitumor efficacy of ACRO in the 3 murine tumors. The VPD control treatments were inactive in each tumor model. Against

![Fig. 1. Structure of acronyine.](image-url)

<table>
<thead>
<tr>
<th>Model</th>
<th>Tumor inoculum</th>
<th>Location</th>
<th>ACRO i.p. treatments</th>
<th>Model Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>MOPC 315</td>
<td>10^5 cells</td>
<td>IP</td>
<td>14</td>
<td>25</td>
</tr>
<tr>
<td>Colon 38</td>
<td>20 mg tumor fragments</td>
<td>Axilla</td>
<td>3, 3-8</td>
<td>15, 30</td>
</tr>
<tr>
<td>MCF-7</td>
<td>5 x 10^6</td>
<td>Axilla</td>
<td>1-5</td>
<td>15, 30</td>
</tr>
</tbody>
</table>

Table 1 Murine tumor models used for parenteral acronyine survival studies

![Table 1](image-url)
ANTITUMOR ACTIVITY AND PHARMACOKINETICS OF PARENTERAL ACRO

Table 2 ACRO cytotoxicity in fresh human tumor cells in vitro

<table>
<thead>
<tr>
<th>Tissue type</th>
<th>1 h exposure</th>
<th>Continuous exposure</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. of evaluable specimens</td>
<td>No. sensitive (%)</td>
</tr>
<tr>
<td>Bladder</td>
<td>2</td>
<td>1 (50)</td>
</tr>
<tr>
<td>Brain</td>
<td>3</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Breast</td>
<td>3</td>
<td>1 (33)</td>
</tr>
<tr>
<td>Uterine</td>
<td>4</td>
<td>1 (25)</td>
</tr>
<tr>
<td>Kidney</td>
<td>7</td>
<td>1 (14)</td>
</tr>
<tr>
<td>Ovarian</td>
<td>23</td>
<td>3 (13)</td>
</tr>
<tr>
<td>Mesothelioma</td>
<td>4</td>
<td>1 (25)</td>
</tr>
<tr>
<td>Testes</td>
<td>3</td>
<td>1 (33)</td>
</tr>
<tr>
<td>Unknown primary (adenocarcinoma)</td>
<td>3</td>
<td>2 (67)</td>
</tr>
<tr>
<td>(Non-adenocarcinoma)</td>
<td>3</td>
<td>2 (67)</td>
</tr>
</tbody>
</table>

*<50% survival of colony-forming cells.
* NT, not tested; NA, not achievable.

Table 3 Comparison of ACRO cytotoxicity in parenteral (sensitive) and MDR tumor cell lines in vitro

<table>
<thead>
<tr>
<th>Tissue type</th>
<th>Original resistance selection agent (Ref.)</th>
<th>Parenteral IC₅₀ values (μg/ml)</th>
<th>MDR sensitive IC₅₀ values (μg/ml)</th>
<th>Cross-resistance ratio</th>
<th>Parenteral IC₅₀ sensitive IC₅₀ resistant</th>
</tr>
</thead>
<tbody>
<tr>
<td>L1210 leukemia</td>
<td>Mitomycin C (16)</td>
<td>2.0</td>
<td>12.0</td>
<td>0.16</td>
<td>12.0</td>
</tr>
<tr>
<td>8226 myeloma</td>
<td>Doxorubicin (17)</td>
<td>0.06</td>
<td>2.6</td>
<td>0.023</td>
<td>0.023</td>
</tr>
<tr>
<td>CCRF-CEM, lymphoblasts</td>
<td>Vinblastine (18)</td>
<td>0.24</td>
<td>6.5</td>
<td>0.037</td>
<td>0.037</td>
</tr>
<tr>
<td>CHO</td>
<td>Colchicine (7)</td>
<td>1.5</td>
<td>0.005</td>
<td>300</td>
<td>0.005</td>
</tr>
</tbody>
</table>

* IC₅₀, inhibitory concentration which reduces colony formation in soft agar to 50% of control after a 1-h ACRO exposure (except for CHO cells wherein ACRO exposure was continuous).

MOPC-315 plasmacytomas, i.p. ACRO produced a median increased life span of 8 days or 33% which was indiscernible from the effect of melphalan (Fig. 2A).

In the MCF-7 model, ACRO was evaluated at 2 dose levels, 15 and 30 mg/kg i.p. for 5 days. Only the latter regimen was associated with significantly enhanced survival after 15 days of observation. This activity was manifested by suppression of MCF-7 cell growth without significant tumor shrinkage from pretreatment tumor volumes (Fig. 2B). A similar dose-dependent inhibition of tumor growth was also noted for ACRO used in Colon 38-bearing mice (Fig. 1C). In this instance, neither single-dose ACRO treatment was active (Treated-Control Survivors, 0% vs. 2% in colon 38). A similar dose-dependent inhibition of tumor growth was also noted for ACRO used in Colon 38-bearing mice (Fig. 2C). In this instance, neither single-dose ACRO treatment was active (Treated-Control Survivors, 0% vs. 2% in colon 38).

In vivo, i.p. ACRO in VPD produced dose-dependent antitumor effects in mice bearing three different solid tumors. Significant antitumor effects were seen in nude mice bearing MCF-7 human breast cancer xenografts and in C57BL mice bearing Colon 38 tumors. In the later case, growth inhibition was comparable to that obtained with an intermediate dose of 15 mg/kg daily regimen, respectively. The daily dose of ACRO was active producing a T-T₅₀ value of 7 days.

ACRO Pharmacokinetics Using HPLC. The detection limit in the HPLC-UV assay was approximately 5.0 ng of ACRO. Separation of ACRO from the solvent front and plasma extraction impurities was also good. The retention time was 4.5 min and only a single peak was observed in the chromatograms from mouse blood extracts. Peak identity and quantitation were verified using external ACRO standards. Drug levels were still detectable for up to 7 h after dosing.

Table 4 compares the pharmacokinetic parameters derived for ACRO in VPD given either i.p. or p.o. at 25 mg/kg. A plot of the mean disposition patterns is shown in Fig. 3. These results show that peak leaks of 1 and 3 μg/ml are achievable with tolerable p.o. or i.p. doses of ACRO, respectively. The terminal disposition of i.p. ACRO was best fit to a two-compartment model with a weighting of 1/concentration. For p.o. ACRO disposition, a one-compartment, unweighted model best described the data. In both, a lag phase indicating the presence of a significant extravascular compartment was evident. The apparent volume of distribution was also quite large at 8.3 liters/kg indicating substantial extravascular distribution of the drug. Nonetheless, plasma clearance was relatively rapid as evidenced by short terminal disposition half-lives of under 2 h. Of interest, the bioavailability of the p.o. ACRO solution was only one-half that of the comparable i.v. dose (Table 4).

DISCUSSION

Acronycine was first tested for anticancer activity in 1966 using a crude, defatted ethanolic extract of the bark of ACRO and its activity was found to be comparable to that obtained with an intermediate dose of 15 mg/kg daily regimen, respectively. The daily dose of ACRO was active producing a T-T₅₀ value of 7 days.

HPLC-UV assay was used to determine drug levels in plasma and other tissues. ACRO was found to be stable in plasma and other tissues. The apparent volume of distribution was quite large at 8.3 liters/kg indicating substantial extravascular distribution of the drug. Nonetheless, plasma clearance was relatively rapid as evidenced by short terminal disposition half-lives of under 2 h. Of interest, the bioavailability of the p.o. ACRO solution was only one-half that of the comparable i.v. dose (Table 4).

In m...
from patients with a variety of solid malignancies. In most instances, ACRO sensitivity was obtained at the lowest concentration tested (0.1 μg/ml). This is an important observation since our pharmacokinetic study of i.p. ACRO showed that peak plasma levels of 3 μg/ml were obtained following tolerable acute doses of 25 mg/kg. These findings demonstrate that ACRO concentrations which are cytotoxic to human tumor cells in vitro can be readily achieved in vivo.

Another important pharmacokinetic observation was that only one-half of a p.o. ACRO solution was systemically available in the mice. The oral bioavailability of powdered ACRO from the human capsule formulation could be even poorer since aqueous dissolution of highly lipophilic drugs in the gut is characteristically very poor (25). Since the i.v. administration of ACRO in the 10% VPD solution is highly toxic (11), a prolonged i.v. infusion might be necessary to deliver active amounts of drug in a clinical trial. Nonetheless, these studies show that ACRO is active against human and murine tumors in vivo, and against fresh human tumors in vitro. Responsive fresh human cancers included ovarian carcinoma and relatively drug-resistant tumors such as kidney cancer and metastatic cancers of unknown primaries. Furthermore, the pharmacokinetic findings document (a) a major increase in bioavailability and (b) the achievement of cytotoxic drug concentrations with the new parenteral formulation.

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