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In Vitro and In Vivo Responses of a Murine Transitional Cell Carcinoma to Doxorubicin, Mitoxantrone and Aclacinomycin-A

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Summary. In vitro and in vivo effects of mitoxantrone, aclacinomycin-A and doxorubicin were examined in a transplantable murine transitional bladder carcinoma, FCB. The in vitro parameters used included monolayer growth kinetics, tumor stem-cell colony formation and autoradiographic analysis of thymidine labeling. Monolayer growth kinetics revealed that both mitoxantrone and aclacinomycin-A resulted in reductions in FCB cell growth, which were significantly higher (41% and 65%, respectively) than those seen with doxorubicin treatment (22%). Similarly, by the stem-cell assay, an increased reduction in colony formation was seen in aclacinomycin-A (98%) and mitoxantrone (91%) treated cultures when compared with doxorubicin (51%) treated cultures. Autoradiographic data revealed that 24-h exposure with both aclacinomycin-A and mitoxantrone significantly inhibited thymidine incorporation (98% and 80% respectively), which was an increase over doxorubicin (19%). In vivo studies revealed that aclacinomycin-A treatment increased the mean life span of C57BL mice by 60.6% when compared with a 33.6% increase in doxorubicin-treated animals and a 19.7% increase in mitoxantrone-treated animals. Both the in vitro and in vivo data suggest that aclacinomycin-A is a superior drug when used against this specific murine bladder tumor cell and that further testing of this agent for its efficacy in other urologic tumors is justified.

Key words: Doxorubicin, Mitoxantrone, Aclacinomycin-A, Bladder cancer.

Carcinoma of the bladder is a serious malady among men in the United States with recurrence being frequent. It has been postulated that seeding of the exfoliated tumor cells and the multifocal nature of the disease are the major contributing factors [12, 17]. In an effort to reduce the recurrence of these tumors, intravesical chemotherapy has been advocated as a prophylatic therapy. Currently, intravesical instillation of chemotherapeutic agents has become a frequent adjunct to endoscopic resection and, in some cases, a reasonable alternative to transurethral resectioning [6].

Among the currently available chemotherapeutic agents, doxorubicin has been shown to be clinically useful against a variety of neoplasms. It has been reported to be, when used in combination therapy, one of the most effective agents available for the treatment of human bladder cancer [14]; however, a recent report has indicated that it may be much less effective [23]. Its high affinity for myocytes has produced a potentially lethal cardiotoxicity in many patients and is itself mutagenic, presumably through a free radical formation in one of its metabolites [9]. Mitoxantrone and aclacinomycin-A are two recently developed antineoplastic agents that have been reported to have reduced cardiotoxicity and in limited trials have been shown to be effective against a variety of neoplastic tissues [7].

In this study we have evaluated the in vitro and in vivo chemotherapeutic efficacies of mitoxantrone, aclacinomycin-A, and doxorubicin at equal molar concentrations on the bladder cell carcinoma, FCB. Experimental parameters used were the growth inhibition of monolayer cultures and tumor stem cells, the inhibition of normal cell cycle progression and the ability to produce permanent cell damage. Additionally, each agent was assessed for its ability to prolong the life span of tumor-bearing mice. Results obtained suggest that both mitoxantrone and aclacinomycin-A are more efficacious than doxorubicin in inhibiting cellular growth in vitro. However, the in vivo data suggest that, when compared with doxorubicin, only aclacinomycin-A was significantly more effective at increasing the survival of the tumor-bearing host.

Materials and Methods

Drug Preparation

Mitoxantrone and aclacinomycin-A were obtained from the Investigative Drug Branch, Cancer Chemotherapy Evaluation Program,

Division of Cancer Treatment, National Cancer Institute (Bethesda, MD). Doxorubicin was supplied by Adria Laboratories (Columbus, OH). All drugs were prepared by dissolving them in sterile normal saline or 0.1 N HCl and diluting them with Eagles Minimum Essential Medium (EMEM) (M.A. Bioproducts, Walkersville, MD). The final pH was maintained at 7.2.

Tumor Cell Preparation

The transplantable bladder tumor FCB was obtained from the Mason Research Institute (Worcester, MA) and was subcutaneously inoculated in C57BL mice (Laboratory Supplies, Indianapolis, IN). All animals were housed unter a 12-h light-dark cycle and maintained on Purina mouse chow (Ralston Purina) and tap water ad lib. Monolayer cultures were prepared by mincing tumors and mechanical dispersion of the minced tumors into a single cell suspension by use of a pippet. The resulting cell suspension was then passed through two nylon sieves, 110 μm and 41 μm pore size, respectively (Nitex, Tekto Co., Elmoford, NY) to produce a single-cell suspension. Monolayer cultures were achieved by seeding 5×10^5 cells per well in 24-well culture plates (16 mm^2 diameter, Falcon Laboratories, Oxnard, CA). Cultures were maintained in EMEM supplemented with 10% fetal bovine serum, antibiotics (100 U/ml penicillin, 100 μg/ml streptomycin, 0.025 μg/ml amphotericin-B), hormones (0.5 μg/ml insulin, 1 μg/ml corticosterone) and incubated in a high humidity incubation chamber at 37 °C with an atmosphere of 95% air-5% CO_2.

Clonogenic assays were performed according to procedures of Salmon et al. [18]. Briefly, 1 ml of sterile molten 0.6% Difco-Bacto agar medium (Difco Laboratories, Detroit, MI) was layered into a 35 mm sterile petri dish (Falcon Plastic, Oxnard, CA) and was allowed it to gel at 37 °C for 1 h. The tumor cells were incubated for 1 h at 37 °C in either drug-free medium (CMRL-1066) or medium containing one of the chemotherapeutic agents at either 10^{-5} or 10^{-7} M concentrations. All soft-agar cultures were maintained in CMRL-1066 supplemented with 15% horse serum, antibiotics, hormones and amino acids. After completion of the above incubation, cells were washed free of all agents and passed through the sieves to again produce a single-cell suspension. One million cells were suspended in 1 ml of sterile molten Difco-Bacto agar (0.3% w/v) prepared in supplemented CMRL-1066. The resulting single-cell suspension was then layered over the earlier prepared agar layer and incubated for 14–21 days in a high humidity (95% air – 5% CO_2) incubation chamber at 37 °C. Colonies containing more than 50 cells were counted by use of an inverted microscope fitted with phase contrast optics. The number of colonies was determined from the average of three cultures, and drug treatment results were expressed as a percentage of the control values.

Dose Response Curves

Monolayer cultures in the log phase of growth were exposed to each agent at concentrations ranging from 10^{-4} M to 10^{-9} M. After 24 h, the drug-containing medium was removed, cultures were washed three times in Ca^{++}-Mg^{++}-free phosphate-buffered saline and all attached cells removed using a trypsine-Versene mixture (M.A. Bioproducts, Walkersville, MD). Cells were counted by use of a Coulter counter (Model Z_F), Coulter Electronics, Hialeah, FI, and the viability of cells was determined by vital dye exclusion.

Sequential Observations of Drug Effects and Cell Recovery

The impact of varying the duration of treatment exposure on the growth potential of monolayer cultures was determined by exposing cultures to 10^{-5} M concentrations of each agent for 12, 24, 48, 72, or 96 h. Additionally, the ability of the cultured cells to recover from drug exposure was examined in growth recovery studies. For this, cells were exposed to the agents for 12 h and then washed, this was followed by a drug-free interval of 12, 36, 60, or 84 h. The average number of cells surviving each drug treatment was determined as previously described. All values were expressed as a percentage of the control values.

Thymidine Labeling Index

Cultures were given a 30 min pulse with 0.5 ml of 1 μCi/ml [^{3}H]thymidine (6.7 Ci/mMole, New England Nuclear, Boston, MA), which was followed by extensive washing with Ca^{++}-Mg^{++}-free phosphate-buffered saline and fixed with 10% neutral buffered formalin. The cultures underwent dehydration using graded ethanols (30%–100%) and the multiwell plates were coated [2] with Kodak NTB-2 nuclear track emulsion (Eastman Kodak, Rochester, NY). The samples were stored at 4 °C for 14 days in a light-proof box. A minimum of 3,000 cells was counted for each data point, and the number of S-phase cells was expressed as a percentage of the control values. Labeled cells were defined as cells containing 7 or more granules in the emulsion overlaying the nuclei.

In Vivo Response of FCB Tumors

Syngeneic C57BL mice supplied by Laboratory Supplies (Indianapolis, IN) were subcutaneously inoculated with approximately 5×10^6 FCB cells into the suprascapular region while the animals were under light ether anesthesia. Animals were randomly divided into groups of 5 animals and each received a 0.5 ml intraperitoneal injection of 2×10^{-4} molar doxorubicin, mitoxantrone or aclacinomycin-A, with control groups receiving a sham injection. It is estimated that the blood concentration of each agent will approximate 10^{-5} M concentration once agents are dissipated into the tissue fluids of the animals. Treatments were initiated one day after cell inoculation and administered every third day for the duration of the experiment. Three separate trials were conducted with a total of 15 test animals in each group.

Results

Induction of the FCB tumors in C57BL mice by subcutaneous inoculation of the transplantable FCB explants resulted in a 93% success rate. Tumors became palpable within 7–10 days and reached maximum size in 14–21 days. Untreated animals survived for 20–31 days with an average of 25.9 days after tumor cell inoculation. Tumors were readily dispersed into a suspension of single cells that yielded stable monolayer cultures and demonstrated logarithmic growth rates for all 5 days of culturing.

A significant reduction in cell number, as defined by the Wilcoxon Rank Order Sum test, was demonstrated after 24 h of treatment with all agents tested at 10^{-5} or 10^{-4} M concentrations. However, both mitoxantrone and aclacinomycin-A were consistently more effective than doxorubicin (Fig. 1). At equal molar concentrations (10^{-5} M) a significantly ($p < 0.05$) greater inhibition in cell growth was exhibited by both mitoxantrone (41%) and aclacinomycin-A (65%) than was exhibited by doxorubicin (22%) (Fig. 1). Similarly, at 10^{-5} M concentra-

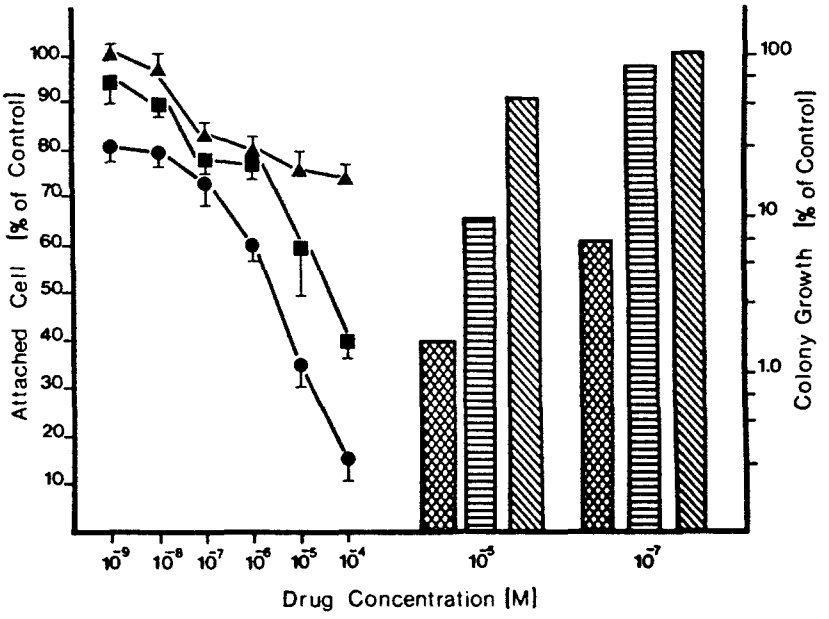

Fig. 1. Dose response of FCB tumor cells to doxorubicin, aclacinomycin-A or mitoxantrone. The line graphs represent the number of attached cells after 24 h of treatment with each agent (▲ = DOX, ● = ACM, ■ = MTN) at various concentrations. Each data point represents triplicate counts from three individual trials. Bar graphs represent the number of colonies formed in soft-agar after 1 h of treatment with each agent (▨ = DOX, ▩ = ACM, ▤ = MTN) followed by 14 days of incubation. The bars represent the average of three trials with each having triplicate plates. For each agent standard deviation was less than 10%

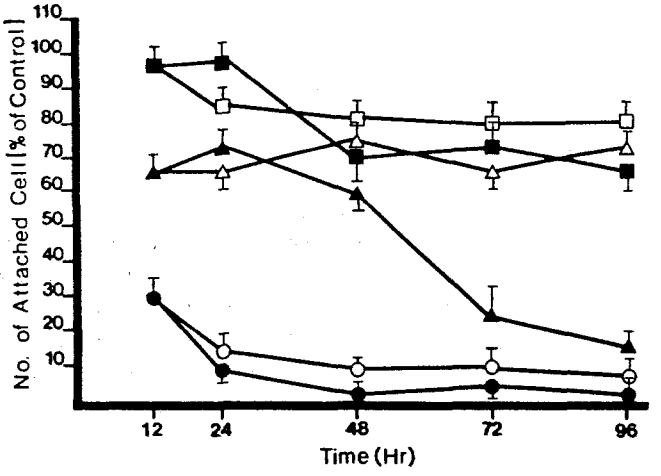

Fig. 2. Time dependent effect of doxorubicin, aclacinomycin-A or mitoxantrone on FCB tumor cells. Cultures were treated with each agent (■ = DOX, ● = ACM, ▲ = MTN) at 10^{-5} M concentrations. After 12 h each culture was washed and replaced with either a drug-free medium (*open figures*) or the appropriate drug-containing medium (*closed figures*). Each point represents the average of triplicate counts of three trials

tions both mitoxantrone and aclacinomycin-A were significantly ($p < 0.05$) more effective than doxorubicin in inhibiting tumor stem-cell colony formation (5 to 40-fold greater).

In experiments where the duration of exposure varied from 12–96 h, the greatest reduction in cell number occurred within the first 24 h of treatment. However, the maximum growth inhibition was often not revealed until 48–72 h later (Fig. 2). In comparing cell survival in monolayer cultures after 96 h of continuous exposure, doxorubicin showed only a 30% inhibition in growth while mitoxantrone and aclacinomycin-A showed growth inhibitions of 80–100%. When cultures were exposed for only 12 h and were followed by an 84-h drug-free interval, all three agents rendered the cultures stagnate with virtually no regrowth (Fig. 2). In addition, it is interesting to note that aclacinomycin-A resulted in a 90% or greater inhibition in the number of attached cells after only 24 h of treatment while mitoxantrone exhibited a time-dependent effect that resulted in only an 80% inhibition after 96 h of continuous incubation. Doxorubicin resulted in only a 20% inhibition of cell growth, which was reached after 48 h and never increased even after 96 h in continuous culture.

Thymidine labeling indices indicated that after 24 h of exposure aclacinomycin-A virtually arrested all DNA synthesis, while mitoxantrone and doxorubicin treatments resulted in an 80% and 18% inhibition, respectively (Fig. 3). Additionally, cells treated with aclacinomycin-A for only 12 h showed a 90% or greater inhibition in DNA synthesis, which continued even after 84 additional h of culturing in drug-free medium. This result differs from that found for mitoxantrone in which complete inhibition of DNA synthesis was observed only after 72 h of continuous exposure. Conversely, after 24 h doxorubicin treatment resulted in only a 20% inhibition of DNA synthesis.

In vivo results (Table 1) revealed that aclacinomycin-A was far more effective than either of the other agents against this specific bladder tumor. Maximum survival increased from 31 days in untreated animals to 52 days in animals treated with aclacinomycin-A. The average length of survival ranged from 25.9 days in control animals to 41.6 days in aclacinomycin-A treated animals; the increase in life span after tumor cell inoculation ranged from 20–34% for mitoxantrone and doxorubicin treatment to 61% for aclacinomycin-A treatment (Table 1). Statistically, the mean survival periods for all three treatment groups were significantly different ($p < 0.05$) from those of the control animals. However, while the mean survival period of animals treated with aclacinomycin-A was significantly

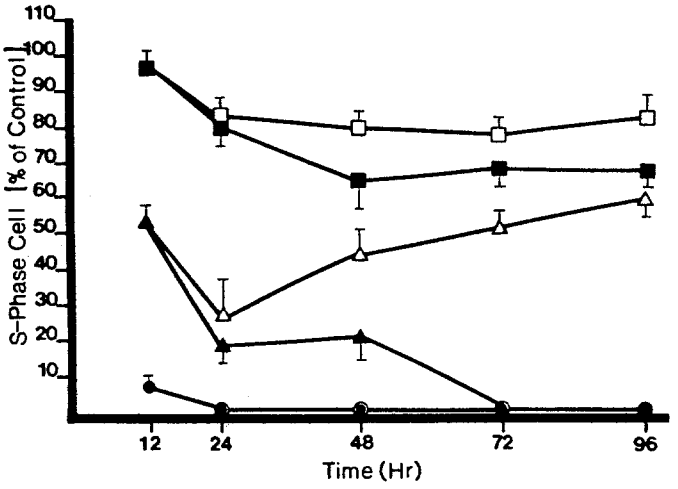

Fig. 3. Thymidine labeling indices of doxorubicin, aclacinomycin-A or mitoxantrone treated FCB tumor cells. Cultures were treated with each agent (■ = DOX, ● = ACM, ▲ = MTN) at 10^{-5} M concentration. After 12 h each culture was washed and replaced with either a drug-free medium (*open symbols*) or the appropriate drug-containing medium (*closed symbols*). Cultures were then pulse labeled with [^{3}H]-thymidine and examined for radiolabeling at 24, 48, 72, and 96 h. Each point represents the average of triplicate counts of three trials

different ($p < 0.05$) from that of those treated with doxorubicin or mitoxantrone, survival for mice treated with either doxorubicin or mitoxantrone was not significantly different.

Discussion

The overall response of advanced bladder cancer patients to doxorubicin as a single agent therapy ranges from as high as 53% to less than 18% [3, 23]. Similarly, for doxorubicin in combination therapy, there have been response rates ranging from as high as 50% to only a partial response [4, 11]. Therefore, the value of doxorubicin in treatment of human bladder cancer remains equivocal, particularly in light of potential cardiotoxicity in some patients. Mitoxantrone, a synthetic anthraquinone, has a planar structure similar to doxorubicin (Fig. 4) but reports of cardiotoxicity have been limited [4]. Preliminary trials indicate that mitoxantrone is highly effective against various neoplastic tissues [20]; however, recent reports of advanced bladder cancer patients have shown vexing results [13, 16]. Aclacinomycin-A is an anthracycline antibiotic and structurally resembles doxorubicin (Fig. 4). Its cellular uptake is approximately twice that of doxorubicin [22] and has been shown to induce fewer side effects [8]. There is a limited number of reports of aclacinomycin-A treatment of bladder cancer, however, a report on a trial using intravesically administered aclacinomycin-A resulted in complete remission in 6 of 9 patients and partial remission in 2 of the remaining 3 patients [19].

While the exact nature of the cytotoxic effects exhibited by these agents is unknown, several postulates exist for their mechanism of action, the most obvious being a direct physical interference with template function during the synthesis of DNA and/or RNA [5]. However, generation of a reactive free radical [1], bioreduction to an alkylating agent [15] and even a recently reported cell membrane interaction [21] have been postulated. In mitoxantrone, the large sugar moieties (Fig. 4) characteristic of most anthracyclines (doxorubicin and aclacinomycin) were absent, thereby eliminating the most probable sites of the proposed free radical formation [23]. Since mitoxantrone shows cytotoxic efficacy comparable to that of doxorubicin, its antitumor properties seem unlikely to be associated with free radical formation but rather to rely on DNA intercalation. Drewinko et al. [7] reported hydroxyl substitutions in the 1 or 4 positions (aclacinomycin-A and mitoxantrone, Fig. 4) that resulted in improved cytotoxicity against a colon cancer cell line. In addition, among the hydroxyl substituted anthraquinones, the chloride salt form (i.e., mitoxantrone) was several times more cytotoxic. Within this context, it is unclear why the chloride salt form would be more effective; however, it is possible that chloridation confers a greater degree of

Table 1. Animal survival after in vivo administration of antineoplastic agents to FCB bearing C57BL mice

Treatment	Number of animals		Maximum survival time (Days)	Mean survival time (Days)	Increase in mean life span after tumor inoculation (Days/%)
	Injected	Bearing tumors			
Control	15	13 (87%)	31	25.9	–
Mitoxantrone	15	11 (73%)	41	31.0[a]	5.1/19.69%
Doxorubicin	15	14 (93%)	46	34.6[a]	8.7/33.59%
Aclacinomycin-A	15	13 (87%)	52	41.6[a,b,c]	15.7/60.62%
Total	60	51/(85%)			

[a] Drug treatment resulting in an increased mean survival time that was significantly higher ($p < 0.05$) than that of the control
[b] Drug treatment resulting in an increased mean survival time that was significantly higher ($p < 0.05$) than that of mitoxantrone
[c] Drug treatment resulting in an increased mean survival time that was significantly higher ($p < 0.05$) than that of doxorubicin

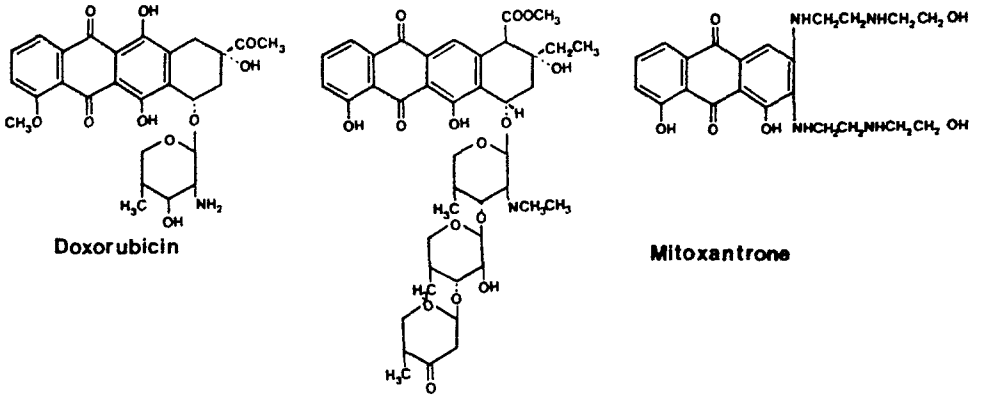

Fig. 4. Chemical structure of doxorubicin, aclacinomycin-A and mitoxantrone

stability to the intercalation. Consequently, mechanisms other than, or at least in addition to, DNA binding exist for the basis of action in the anthracyclines and anthraquinones [7, 21, 24]. Finally, Tritton and Yee [21] treated cultures of L1210 cells with doxorubicin covalently linked to large agarose beads and showed that cytotoxic effects were exhibited while internalization of the drug, due to the size of the beads, was impossible. Therefore, membrane interactions alone may be sufficient to produce cytotoxicity.

The relative efficacy of doxorubicin, mitoxantrone or aclacinomycin-A is difficult to determine directly from the existing literature since tumor origins and drug concentrations often differ drastically. In this study we have controlled both the concentrations and the duration of exposure in an attempt to compare the response of a murine bladder tumor to these agents. Using multiple in vitro evaluation parameters both aclacinomycin-A and mitoxantrone showed superior growth inhibition in monolayer cultures, reduced stem-cell colony formation and reduced thymidine labeling indices more than did doxorubicin. However, the in vivo data suggest that only aclacinomycin-A exhibited a higher potential for increasing the survival period in the animals tested. This may be simply a phenomenon of the route of drug administration. As reported by Wallace et al. [23], when Adriamycin and mitoxantrone were intraperitoneally administered to mice subcutaneously bearing tumors, mitoxantrone was no more effective than Adriamycin. However, other investigators have shown that mitoxantrone was moderately more effective than Adriamycin against one of these three tumor when administered intravenously [10]. Hence, mitoxantrone's effectiveness is somewhat route dependent. However, regardless of the administration route aclacinomycin-A was significantly more effective in increasing the mean life span of the animals tested. Collectively, these data suggest that aclacinomycin-A may be useful as an intravesicle chemotherapeutic agent against superficial bladder tumors; however, additional research in this area is required before beginning any phase II evaluations. Currently, we are attempting to evaluate the efficacy of this and other agents on intravesically produced murine bladder tumors as well as evaluating various administration routes of the agents.

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