Effects of N-trifluoroacetyladriamycin-14-valerate (AD-32) on human bladder tumor cell lines

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Summary. We have compared the in vitro activity of N-trifluoroacetyladriamycin-14-valerate (AD-32) and doxorubicin hydrochloride (ADR) on the clonal growth of human bladder tumor cell lines (HBTC). In order to determine the relative toxicity of ADR and AD-32 on hematopoietic stem cells, CFU-GM assays were set up using 10 normal human bone marrow samples. The mean lethal dose for 50% of the colonies (LD_{50}) for ADR was 1.6 ± 1.4 μM and that for AD-32, 3.9 ± 4.9 μM (P < 0.55), suggesting that these agents have similar bone marrow toxicity. Both drugs produced enhanced inhibition of clonal growth of HBTC with increasing C × Ts. The spectrum of activity of the two drugs was similar against a panel of seven HBTC. The activity of ADR was inhibited at 4 °C while the activity of AD-32 was unaffected by temperature. ADR was more effective against HBTC in the log growth phase than the plateau phase while the reverse was found using AD-32. Verapamil was found to enhance the activity of both ADR and AD-32 against a HBTC cell line (T24), found to be resistant to both agents. The lipophilic properties of AD-32, along with its enhanced activity when used over prolonged periods of time and its activity against tumor cells in the plateau phase, suggest that AD-32 could be useful in the management of patients with superficial bladder cancer.

Introduction

Doxorubicin, administered systemically, has been shown to have limited activity in metastatic carcinoma of the bladder. The objective response rate in advanced disease is reported to be between 10%–25% (20, 27, 33, 35). From 70%–80% of individuals with bladder cancer, however, have their initial tumor confined to the mucosa or lamina propria; at least one-half of them will develop a true recurrence or new occurrence despite resection of their initial tumor. Anticancer agents have been instilled directly into the bladder in an attempt to inhibit further growth of the tumor, reduce the requirement for repeated resections, and decrease the chance of subsequent invasive bladder cancer. ADR has been shown to be active intravesically in the treatment of superficial bladder cancer with response rates of 50%–70% [4, 22]. The high molecular weight of ADR and its polar character result in minimal systemic absorption and little systemic toxicity [8].

N-Trifluoroacetyladriamycin-14-valerate (AD-32) is a semisynthetic analogue of doxorubicin (ADR), differing from its parent compound in having a five-carbon straight-chain ester function at the 14-carbinol position and a trifluoroacetyl substituent on the amino group of the glycoside [12]. AD-32 was found to be superior to ADR in the L1210 and P388 leukemia models, both in terms of increasing the lifespan and in percentage of animals achieving long-term survival [12, 26]. AD-32 was also found to have superior activity to ADR in an ADR-resistant P388 subline. AD-32 has been shown to be active in the Ridgway osteogenic sarcoma, Lewis lung carcinoma, and B16 melanocarcinoma solid tumor models [13, 26, 32], thereby demonstrating the effectiveness of drug when administered at sites distant from the tumor mass. Certain mechanistic properties of this drug are markedly different from those of ADR. AD-32, for example, does not bind to double helical DNA [28] and shows drug-associated fluorescence in the cytoplasm, as opposed to the nuclear fluorescence seen with ADR [15]. Other properties, such as its ability to inhibit DNA and RNA synthesis [16] and to produce DNA lesions [17], are similar to those of ADR.

In early clinical trials, i.v. infusion of AD-32 showed activity against bladder cancer [7]. The high antitumor activity of this compound, and its lipophilic nature, which allows for easy tumor mass penetration, suggest that AD-32 may be of value as an intravesicular agent. In order to evaluate the potential usefulness of AD-32 in bladder cancer, we have compared its activity with ADR in vitro using a panel of human bladder tumor cell lines (HBTC).

Material and methods

HBTC. The following HBTC were used for the in vitro studies: CUB-2 [6], CUB-3 [6], RT-4 [29], ScABER [23], J82 [24], T24 [2], and 488P [5]. All cell lines were obtained from the laboratory of Dr. Jorgen Fogh (Sloan-Kettering Institute for Cancer Research, Rye, NY). These cell lines have been characterized by ultrastructural morphology, karyo-
Monolayer culture of HBTCL. All cell lines were maintained in monolayer culture in RPMI 1640 (GIBCO, Grand Island, NY) with 10% fetal calf serum (Flow Laboratories, Newbury Park, Calif) in 100-mm plastic petri dishes incubated at 37 °C in 6% CO₂ and 100% humidified atmosphere.

Tumor cells were harvested from monolayer culture by incubation for 10 min at 37 °C in 0.25% trypsin in Hank’s balanced salt solution (HBSS). The resulting cell suspensions were counted in a hemocytometer; viability, as determined by trypan blue exclusion, was usually greater than 90%. Tumor cell suspensions produced in this manner were either drug tested immediately or subcultured at a 1/10 dilution to monolayer culture for subsequent study.

Soft agar assay. After trypsinization, tumor cells were plated into the upper layer of a two-layer agar culture system. The underlayer consisted of McCoy’s medium (Gibco, Grand Island, NY) with 15% fetal calf serum (FCS) and 0.5% agar. The overlayer consisted of CMRL 1066 medium (Gibco, Grand Island, NY) with 15% horse serum, 2 μg/ml insulin, 5 μg/ml transferrin, and 0.3% agar along with the tumor cells. Cultures were incubated at 37 °C in 6% CO₂ and 100% humidified atmosphere. Plates were examined with an Olympus CK inverted microscope. Final colony counts were made from 10–14 days after plating. Aggregates of 50 or more cells were scored as colonies.

CFU-GM assay. Bone marrow samples were aspirated into preservative-free heparinized syringes from patients with solid tumors and normal marrows. Marrow cells were passed through a 22 gauge needle, diluted 1:2 in HBSS and layered onto a Ficoll-Hypaque gradient (Sigma Diagnostics, Histopaque-1077). Mononuclear cells were recovered from the interface, washed three times in HBSS and suspended in Iscove’s Modified Dulbecco’s medium (Gibco, Grand Islands, NY) containing 20% FCS. Quadruplicate cultures of 2 × 10⁵ cells were plated into the upper layer of a two-layer agar system. The underlayer consisted of 1 ml of Iscove’s medium supplemented with 30% FCS, 0.5% agar, and 20% human placental conditioned medium as a source of colony stimulating factor [3]. The upperlayer contained bone marrow cells in 1 ml Iscove’s medium supplemented with 30% FCS, 0.3% agar, 100 Units penicillin, 200 μg streptomycin, and 2 mM of glutamine. Culture plates were incubated in a 6% CO₂ humidified incubator at 37 °C CFU-GM colonies (> 40 cells) were scored after 7–8 days of culture.

Drug preparation. The chemotherapeutic drugs used in these experiments were ADR (doxorubicin hydrochloride), obtained from Adria Laboratories, Dublin, Ohio, and AD-32 (N-trifluoroacetyladriamycin-14-valerate), obtained from the Division of Cancer Treatment, NCI. ADR was diluted to the desired concentration in 0.9% NaCl. AD-32 was put into solution with 1% DMSO and 15% FCS with plating medium. The drug was then further diluted to the desired concentration in 0.9% NaCl. Both drugs were aliquoted in 1.5-ml plastic Falcon centrifuge tubes and stored at −80 °C. Prior to use, drugs were thawed in a 37 °C water bath. Equimolar concentrations of drugs were used for all experiments, ranging from 0.01–20 μM. Verapamil hydrochloride (Searle Pharmaceuticals) was prepared in the appropriate diluent just prior to each experiment and solutions of 1, 6 and 20 μM were prepared for use in culture.

In vitro drug testing. For most experiments tumor cells were incubated in the presence of drug, along with a control tube, for 1 h in a shaking water bath at 37 °C. Cells were then centrifuged at 150 g for 10 min, washed twice in RPMI 1640 without horse serum and plated in culture. Control and treated cells were plated with four plates at each drug level and scored at 10–14 days. Continuous drug exposure was performed by plating the tumor cells with drug in agar without washing the drug off prior to incubation.

In separate experiments, 1-h drug incubations were carried out in a 37 °C water bath and simultaneously at 4 °C in a refrigerator. To insure equivalent drug mixing during drug incubation, the tubes were shaken by hand at 10-min intervals.

Log versus plateau phase drug studies. Tumor cells were harvested in the log growth phase (2–3 days) and during the plateau growth phase (6–7 days) and exposed to each drug for 1 h prior to plating. The proliferative state of cells in both the log and plateau phase of growth were evaluated by DNA labeling with tritiated [methyl-³H] thymidine (2 Ci/mm sp. act., Research Products International, Mt. Prospect, III). HBTCL in monolayer culture were trypsinized, counted using trypan blue exclusion as a marker for viability and cultured in quadruplicate in microtiter wells (Linbro Chemical Co., Hamden, Conn) at 1 × 10⁵ cells/well. Culture medium consisted of RPMI 1640 supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, 2.5 mM Hepes buffer, and 10% newborn calf serum. Immediately after placing the cells in the microtiter wells, they were pulsed with 1 μCi of thymidine. Cells were harvested onto glass filter paper at various time intervals after pulsing using a 24-well semi-automated harvester (Microbiological Associates, Walkersville, Md). The paper disks were counted in Packard liquid scintillation counter.

Results

In order to determine the relative toxicity of ADR and AD-32 on hematopoietic stem cells, CFU-GM cultures were set up using bone marrow samples from 10 solid tumor patients with normal bone marrows. Figure 1 represents a colony survival curve formed by using the mean level of colony survival at each drug concentration from the ten CFU-GM assays. The mean LD₅₀ of all ten CFU-GM assays for ADR was 1.6 ± 1.4 μM, while the LD₅₀ of AD-32 was 3.9 ± 4.9 μM. The difference between LD₅₀ levels was not statistically significant (P<0.17). The LD₇₀ for each drug was 3.6 ± 4.5 μM for ADR and 4.9 ± 4.6 μM for AD-32 (P<0.55).

We next compared the activity of the two drugs against a panel of HBTCL in a clonogenic assay using a drug exposure time of 1 h. Simultaneous assays were performed using continuous drug exposure to determine the effect of increasing the time of drug exposure on the clonal growth