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Pharmacologic rationale for intravesical *N*-Trifluoroacetyladriamycin-14-valerate (AD 32): a preclinical study

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Summary. Based on previous clinical findings following systemic administration, as well as appropriate laboratory evidence, the novel lipophilic anthracycline analogue *N*-Trifluoroacetyladriamycin-14-valerate (AD 32) has been identified as an agent of potential value in the intravesical therapy of superficial bladder carcinoma. Toward this end, using a rat model, the present study was designed to evaluate the potential for toxicity of a therapeutic dose of AD 32 given intravesically. With regard to systemic toxicity, following a single intravesical dose of AD 32 (20 mg/kg), the total systemic drug exposure (0–6 h), expressed as the area under the plasma concentration-time curve, was 14.2 μg min ml^{-1}, or <1% of the corresponding value obtained when the identical dose was injected intravenously (2,392 μg min ml^{-1}). In separate studies, a single intravenous dose of AD 32 (20 mg/kg) given to normal animals produced only a 20% reduction in white blood cell counts as compared with a 60% reduction following the administration of a therapeutic dose of Adriamycin (5 mg/kg); no effect was seen for either drug on red blood cell production. Taken together, these results imply that systemic drug exposure following the intravesical instillation of a therapeutic dose of AD 32 would result in negligible (~ 0.2%) hematotoxic potential. Furthermore, intravesical instillation of AD 32 (20 mg/kg) at a concentration (10 mg/ml) greater than that projected for use in humans resulted in no evidence of contact toxicity to the rat bladder urothelium. Thus, based on experimental and clinical considerations of safety and efficacy, AD 32 appears to be an excellent candidate for the intravesical treatment of superficial bladder cancer.

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Introduction

On initial diagnosis, the majority of patients with bladder cancer have superficial carcinoma, with neoplastic cells being confined to the mucosa and adjacent lamina propria. Although such local disease may initially be controlled by resection, the high likelihood of tumor recurrence is well recognized. Thus, in recent reviews Soloway [16] and Herr et al. [7] noted recurrence rates of 30%–40% and 70%–90% for single and multiple papillary tumors, respectively, and of 80% for carcinoma in situ (CIS), a flat, high-grade epithelial malignancy. Although most recurrences take place at the same initial stage and grade, a significant proportion of high-grade tumors and CIS show tumor progression to invasive disease that is generally unresponsive to systemic chemotherapy. Clinical studies have shown that intravesical chemotherapy given prophylactically or as an adjunct to transurethral tumor resection may be effective in reducing both the frequency of tumor re-emergence and progression of disease. By virtue of its structure and function, the urinary bladder is well suited to intravesical therapy. This treatment modality offers several potential advantages over systemic drug therapy in that it enables a high concentration of chemotherapeutic agent to come into intimate contact with the urothelial tumor site while generally producing minimal systemic drug toxicity, since the urothelium normally prevents extensive absorption of drugs and other substances with masses that are greater than about 270 Da. Thus, for example, significant tumor response rates have been noted for mitomycin (molecular weight, 334 Da) and Adriamycin (doxorubicin; molecular weight, 544 Da) [7, 19], with little evidence of circulating drug levels [2, 4, 11, 14].

Lack of tumor response to intravesical therapy may relate to drug insensitivity as well as to the inability of such relatively hydrophilic agents as mitomycin and Adriamycin to penetrate the urothelium adequately and reach more deeply seated neoplastic cells. A need exists for the development of new intravesical agents that combine broader activity against bladder tumors with an improved ability to penetrate urothelial tissue. Enhanced tumor pene-

tration may be accomplished with highly lipophilic drug substances that can pass into cells and tumor masses by simple diffusion. Accordingly, such agents should exhibit low systemic toxicity so as to minimize the potential effects of increased transurothelial passage. Toward this end, we have identified a novel, highly lipophilic anthracycline analogue, *N*-Trifluoroacetyladriamycin-14-valerate (AD 32), as a potential candidate for intravesical therapy based on the following observations: rapid and extensive cellular penetration as compared with Adriamycin [10]; marked superiority to Adriamycin in transplantable murine tumor systems [6, 8, 13, 20]; demonstrated in vitro activity against human bladder-tumor cell lines [12]; documented systemic activity against advanced bladder tumors in phase I/II clinical trials [1, 3; unpublished results]; and low systemic toxicity both in animal model systems [8, 13, 20] and in humans [1, 3], including the absence of clinical cardiotoxicity and contact toxicity. The present studies were undertaken to examine the plasma pharmacology of AD 32 in rats following intravesical drug instillation and to compare the ensuing plasma drug levels with those seen following an identical intravenous drug dose. To gauge the significance of drug levels arising from intravesical treatment, the hematotoxicity of a comparable intravenous dose of AD 32 was determined separately. In addition, the histologic effects of intravesical AD 32 on the rat urothelium were evaluated.

Materials and methods

Drugs and drug formulation

The AD 32 used in this study was derived from sterile formulated vials originally prepared under contract from the National Cancer Institute for use in systemic clinical trials; drug purity was ⩾97% as assayed by high-performance liquid chromatography against a pure laboratory reference standard. For in vivo use, AD 32 was dissolved in the appropriate volume of NCI Diluent 12 [Cremophor EL (polyethoxylated castor oil) : ethyl alcohol, 1 : 1 v/v; Pharmaceutical Resources Branch, Division of Cancer Treatment, National Cancer Institute, Bethesda, Md.] and diluted with 4 vol. 0.9% saline. Adriamycin and daunorubicin as hydrochloride salts were kindly provided as bulk material by Farmitalia Carlo Erba S.p.A. (Milan, Italy); the Adriamycin was reconstituted in 0.9% saline for use in animal studies. Gentamicin sulfate was obtained from Fermenta Animal Health Co. (Kansas City, Mo.).

Pharmacology studies

Intravesical drug administration. AD 32 (10, 20, or 30 mg/kg in 0.2, 0.4, or 0.6 ml vehicle, respectively) or Adriamycin (7 mg/kg in 0.2 ml saline) was instilled into the previously emptied urinary bladders of pentobarbital-anesthetized female Harlan Sprague-Dawley rats (200–250 g) via a catheter (PE20) inserted through the urethra. Drug was held in the bladder for 2 h, a period often employed in human therapy, after which time the contents as well as 4 × 0.3 ml saline used subsequently to rinse the bladder were recovered and frozen for later analysis. Thereafter, the bladders were rinsed via the catheters with 0.3 ml saline at 30-min intervals for the duration of the study. Blood samples (50–100 µl) were obtained from an indwelling carotid-artery cannula at various times during the drug dwell time (15, 30, 45, 60, 75, 90, 105, and 120 min) and following drug removal and washout (15, 30, 60, 90, 120, 150, 180, 210, and 240 min). For animals receiving Adriamycin, the number of sample times was reduced (30, 60, 90, 120, 150, 210, 270, and 360 min) and the volume of plasma was increased (250 µl) to facilitate drug detection and quantitation. Plasma samples were kept frozen (–70° C) pending analysis.

Intravenous drug administration. Pentobarbital-anesthetized female Sprague-Dawley rats that had been fitted surgically with femoral-vein catheters for drug administration and with carotid-artery cannulae for blood collection received a single bolus dose of AD 32 (20 mg/kg) or Adriamycin (7 mg/kg). The drug doses used were based on the therapeutically active single doses of 40 and 14 mg/kg for AD 32 and Adriamycin, respectively, in $B6D2F_1$ tumor-bearing mice [1, 8, 13]. Blood samples were obtained at various intervals through 6 h. As before, plasma samples (20–200 µl) were kept frozen (–70° C) pending analysis.

Sample processing. Plasma samples from animals receiving 30 mg/kg AD 32 intravesically or 20 mg/kg intravenously, augmented with internal standard (*N*-Trifluoroacetyladriamycin-14-octanoate), were extracted from TRIS buffer (pH 8.5) using ethyl acetate : 1-propanol (9 : 1, v/v). For each of the remaining treatment groups (10 or 20 mg/kg AD 32 given intravesically and 7 mg/kg Adriamycin given intravenously or intravesically), plasma samples were pooled for each time point ($n = 3$) and processed as above, except that in animals receiving Adriamycin, daunorubicin was used as an internal standard and samples were processed using C_{18} Sep-Paks (Waters Associates, Milford, Mass.) according to a previously published procedure [5]. Extracted samples were dried under a stream of nitrogen and reconstituted in methanol (100 µl) for analysis. The analytical method involved reversed-phase high-performance liquid chromatography using flow fluorescence detection essentially as described elsewhere [9]. Samples were quantified by reference to standard curves for pure authentic materials added to blank rat plasma and processed in a similar manner.

Toxicity studies

Hematotoxicity. Groups of five female Sprague-Dawley rats were anesthetized momentarily with methoxyflurane and AD 32 (20, 30, or 45 mg/kg) was given as an intravenous bolus via the tail vein. Two further groups ($n = 5$) received either Adriamycin (5 mg/kg; positive control) or 20% NCI Diluent 12 : 80% saline (0.3 ml/100 g body weight; vehicle control). The Adriamycin dose used represents 50% of the LD_{20} for this system and is unassociated with lethality or morbidity. Blood samples (20–30 µl) were obtained by retrobulbar bleeding of the animals under momentary anesthesia both prior to drug administration and at various times throughout the subsequent 21-day period. Samples were immediately diluted with isotonic buffered saline, and red and white blood cell counts were determined using a Model ZM Coulter counter (Coulter Counter Electronics Ltd., Luton Beds, UK).

Urothelial contact toxicity. A total of 35 female Sprague-Dawley rats weighing 230–260 g were used in 2 studies to evaluate the short- and intermediate-term effects of drug and vehicle on the urothelium. For investigation of the intermediate-term effects (28 days, study 1), five test groups were established at five animals per group as follows: group 1, control; group 2, 1 ml/kg saline; group 3, 1 ml/kg, 20% NCI Diluent 12 : 80% saline; group 4, 2 ml/kg 20% NCI Diluent 12 : 80% saline; group 5, 20 mg/kg (10 mg/ml) AD 32 in 20% NCI Diluent 12 : 80% saline. For evaluation of the short-term effects (3 days, study 2), two test groups at five animals per group were established as follows: group 1, 2 ml/kg 20% NCI Diluent 12 : 80% saline; group 2, 20 mg/kg (2 ml/kg) AD 32 in 20% NCI Diluent 12 : 80% saline.

All animals except the 28-day nonintervention controls were anesthetized with pentobarbital sodium (45 mg/kg i.p.) and maintained on isothermal heating pads for the duration of the instillation procedure. Urinary bladders were catheterized aseptically via the urethra using sterile catheters (PE 20) through which the appropriate drug and/or vehicle was introduced. Instillate was again maintained in the bladder for 2 h after which time the bladder contents were recovered and the bladders were irrigated with saline. Animals were returned to their cages to recover consciousness and were thereafter maintained in the rat colony

with acess to food and water ad libitum. To reduce the chance of non-specific bladder infection associated with the catheterization procedure, each animal received one dose of gentamicin sulfate (4.5 mg/kg i.p.) at 1 day prior to instillation and another immediately following drug removal and bladder irrigation; control animals also received gentamicin but otherwise remained without manipulation. At 3 or 28 days, the animals were euthanized and the urinary bladders were removed, examined grossly, and inflated with 70% alcoholic formalin. Following fixation for 24 h, the bladders were longitudinally bisected and the mucosal surfaces were examined. Paraffin embedding, sectioning (thickness 5–6 μm), and hematoxylin-eosin staining were performed according to standard practice. Animal sacrifice and pathologic gross and microscopic examination for evidence of inflammatory response or overt cellular damage were conducted without knowledge of the specific group assignment.

Results

Figure 1 shows the decay of parent drug and metabolites in rat plasma following the intravenous administration of 20 mg/kg AD 32. As previously described [17], levels of parent drug showed a biphasic decline, with concentrations falling from 20 μg/ml at 2.5 min to barely detectable levels by 4 h (limit of quantitation, 2 ng/ml). By contrast, plasma levels of the principal circulating AD 32 metabolite, *N*-Trifluoroacetyladriamycin (AD 41), which were detectable from the earliest time point (14 μg/ml at 2.5 min), declined more slowly than the parental agent and persisted throughout the study period. Lower levels of *N*-Trifluoroacetyladriamycinol (AD 92; 4 μg/ml) and barely detectable levels of aglycones and Adriamycin were evident at the early intervals but were not seen at later times. Total anthracycline fluorescence in plasma declined from 40 μg/ml at 2.5 min to 1.6 μg/ml at 6 h. Mean half-lives for the distribution and elimination phases were estimated to be 9 and 99 min, respectively. Using the trapezoidal rule, the total area under the concentration-time curve ($AUC_{0-6\,h}$) was calculated to be 2,392 ± 831 μg min ml^{-1}. Following an intravenous bolus dose of Adriamycin (7 mg/kg), parental drug was the only detectable anthracycline material in the limited plasma volumes obtained (data not shown). The drug showed a biphasic decline, with mean distribution and elimination half-lives being estimated at 4 and 89 min, respectively. The total $AUC_{0-6\,h}$ value was 178 ± 22 μg min ml^{-1}.

Plasma anthracycline levels measured following the intravesical administration of AD 32 at several different doses are shown in Fig. 2. Only two fluorescent species, principally AD 41 and trace levels of parental drug, were seen. Total anthracycline levels were essentially constant throughout the instillation period at each dose level (10–30 mg/kg). These concentrations persisted through 6 h despite removal of the drug dose at 2 h and subsequent irrigation of the bladder. Although the plasma levels resulting from 10 or 20 mg/kg AD 32 were similar, animals receiving 30 mg/kg showed slightly elevated concentrations and greater standard deviations. The total $AUC_{0-6\,h}$ value for the 20-mg/kg dose was 14.2 μg min ml^{-1}. Following the intravesical instillation of Adriamycin (7 mg/kg), the drug was undetectable in plasma until 60 min, at which time low levels (~15 ng/ml) became evident. Similar low levels of Adriamycin were seen at 90 and 120 min after drug dosing.

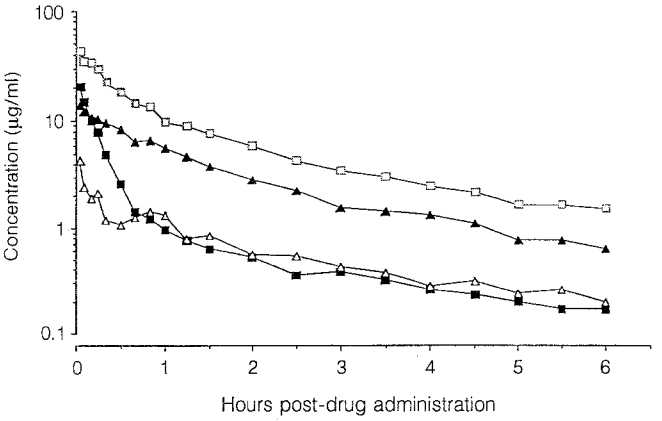

Fig. 1. Appearance and disappearance of parent drug and metabolites in the plasma of female Sprague-Dawley rats following the intravenous bolus administration of 20 mg/kg AD 32. ■, AD 32; ▲, AD 41; △, AD 92; □, total anthracycline fluorescence ($n = 5$)

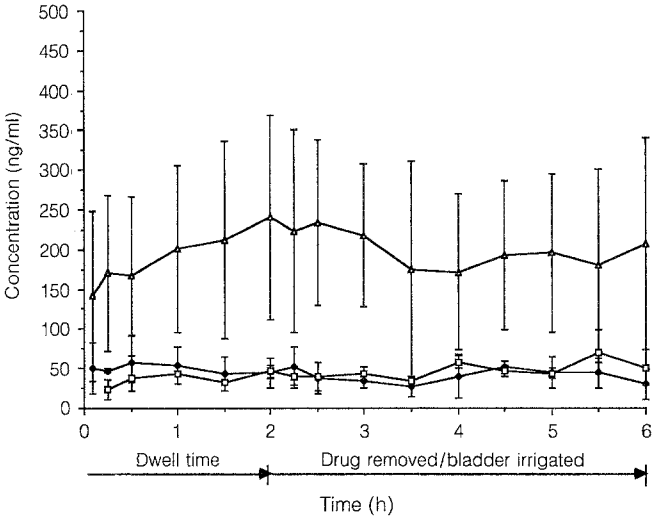

Fig. 2. Total anthracycline fluorescence in rat plasma following the intravesical administration of AD 32 at doses of 10 (□, $n = 9$), 20 (◆, $n = 9$), and 30 mg/kg (△, $n = 11$)

No anthracycline was detectable in any plasma samples obtained after drug removal from the bladder. The total $AUC_{0-6\,h}$ value was 1.7 μg ml min^{-1} (data not shown).

Analysis of the bladder contents at the end of the 2-h drug dwell time revealed a high recovery (>91%) of the AD 32 dose, with >95% of the drug fluorescence being attributable to unchanged drug and the balance AD 41. Overall, 97% of the Adriamycin dose was recovered from the bladder at 2 h; no other fluorescent species was evident.

The hematotoxicity of a single intravenous dose of AD 32 (20, 30, or 45 mg/kg) as compared with Adriamycin (5 mg/kg) is shown in Fig. 3. Intravenous administration of Adriamycin to rats resulted in a marked reduction in white blood cell counts, which reached a nadir of 40% of initial levels by day 10 and had not fully recovered by day 21. In contrast, the decline in white blood cell counts following AD 32 was only 20% for the 20-mg/kg dose. Moreover, a >2-fold escalation of the dose (to 45 mg/kg) produced only a 40% reduction in the number of white blood cells. None of the treatments had any effect on red

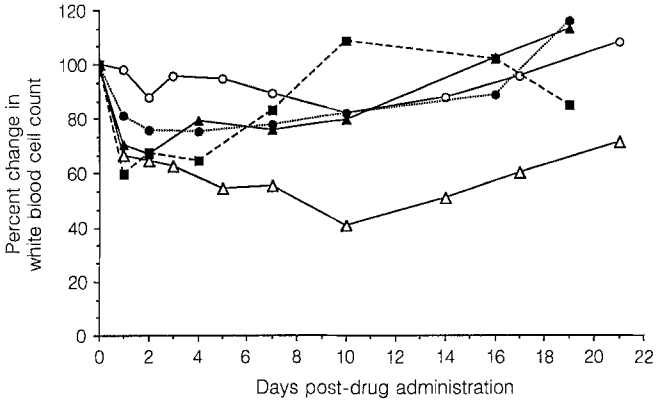

Fig. 3. Change in white blood cell counts following the administration of a single intravenous dose of AD 32 at 20 (●), 30 (▲) and 45 mg/kg (■) and of Adriamycin at 5 mg/kg (△) to healthy female Sprague-Dawley rats. Vehicle control animals (○) received 20% NCI Diluent 12: 80% saline at 0.3 ml per 100 g body weight

blood cell counts. Furthermore, Adriamycin treatment was associated with a 10% weight loss by day 3, whereas AD 32-treated animals showed no significant changes in weight relative to untreated controls throughout the experiment.

Studies to determine the contact effects of AD 32 on the rat bladder urothelium included an evaluation of the surfactant-containing vehicle alone and of the drug in its final formulation. Following animal sacrifice and bladder removal on days 3 or 28, no abnormalities were seen in any of the test groups on gross examination, except for the presence of a large bladder stone (approximately 1 cm in diameter) in one of the 28-day control animals. Moreover, no significant treatment-related histopathology was seen in any of the bladders on microscopic examination, except for mild subacute cystitis in one of the 28-day AD 32-treated animals, presumably due to trauma associated with catheterization.

Discussion

Intravesical use of the low-molecular-weight lipophilic alkylating agent thiotepa is associated with significant transurothelial drug absorption and resultant suppression of bone marrow activity. Based on this model, conventional wisdom has held that agents selected for intravesicular use should have limited potential for resorption from the bladder. Unfortunately, this property correlates with minimal drug penetration of the urothelium and an inability of the drug to reach more deeply seated superficial bladder tumors. In contrast, we believe that potentially useful intravesical agents should be highly lipophilic in nature and, thus, be capable of easily penetrating tumor masses. However, such agents should also exhibit a wide therapeutic safety margin such that measureable systemic drug levels that might result from transurethelial absorption would not constitute a toxic burden to the host. Accordingly, the present study provides a strong pharmacologic rationale for the intravesical use of the novel lipophilic anthracycline analogue AD 32 in the treatment of superficial bladder carcinoma.

Studies using AD 32 in laboratory animals and humans have clearly demonstrated significant antitumor activity for this agent, including specific activity against bladder carcinoma [1, 3, 6, 8, 13, 20]. In other studies, AD 32 has been shown to be active against a range of human bladder-tumor cell lines [12]. In this regard, AD 32 showed activity in cells in plateau-phase growth as well as in more rapidly dividing cultures, whereas Adriamycin showed activity against only proliferating cells [12]. Furthermore, AD 32 activity was found to increase with increased cellular drug exposure ($c \times t$), a pharmacologic parameter that can most readily be achieved in intravesical therapy by virtue of the direct exposure of tumor to a high drug concentration. These factors suggested to us that AD 32 may be of value in intravesical therapy, with additional advantage being taken of the drug's highly lipophilic character for improved tumor-mass penetration. However, as with thiotepa, this high lipophilicity could potentially result in significant transurothelial absorption, with the expression of systemic toxicity. The present study was thus designed to assess the potential for systemic toxicity in a rat model following the intravesical instillation of AD 32 under conditions similar to those used clinically for intravesical drug therapy. The doses of AD 32 used in this study reflect the rat equivalent of the daily AD 32 doses found to be optimally active in various rodent tumor models. Parallel studies conducted using Adriamycin confirm that negligible levels of this drug are absorbed systemically following intravesical instillation.

The present study demonstrates that detectable levels of AD 32 and its principal metabolite AD 41 are seen in plasma after intravesical drug instillation. However, over the period of 0–6 h post-administration, these plateau drug concentrations result in a total systemic drug exposure as determined by AUC that is significantly lower (~0.6%) than that produced by the intravenous administration of the same drug dose. Since plasma anthracycline levels at 6 h were approximately equal when the same dose was given intravenously or intravesically, these levels can be expected to decay in parallel through later times. Thus, the difference in tissue exposure as defined by the $AUC_{0-6\,h}$ value for intravenous vs intravesical drug administration clearly represents the pharmacologic advantage for intravesical AD 32.

Based on the noted differences in drug processing, the level of systemic drug exposure following intravesical administration of AD 32 is apparently lower than that seen in animals receiving intravesical Adriamycin (1%). However, the lower recovery of AD 32 from the bladder at 2 h (91%), relative to that of Adriamycin (97%), indicates a somewhat higher systemic absorption of drug in the AD 32-treated animals. Although drug levels were detectable in AD 32-treated animals following the removal of drug from the bladder lumen, their persistence in plasma is likely due to continued absorption from within the bladder tissue, together with redistribution from other organ sites. For all of these studies, animals were functioning with intact ureters so as to minimize unnecessary surgery. Previous studies using other agents in rats have shown that the urinary

bladder can exhibit marked changes in permeability and drug absorption following surgical manipulations [15]. The possible increase in bladder volume resulting from continuing ureteral urinary flow did not appear to produce any significant changes in permeability in the groups that received 10 or 20 mg/kg AD 32. However, data for the 30-mg/kg treatment group indicate increased absorption for some of the animals, resulting in a larger standard deviation for this group. This dose of AD 32 required an instillate volume of 0.6 ml, which, combined with the presumed increase in intraluminal pressure, may have exceeded normal physiological parameters and produced some loss of urothelial integrity. However, no such problem is anticipated in the clinical use of AD 32 since the human bladder can hold a much larger volume. In view of the clinical experience with intravesical Adriamycin, the systemic drug levels seen in this rat model for the less toxic AD 32 relative to those of Adriamycin suggest that no significant pharmacologic problems would be anticipated from the intravesical use of AD 32. Companion studies have recently established that the NCI Diluent 12 formulation has no significant effect on transurothelial drug absorption [18].

The present hematotoxicity studies using AD 32 further confirm the high therapeutic margin of safety for this agent relative to Adriamycin that has been seen in phase I/II clinical trials and in previous animal studies [3, 8, 13]. Thus, a single therapeutically active systemic dose of AD 32 (20 mg/kg) produced myelosuppression of only one-third the level induced by a comparable single dose of Adriamycin (5 mg/kg). Extrapolation of the current rat data implies that intravesical administration of AD 32, which produces <1% of the circulating plasma drug levels found following intravenous administration of the same dose would thereby produce only 1% of the corresponding hematotoxicity seen following intravenous dosing. Thus, one would expect a neglible (~ 0.2%) decline in white blood cell count when a full therapeutic dose of drug is given by intravesical instillation. Since AD 32 at its full systemic dosage is unassociated with clinical cardiac toxicity, systemic levels following intravesical administration are of no concern in this regard.

Although a major advantage of intravesical therapy is that it enables superficial tumor to come into contact with drug at concentrations greater than those achievable by systemic administration, currently used agents, including Adriamycin, are compromised by drug-associated local irritative symptoms and chemical cystitis, which serve to limit the utility of this therapeutic modality [19]. For AD 32 this was considered to be unlikely, for among its other advantages, this agent does not produce contact toxicity, as evidenced, for example, by the absence of peritoneal histopathology in animals receiving the drug by the intraperitoneal route [13] and by the lack of local tissue reaction in patients subjected to accidental drug extravasation during intravenous treatment [1, 3]. The present studies further confirm the absence of local tissue toxicity in the bladder when a single dose of drug is instilled intravesically, even at a concentration greater than that expected to be achieved in patients (500–600 mg/75 ml).

The ability of the lipophilic AD 32 compound to penetrate into and through the urothelium is indicated both by the appearance of low circulating anthracycline levels and by a reduction in the recovered amount of instilled drug following intravesical drug administration. Therefore, based on the penetrant qualities of this agent, the low systemic toxicity of both intravenously and intravesically delivered drug, its lack of urothelial contact toxicity, and the documented profile of in vitro and in vivo activity for this agent against bladder tumors, we suggest that AD 32 is a worthy candidate for clinical evaluation as an intravesical treatment for superficial bladder carcinoma.

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