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INCREASED ANTI-TUMOR EFFECT OF ADRIAMYCIN-LOADED ALBUMIN MICROSPHERES IS ASSOCIATED WITH ANAEROBIC BIOREDUCTION OF DRUG IN TUMOR TISSUE

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Abstract—Anti-tumor activity and fate of adriamycin incorporated into biodegradable albumin microspheres was examined *in vivo* after direct intratumoral injection. Adriamycin in microspherical form displayed superior anti-tumor activity to a comparable dose of drug in solution. This was associated at later time points (40 hr, 50 hr and 72 hr after injection) with higher median parent drug concentrations in tumor tissue (4.1, 3.6, 2.6 μg/g respectively for microspheres and 1.6, 1.7 and 1.0 μg/g for solution) and the consistent detection of 7-deoxyaglycone metabolites, end products of reduction of adriamycin under anaerobic conditions (1.1, 1.0, 1.0 μg/g respectively for microspheres and <0.1 μg/g at all time points for solution). It is generally considered that the redox properties of anthracyclines are responsible for their toxicity to normal tissues whereas other mechanisms are responsible for antineoplastic activity. In this study we show that inducing metabolism of Adriamycin via reductive pathways is associated with increased anti-tumor effect.

Microspheres are solid, internally homogeneous particles containing drug within the matrix [1]. By virtue of their size they become trapped in capillaries and produce high local concentrations of therapeutic agents in selected organs following systemic or regional administration. We have recently succeeded in incorporating adriamycin (ADR) into biodegradable albumin microspheres prepared by glutaraldehyde crosslinking, characterized them with regard to particle size and drug content [2, 3] and demonstrated incorporated ADR in its original chemical form [4]. After i.v. administration in rats they became trapped in the lung and altered drug serum pharmacokinetics, avoiding the high peak levels normally associated with i.v. administration of ADR [3]. *In vitro* studies have shown that ADR release can be prolonged by incorporation into microspheres [5]. Consequently, in this study we have examined both the anti-tumor effect and fate of comparable amounts of ADR in solution and in microspherical form after direct intratumoral injection.

MATERIALS AND METHODS

Rats. In all experiments male Wistar-derived Nottingham (WAB/NOT) rats were used, bred in Glasgow from stock animals kindly provided by Professor Baldwin of the Cancer Research Laboratories, Nottingham in 1979.

Tumor. The tumor, designed Sp107, used in this study is a transplantable, non-immunogenic mammary carcinoma that originally arose spontaneously in a female rat of the WAB/NOT strain. Following SC injection of syngeneic rats with 2×10^4 viable Sp107 cells prepared by trypsin digestion of solid tumor fragments, animals were left until tumors became palpable. At this point they were randomized into experimental groups and the various treatments administered intratumorally (i.t.) in a volume of 0.5 ml of phosphate buffered saline plus 0.5% Tween 80 (PBS/Tween). For the growth delay experiment two tumor diameters at right angles were measured twice weekly and a weight in grams calculated from the formula $\frac{1}{2} \times$ lowest diameter $(\text{cm})^2 \times$ highest diameter (cm). Animals were killed when mean tumor diameter exceeded 4 cm. To obtain concentration–time profiles of ADR and metabolites in tumor tissue rats were sacrificed at intervals, tumors excised and weighed, then the content of ADR and metabolites determined. Blood samples were also taken for analysis.

Microsphere preparation. Microspheres containing ADR were prepared by stabilization, through crosslinking of albumin by glutaraldehyde, of water in oil emulsion droplets containing the protein and drug. Following suspension in PBS/Tween, samples were taken to obtain particle size, weight per ml of microspheres and amount of ADR per mg of microspheres. Techniques are described in detail elsewhere [2, 3, 5]. Microspheres of 14 and 17 μm diameter (50% weight average) were used in these experiments and contained approximately 1% by weight of ADR.

Quantitation of ADR and Metabolites. Serum samples and samples from microspheres solubilized by trypsin digestion were extracted with chloroform/isopropanol (2/1). The organic layer was evaporated to dryness, samples reconstituted in methanol and analysed using reversed phase HPLC with fluorescence detection (excitation 480 nm, emission 560 nm). Tissue samples were homogenized and treated with silver nitrate to release intercalated ADR prior to extraction with chloroform/iso-

propanol [6, 7]. In an attempt to assess extraction efficiency of ADR from tumor tissue the recovery of known amounts of drug was determined after *ex vivo* injection, either in microspherical form (N = 10) or in solution (N = 10), into 1–2 g pieces of freshly excised tumor tissue. In both cases recovery was approximately 60% and no significant difference between groups was found (unpublished observation).

RESULTS

Anti-tumor effect of ADR-loaded microspheres

The potential of ADR-loaded microspheres for loco-regional cancer chemotherapy was assessed by direct injection into SC growths of Sp107. Controls were empty microspheres and a comparable dose of ADR in solution administered in the same way. Figure 1 shows that ADR in solution exhibited significant anti-tumor activity, although drug in microspherical form was even more effective in inhibition of tumor growth. Empty microspheres were completely without effect in this system. Intratumoral injection of ADR along with empty microspheres was no more effective than the equivalent amount of drug alone (not shown in Fig. 1).

Fate of ADR in microspherical form following i.t. injection

Groups of animals were killed at intervals after i.t. injection of ADR either in microspherical form or in solution, and parent drug and metabolites in tumor tissue and serum separated, identified and quantitated as described in Materials and Methods. Figure 2 shows the concentration–time profile of ADR after i.t. administration in the two different

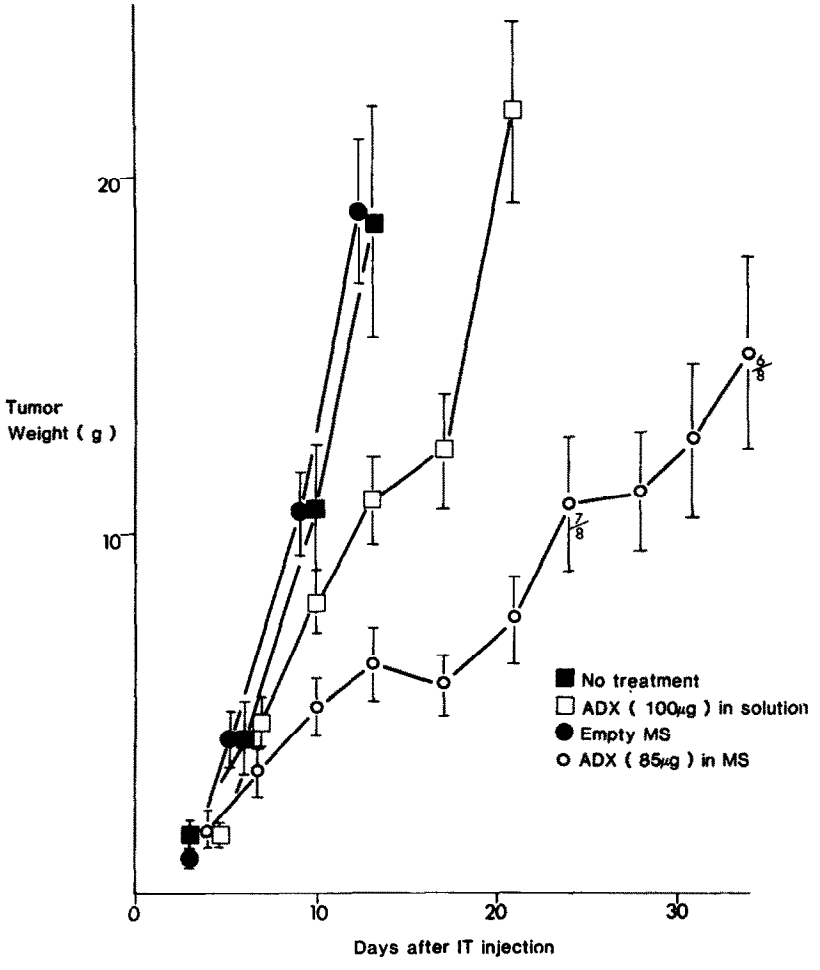

Fig. 1. Anti-tumor effect of ADR-loaded microspheres. Fifteen days after s.c. injection of 2×10^4 viable Sp107 cells tumor-bearing rats were randomized into 4 groups (6–8 rats per group) and injected i.t. in a volume of 0.5 ml with: ADR in solution; ADR in microspherical form (14 μm dia. prepared with 1% glutaraldehyde); empty microspheres or left untreated (see figure for key). At intervals two tumor diameters at right angles were measured and a weight in g calculated from the formula $\frac{1}{2} \times$ lowest dia. $(cm)^2 \times$ highest dia. (cm). Error bars represent mean ± ISE. Animals killed when mean tumor diameter >4 cm. Numbers on curves are animals remaining/animals in group. Mean tumor weight after administration of ADR in solution significantly different to that of untreated group on day 13. Mean tumor weights after administration of ADR in microspherical form significantly different to those of group treated with ADR in solution on days 13, 17 and 21 by two-tailed Student *t*-test ($P < 0.05$).

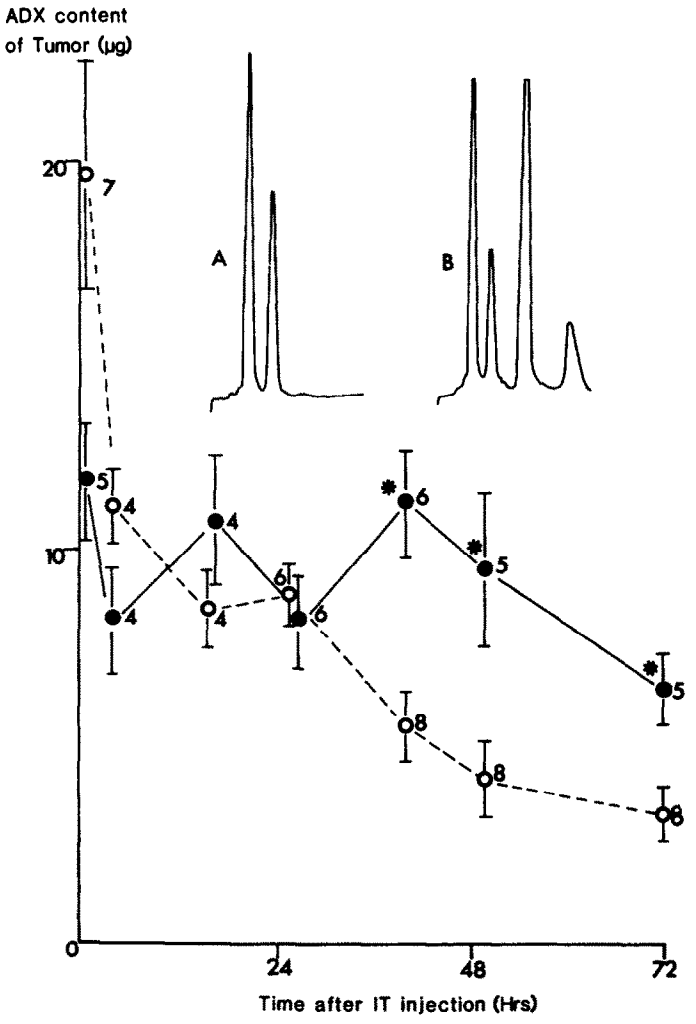

Fig. 2. Concentration–time profile of ADR in tumor tissue. ADR in solution or in microspherical form (17 μm dia. prepared using 1% glutaraldehyde) was injected i.t. in a volume of 0.5 ml into SC growths of Sp107 induced as in Fig. 1. At intervals rats were sacrificed, tumors excised and weighed, then the content of ADR and metabolites determined. Tumor weight varied from 1 g (lowest value at time 0) to 5 g (highest value at 72 hr). Doubling time of untreated tumors was 3.5 days. ●—● ADR content of tumors following administration in microspherical form (75 μg); O---O ADR content of tumors following administration in solution (80 μg). Each point is mean ± ISE and number of animals examined is adjacent to the symbol. Asterisk denotes significant difference by two-tailed Student *t*-test ($P < 0.05$) between ADR content of tumors sampled at the same time point. Inset A: HPLC chromatogram of fluorescent species extracted from tumor tissue 50 hr after injection of ADR in solution. From left to right peaks are ADR and daunorubicin (internal standard). Inset B: HPLC chromatogram of fluorescent species extracted from tumor tissue 50-hr after injection of ADR in microspherical form. From left to right peaks are ADR, daunorubicin (internal standard), AOL-DONE and ADR-DONE. Chromatographic conditions and detector attenuation were identical for both chromatograms.

forms, tumor content of parent drug being expressed as μg per whole tumor. Levels of ADR in tumors were significantly higher between 40 and 72 hr after administration in microspherical form. Differences between treatments at other points on the concentration–time profiles were not significant at the 5% level. Low but detectable concentrations of ADR were detected in serum for up to 4 hr indicating substantial early clearance. From 4 to 72 hr, neither parent drug nor its metabolites were detected in serum. Two metabolites, ADR 7-deoxyaglycone (ADR-DONE) and adriamycinol 7-deoxyaglycone (AOL-DONE) were identified in tumor tissue by HPLC using known standards and were identical to the two 7-deoxyaglycones previously isolated from mouse liver and heart and characterized by TLC and mass spectrometry [6].

Table 1 contains tumor AOL-DONE levels (μg per whole tumor). Whole tumor contents of ADR-DONE were generally lower and at times undetectable; moreover, no discernible pattern was apparent over the time span studied. Because of the discontinuous distribution of metabolite levels following administration of ADR in solution indi-

Table 1. AOL-DONE levels in tumor tissue following i.t. injection of ADR either in microspherical form or in solution

Time after i.t. injection:	10 min	4 hr	16 hr	26 hr	40 hr	50 hr	72 hr
AOL-DONE levels per tumor after i.t. injection of ADR in microspherical form (μg)	0	0.3	0.2	1.5	1.6	0.8	0.5
	0	0.4	0.4	2.0	1.7	1.6	1.8
	0	0.4	1.0	2.4	2.9	2.6	2.2
	0	1.6	9.7	3.3	3.1	5.6	2.6
	0			9.5	3.5	16.7	6.3
				17.9	4.5		
(Median)	(0)	(0.4)	(0.7)	(2.9)	(3.0)	(2.6)	(2.2)
AOL-DONE levels per tumor after i.t. injection of ADR in solution (μg)	0	0	0.2	0	0	0	0
	0	0.3	0.3	0	0	0	0
	0.2	0.3	0.5	0.4	0	0	0
	0.3	0.6	3.7	0.9	0	0	0
	0.4			2.8	0.3	0	0.2
	0.6			8.5	0.8	0	0.6
	0.7				1.0	0	0.6
					6.9	0.3	1.2
(Median)	(0.3)	(0.3)	(0.4)	(0.7)	(0.2)	(0)	(0.1)
Significance of difference between treatments	$P = 0.01$	$P > 0.1$	$P > 0.1$	$P = 0.1$	$P = 0.02$	$P < 0.001$	$P < 0.02$

Following the two treatments animals were killed at the time points shown and tumors analysed for ADR and metabolites. Concentration–time profiles of ADR in tumor tissue are shown in Fig. 2. Metabolite levels were more variable than levels of ADR; therefore, the individual values are shown above. Differences between treatments at all time points were assessed by two-tailed Wilcoxon Rank-Sum test. Each value in the columns under the time points represents the AOL-DONE level of an individual tumor.

vidual values are presented in Table 1, and it can be seen that, despite variability, median levels of AOL-DONE in tumors were significantly higher between 40 and 72 hr after i.t. injection of ADR in microspherical form. ADR-loaded microspheres incubated *in vitro* for 48 hr at 37° showed no trace of 7-deoxyaglycone formation.

An alternative way of presenting the results is as μg of ADR or AOL-DONE per g of tumor tissue. Expressed in this way the median ADR concentrations at 40, 50 and 72 hr after injection were 4.1, 3.6 and 2.6 μg per g respectively when administered in microspherical form and 1.6, 1.7 and 1.0 μg per g when administered in solution. Median metabolite concentrations in tumor tissue were 1.1, 1.0 and 1.0 μg per g for ADR administered in microspherical form and <0.1 μg per g at each time point for ADR administered in solution. All comparisons at these time points showed the median concentrations of ADR and AOL-DONE in tumor tissue to be significantly higher ($P < 0.05$ by two-tailed Wilcoxon Rank-Sum test) after injection of drug in microspherical form.

The two tumors with highest AOL-DONE levels (8.5 and 6.9 μg per whole tumor) following administration of ADR in solution were in the large region of the range studied (3.4 and 2.6 g respectively), a factor that may be important in explaining this result *(vide infra)*. However, not all large tumors in this group contained high AOL-DONE levels and, moreover, no significant difference in tumor weight between treatment groups was apparent at this stage.

DISCUSSION

The significance of increased 7-deoxyaglycone production by tumor tissue lies in the proposed mechanism of biotransformation of ADR leading to this particular end product (Fig. 3). In this scheme ADR is converted by an enzyme catalysed one or two electron reduction of the quinone nucleus [8, 9] to a series of electrophilic intermediates capable of binding covalently to DNA [10] and that subsequently degrade to 7-deoxyaglycones.

In our study the 7-deoxyaglycone present in greatest abundance in tumor tissue was AOL-DONE rather than ADR-DONE (e.g. see Fig. 2): on occasions ADR-DONE was below the limit of detection even though AOL-DONE was present. This is consistent with *in vitro* studies showing that rat liver microsomes convert ADR to 7-deoxyaglycones via a linear sequential pathway which obeys first order kinetics: ADR is first transformed rapidly to ADR-DONE which is then transformed to AOL-DONE, with higher concentrations of the latter always being achieved [11, 12]. From this and other evidence [13–15] it appears that 7-deoxyaglycones are obligatory end products of anaerobic bioreduction of anthracyclines such as ADR. It is envisaged that anaerobosis prevails either *ab initio*, as in hypoxic areas of tumors [16] or is brought about by redox cycling of electrons from NADH/NADPH to molecular oxygen, thereby removing it in the form of toxic oxyradicals [15].

Data from different sources is in agreement that there are two broad mechanisms by which anthracyclines may exert their deleterious effects on cells; firstly, one that is dependent on the redox properties conferred on these drugs by the quinone moiety and, secondly, one that is independent of redox properties. Thus, agents that prevent reduction of ADR have been shown *in vivo* to reduce its toxicity without affecting its anti-tumor activity [17]. In addition, structure–activity studies show that when

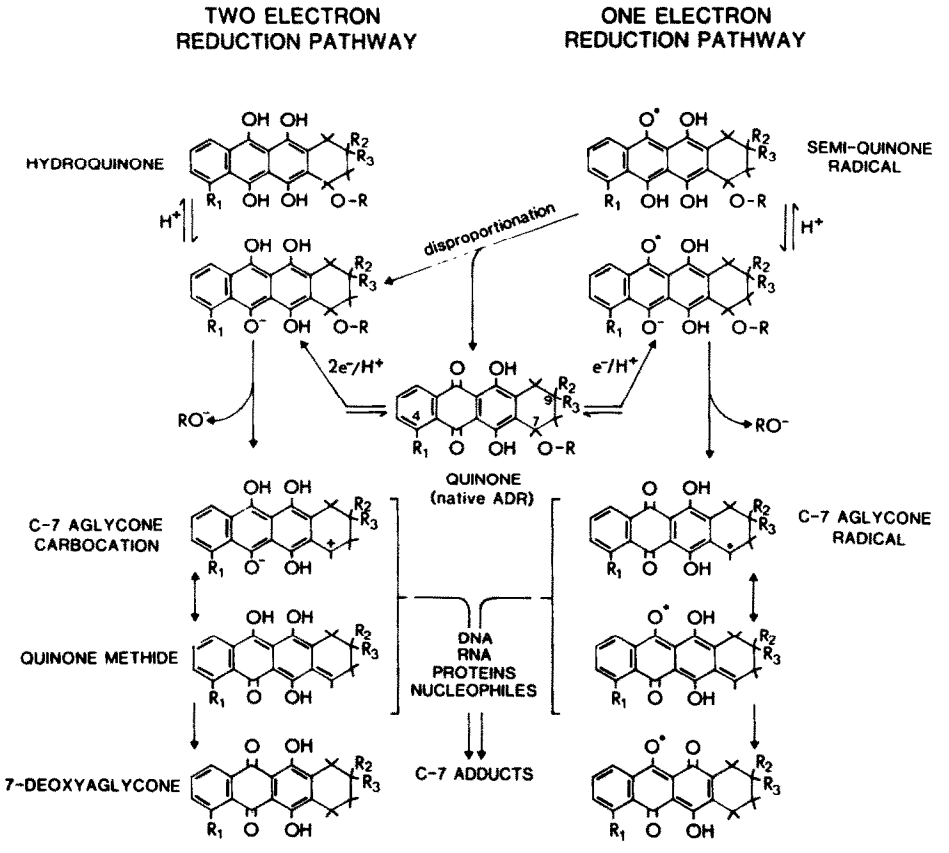

Fig. 3. Proposed pathways of metabolic activation of ADR to reactive intermediates (taken from a figure in ref. 9). 7-Deoxyaglycones occur as pharmacologically inactive end products.

the redox properties of anthracyclines are abrogated, as in the 5-imino analogues, then the drug is incapable of participating in cycling of electrons to oxygen to give oxyradicals, nor is it bioreduced to 7-deoxyaglycones [18]. Interestingly, it is reported that cardiotoxicity is diminished but antineoplastic activity is retained in this analogue [19]. A further consideration for anthracyclines that can participate in redox reactions is the tissue distribution of enzymes that permit expression of the latent potential for cytotoxicity inherent in the quinone structure. In animal studies reduction of anthracyclines by subcellular fractions from liver, heart, kidney, lung, spleen and red blood cells has been demonstrated [15]. Similarly, we have shown *in vivo* that liver and heart tissue can metabolize ADR via reductive pathways to 7-deoxyaglycones. On the contrary, tumor tissue was unable to metabolize ADR via this route [6]. The *in vitro* cytotoxicity of ADR towards a mouse tumor cell line also appeared to be independent of the redox properties of the drug [20]. In this present study we again interpret the generally low levels of 7-deoxyaglycones in tumor tissue after administration of ADR in solution (Table 1) as reflecting its low innate capacity to metabolize the drug via reductive pathways. It is therefore of interest that, in addition to increasing drug potency (Fig. 1), incorporation of ADR in albumin microspheres promoted reductive metabolism of the drug in tumor tissue (Table 1), perhaps accompanied by the generation of electrophilic intermediates of ADR (Fig. 3) and oxyradicals [15]. The anti-tumor effect of ADR in solution (Fig. 1) was presumably mediated by mechanisms largely independent of the redox properties of the drug.

An alternative explanation for our results could be that reductive metabolism of ADR to deoxyaglycones is purely an inactivating process and that drug incorporated into microspheres is inactivated to a greater extent *in vivo* than drug in solution. This hypothesis would then need to account for the increased anti-tumor effect of ADR-loaded microspheres by postulating some other factor residing in the delivery system that more than compensates for drug inactivation. Whilst this cannot be ruled out, especially in view of the higher drug levels in tumor tissue at 40, 50 and 72 hr following administration of ADR-loaded microspheres (Fig. 2), the simplest, most elegant hypothesis, requiring the fewest unsubstantiated entities, is that invoking cytotoxicity consequent on reduction of ADR. Moreover, in our system empty microspheres co-administered with ADR in solution did not exhibit a significantly different anti-tumor effect to the same amount of ADR alone (unpublished observations): this argues against a microsphere induced hypoxia causing drug inactivation.

Why incorporation of ADR into albumin micro-

spheres causes a change in the metabolic fate of the drug is at present unknown; however, it is of interest that there was a delay of 16–24 hr before a substantial level (>1 μg) of AOL-DONE was consistently detected, in marked contrast to its formation within minutes by anaerobic rat liver microsomes *in vitro* [12] and mouse liver and heart *in vivo* [6]. This lengthy interval corresponds approximately to the time that albumin microspheres begin to be biodegraded in the rat lung [3] and liver [21] accompanied by an inflammatory response involving phagocytic cells. Furthermore, it is known that activated phagocytic cells display increased plasma membrane NADPH-dependent cytochrome c reductase activity [22] that can catalyse anaerobic bioreduction of anthracyclines [23]. Thus, in our system the increased anti-tumor effect of ADR-loaded microspheres may be associated with metabolic conversion of drug to a reactive intermediate, perhaps at the plasma membrane of activated phagocytic cells involved in biodegradation of microspheres and in close proximity to tumor cells. Thus, the large variation in AOL-DONE levels (especially in comparison to variation in ADR levels from which they were derived) may reflect the fact that they represent only one point of a dynamic series of events involving ADR metabolism, accumulation and activation of host phagocytic cells (itself a function of tumor size) and diffusion of lipophilic 7-deoxyaglycones away from the site of formation [6].

Whatever the underlying mechanism of these results, they are consistent with and offer experimental support for a hypothesis advanced by Moore [8] that linked the occurrence of 7-deoxyaglycones to production of an ADR-derived alkylating moiety and increased drug potency.

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