Cancer Chemother Pharmacol (1987) 20: 109–114

Cancer Chemotherapy and Pharmacology

A comparison of adriamycin and mAMSA

II. Studies with V79 and human tumour multicellular spheroids

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Summary. Multicellular spheroids were used to compare the two chemotherapeutic agents adriamycin (ADM) and 4′[(9-acridinyl)-amino] methanesulphon-m-anisidide (mAMSA). Chinese hamster cells, V79 379A, a human small cell lung carcinoma, designated ME/MAR, and a human melanoma xenograft, HX117, were grown as spheroids (200 or 400 μm in diameter) and treated with either drug for 1 h, at 37° C, in air. Cytotoxicity was assayed using both cell survival and growth delay.

Both drugs were highly toxic towards V79 but showed less activity toward the human tumour single cell suspensions; ADM was more effective towards HX117 and ME/MAR than mAMSA. When grown as spheroids, the cells developed marked resistance to both drugs. In all cases, cytotoxicity was drug dose and spheroid size dependent. The response of HX117 spheroids to both drugs was similar. In contrast, ADM was more effective toward 200 μm diameter ME/MAR spheroids, and mAMSA showed greater activity than ADM against V79 spheroids. Both endpoints gave qualitatively equivalent results, and a comparison of the two showed relatively long growth delays for a given level of cell kill, for both drugs and with all three cell lines. The greater cytotoxicity of ADM toward ME/MAR spheroids is consistent with the clinical finding that ADM has a use in the treatment of small cell carcinoma of the lung, while mAMSA has not demonstrated any activity in the treatment of lung cancer.

Introduction

There are many similarities between the two chemotherapeutic agents ADM and mAMSA. Work with Chinese hamster cells in vitro indicated the two drugs to be equitoxic when cell lethality and sister chromatid exchange were used as endpoints [43]. The cellular uptake of both drugs is rapid [14, 29, 47], intercalative binding to DNA occurs [11, 42], and single- and double-strand DNA breaks are produced [47]. Both are effective, at low doses, in producing chromosome aberrations, chromatid aberrations, chromatid fragments and sister chromosome exchanges [1, 6, 40, 43]. Also, both cause cell progression delay [2, 35]. Activity has been demonstrated against a number of experimental murine tumours [5, 12]. In contrast, studies with human tumour xenografts have proved disappointing for both ADM [32] and mAMSA [24, 31]. Given their similarities, one might predict that mAMSA would be equally, if not more effective, than ADM in the clinical treatment of solid tumours, mainly because much higher peak plasma concentrations have been obtained with mAMSA (12 μ*M*, [39]) than with ADM (1 μ*M*, [18]). However, while ADM has proved clinically useful in the treatment of some solid tumours, mAMSA has demonstrated activity only toward hematological malignancies, in particular acute myeloblastic leukemia [21].

There are several possible factors involved in determining the heterogeneity of drug sensitivities between neoplasms: differences in drug pharmacology, differences in the cells' ability to repair drug damage, the efficiency of drug diffusion, the presence or absence of cell contact effects, and differences in the response of the various cell subpopulations that develop within tumours. The work reported here was undertaken to provide some idea of the basis of the difference in the response of tumours to ADM and mAMSA. To do this we assayed the response of three-dimensional multicellular spheroids to the two intercalating agents. Multicellular spheroids show an intermediate complexity between single cells in vitro and solid tumours in vivo. They are a useful model for studying the influence of cell contact and cellular heterogeneity on drug response. In addition, drug sensitivity can be assayed by growth delay as well as by cell survival, and a comparison of the two can provide information on the ability of the cells to repair drug-induced damage and the ability of the drugs to cause cell-progression delay. Finally, in order to address the importance of cell type in determining drug sensitivity, spheroids were grown from Chinese hamster cells (V79), from cells derived from a human melanoma xenograft (HX117), and from an early-passage human small cell lung carcinoma cell line (ME/MAR).

Materials and methods

mAMSA (4′[(9-acridinyl)-amino] methanesulphon-m-anisidide, NSC 249992) was supplied by the late Dr. B. F. Cain and made up in distilled water at 1 mg/ml immediately prior to use. Adriamycin (ADM) was obtained in 10-mg vials and reconstituted with 5 ml of distilled water; the resulting solution was stored frozen. Dilutions of stock suspensions were made in phosphate-buffered saline (PBS).

The cells used in this study – their culture, formation and growth as spheroids – have been described previous-

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Table 1. Spheroid characteristics

Cell line	Mean plating efficiency (%)	Mean initial volume-doubling time of 200/μm spheroids (days)	Median diameter of cells from 200/μm spheroids[a] (μm)	Estimated number of cells per spheroid[b] 200 μm	400 μm
V79	76	0.7	11.5	3.6×10^3	2.2×10^4
ME/MAR	30	2.5	10.5	3.5×10^3	2.7×10^4
HX117	5	2.2	18	2.4×10^2	4.2×10^3

[a] Determined using a Coulter Channelizer

[b] One hundred spheroids of appropriate size were selected and trypsinized, and the number of cells in suspension was counted using a haemocytometer

ly [44]. Spheroids were harvested, washed, selected for size, divided into equal aliquots and placed in 6-cm plastic petri dishes in 4.5 ml of medium. Drug (0.5 ml) at ten times the required concentration or 0.5 ml PBS for controls was added and the dishes were incubated for 1 h at 37° C in a humidified atmosphere of air + 5% CO_2. At the end of this time the spheroids were transferred to universal containers, allowed to settle and then washed twice with PBS. Each group of spheroids was subsequently subdivided into two portions in order to assay drug response by both clonogenic cell survival and growth delay, as described previously [44].

Results

Cell characteristics

Table 1 lists the plating efficiencies, volume-doubling times, number of cells per spheroid and average cell diameters for the spheroids used in these experiments.

Cell survival

Figure 1 illustrates the drug response of single cells from disaggregated 200 μm diameter spheroids. Spheroids were trypsinized and the cells exposed to ADM or mAMSA for 1 h at 37° C in air; they were then washed, serially diluted and plated for survival. ADM and mAMSA showed dose-dependent cytotoxicity towards each of the cell types. Under these experimental conditions, both drugs were most cytotoxic toward V79 cells. ME/MAR and HX117 cells showed similar sensitivities to ADM, but mAMSA was less effective toward the human tumour cells.

The response of cells from 200, 400 or 600 μm spheroids treated with ADM or mAMSA is given in Fig. 2. For each cell type and for each drug there was a progressive decrease in response as the spheroids were treated at larger sizes. The responses of 200 μm ME/MAR spheroids and single cells from dissociated spheroids exposed to ADM were similar. This was not the case for the other cell types or for any of the cells exposed to mAMSA. The change in sensitivity from single cells through different sizes of spheroids has been reported previously for V79 cells with both ADM [9] and mAMSA [46]. It is note worthy that 200 μm HX117 spheroids contain only approximately 250 cells; yet when treated with 5 μ*M* ADM, survival increased ten fold compared with that seen with single cells.

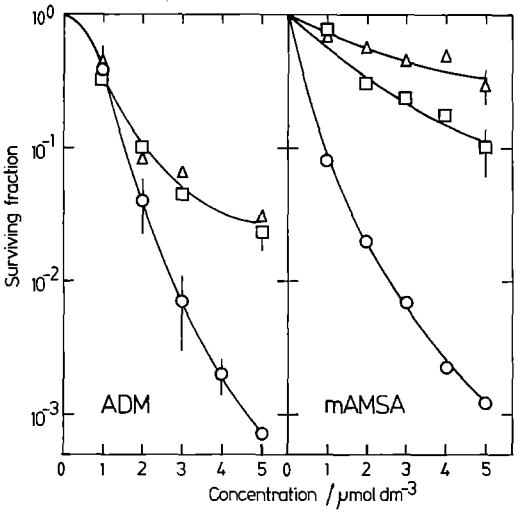

Fig. 1. The response of V79 379A (○), HX117 (□) and ME/MAR (△) cells from disaggregated 200 μm diameter spheroids to graded doses of ADM and mAMSA. Cells were exposed for 1 h at 37° C. The *points* represent the mean of a minimum of three experiments and *error bars* the SEM

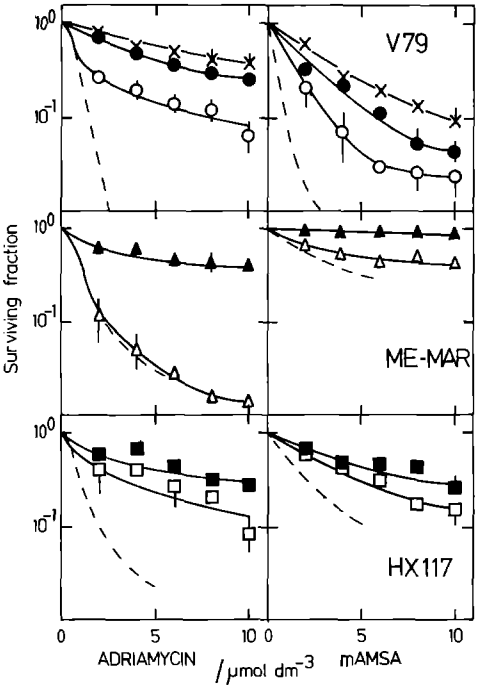

Fig. 2. The effect of varying concentrations of ADM or mAMSA, exposed for 1 h at 37° C, on the response of V79 379A, ME/MAR and HX117 spheroids 200 μm (*open symbols*), 400 μm (*closed symbols*) or 600 μm (*crosses*) in diameter. The averages of three to five experiments are shown and *error bars* indicate SEM. *Dashed lines* are the curves from Fig. 1.

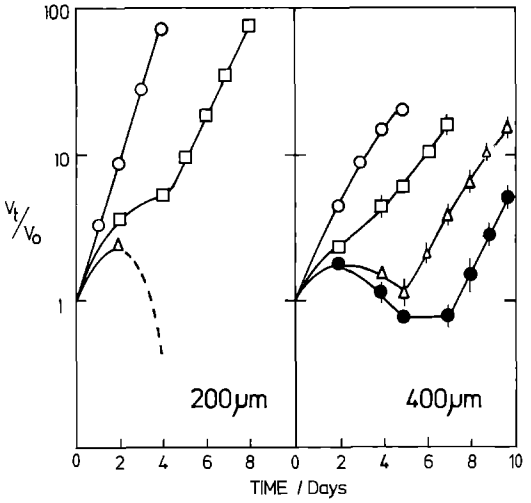

Fig. 3. The response of V79 379A spheroids (200 or 400 μm in diameter) to a 1-h exposure of 0 (○), 1 μ*M* (□), 5 μ*M* (△), and 10 μ*M* (●) mAMSA in air at 37°C. *Points* represent the mean and SEM of 12 spheroid volumes

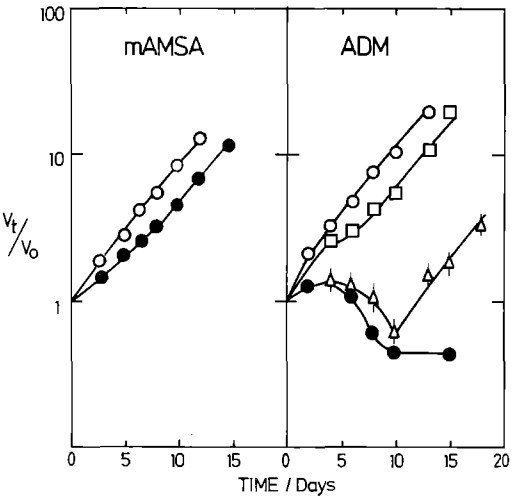

Fig. 4. The response of 200 μm diameter ME/MAR spheroids after a 1-h exposure to 0 (○), 1 μ*M* (□), 5 μ*M* (△), 10 μ*M* (●) drug. The mean and standard error of 12 spheroid volumes are shown

Growth delay

Data from individual sets of experiments showing growth of 200 or 400 μm diameter V79 spheroids exposed to mAMSA and of 200 μm diameter ME/MAR spheroids following treatment with ADM or mAMSA are illustrated in Figs. 3 and 4 respectively. In these figures the data are normalized to the initial treatment volume. Curves generally showed some delay in growth after treatment, followed by an increase in spheroid volume at a rate similar to that for untreated controls.

From the data in Figs. 3 and 4 and from many additional experiments with each cell type at each spheroid size, we have determined the specific growth delay (SGD) as a function of drug dose, where:

$$\text{SGD} = \frac{\text{T (treated)} - \text{T (control)}}{\text{TD (control)}}$$

T is the time taken to reach four times the initial treatment volume and TD is the initial volume-doubling time. The results, illustrated in Fig. 5, are consistent with the cell-survival findings and show that ADM was more effective than mAMSA toward the human small cell carcinoma spheroids and that the response of HX117 to both drugs was similar. In addition, as with cell-survival studies, a size-dependent drug response was seen for each cell type, with large spheroids being most resistant.

Finally, in order to study the relationship between the two endpoints, the SGD has been plotted against the measured cell survival for data when both endpoints were determined within the same experiment. If cell survival and growth delay were well correlated, one decade of cell kill would require 3.32 doublings of the surviving cells for regrowth to the original volume; that is, there should be a SGD of 3.32 for one decade of cell kill. The determined value is 8.5 for both drugs in all three cell lines. This implies that there were long growth delays for a given level of cell kill.

Discussion

The development of drug resistance in cells, when grown as spheroids, is well documented and several factors have been implicated to be involved. Problems associated with drug diffusion may, in part, explain the observed resistance [9, 46]. Cellular heterogeneity and cell contact effects may also be important [8].

The results presented here suggest that the roles of drug diffusion and cellular heterogeneity in dictating the response of spheroids are likely to be due to differences in cell type rather than to intrinsic differences between the drugs. There are no systematic differences in the drugs' cytotoxicities towards single cells and spheroids that would strongly indicate the importance of diffusion barriers to either of the drugs. For both agents the greatest difference in the response of single cells and spheroids is seen in V79 and the smallest in ME/MAR. Also, both drugs have been shown to be relatively resistant towards plateau-phase, chronically hypoxic cells and cells cultured at reduced pH [43]; the cell subpopulations that have been shown can occur in spheroids [3, 34]. Any effect of cell contact in the development of resistance to ADM and mAMSA also appears to be cell type and not drug related. Electron-microscope studies revealed that, when grown as spheroids, V79 379A cells are characterized by the possession of desmosome-type junctions as well as tight junctions, HX117 cells have many fewer demosome-type junctions, and ME/MAR cells lack any recognizable junctions whatsoever [41]. From Fig. 2 it is apparent that there is a marked difference in the response of V79 single cells and 200 μm diameter spheroids; the difference is less for HX117 and very small for ME/MAR. This suggests a possible relationship between possession of cell junctions and the development of spheroid drug resistance.

In these studies drug response has been assayed using two endpoints; cell survival and growth delay. A comparison of the two is useful, as it helps to eliminate artifacts which may occur when data are interpreted from a single endpoint. With some drug treatments and in some cell lines considerable repair of potentially lethal damage (PLD) can occur up to 24 h after treatment [38]; also, cell damage may occur during disaggregation. These could both lead to an overestimation of cell kill if cell survival were the sole method of assay. Conversely, if drug treat-

ment caused cell-cycle delay, cell survival measurements would be artificially low. From these studies it is apparent that for both drugs, in all three cell lines, there were relatively long growth delays for a given level of cell kill. This observation confirms the work of others, who have found relatively long growth delay for a modest cell kill with ADM in EMT-6 spheroids [22, 38] and appreciable delay without a detectable cell kill in an experimental rat tumour [26]. Host tumour effects have been implicated in the long delays caused by ADM in tumours [27]. These spheroid studies illustrate that long delays can be found in the absence of host tumour interactions, suggesting that both drugs can cause considerable cell-cycle delay in both V79 and human tumour systems.

We have shown that ADM is effective whereas mAMSA is ineffective towards the oat cell carcinoma spheroids. On the other hand, the response of the melanoma spheroids to either drug is partial. Clinically, mAMSA has not shown any activity towards lung carcinomas [28] or melanomas [10]. ADM is not used in the treatment of melanoma but is regularly used for small cell carcinoma of the lung. The basis for the difference between ADM and mAMSA toward the ME/MAR cells probably has its origin in the cellular pharmacokinetics of two drugs. These would include the efficiency of drug uptake and efflux, the affinity for intracellular binding sites, and physicochemical factors such as drug pKa and the intra- and extracellular pH.

The uptake of ADM and mAMSA into cells is rapid and is thought to occur by passive diffusion of the un-ionized form of each drug [14, 29, 47]. ADM and mAMSA are weak bases, with pKa's of 8.22 [29] and 8.88 [40] respectively. ADM efflux is by an energy-dependent active transport mechanism [30]. In contrast, mAMSA effluxes more rapidly from cells and is not energy dependent [47, 48]. Following drug exposure and washing in sensitive cells, drug retention is greater than 75% in ADM-treated cells [4, 29], while less than 20% mAMSA remains intracellularly [47, 48]. In addition, ADM cytotoxicity can be modulated by modifiers of extracellular calcium [17, 37]. ADM has a high affinity for intracellular binding sites [30] but mAMSA has a low degree of intracellular binding [16, 47]. Around 50% of the accumulated drug has been shown to be retained by nuclei for ADM [4], while less than 20% is retained following exposure to mAMSA [25, 47]. Although there are many instances of cellular cross-resistance, the mechanism involved in the development of these resistances is different. Resistance to mAMSA has been associated with impaired nuclear accumulation although net cell accumulation remains similar [15], while ADM resistance is associated with an energy-dependent enhanced drug efflux and decreased drug retention [14, 25].

It is now generally accepted that for both drugs, DNA intercalation and subsequent inhibition of polynucleotide syntheses cannot sufficiently account for the drugs' cytotoxicities. There are many biochemical and morphological changes associated with ADM treatment. ADM has been shown to interfere with cellular respiration [20], to produce free radicals [19] and to damage membranes [23, 36]. The structure of the cell membrane may be an important determinant of the therapeutic efficiency of ADM. ADM has been shown to interact specifically with membranes containing a high proportion of negatively charged phospho-

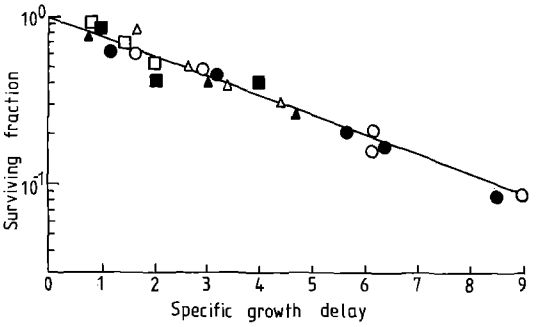

Fig. 6. Specific growth delay plotted against surviving fraction for ADM-(*solid symbols*) and mAMSA-(*open symbols*) treated spheroids. The *points* were taken from individual experiments when cell survival and growth delay were measured on the same day. V79 379A (○, ●), ME/MAR (□, ■) and HX117 (△, ▲)

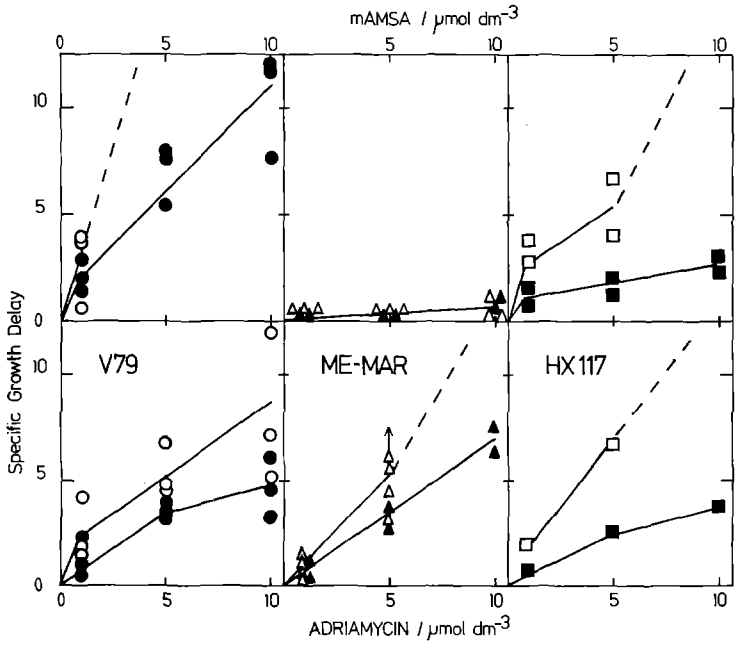

Fig. 5. Specific growth delay (growth delay ÷ control doubling time) versus drug concentration. Each *point* represents a single experiment and the average of 12 spheroid volumes. Spheroids, either 200 (*open symbols*) or 400 μm (*closed symbols*), were exposed to drug for 1 h at 37° C

lipids (e. g. cardiolipin), and this could lead to a high affinity for or a more persistent binding to membranes [7, 23, 36]. It is likely that mAMSA, too, will have alternative/additional mechanisms for exerting its toxicity. The sequestering of mAMSA into non-cytotoxic intracellular loci has been implicated in the drug's mode of action [45, 48]. Ruthenium red has been shown to protect tumour cells in vitro from ADM and mAMSA toxicities [45]. With mAMSA this is associated with an increase in drug accumulation, presumably to a non-cytotoxic cell loci [45], and with ADM it is associated with a decreased drug accumulation. Cell lines with the ability to sequester mAMSA may be resistant to the drug's action [33].

In conclusion, we suggest that drug diffusion, cell heterogeneity and cell-contact effects are not the overriding factors determining tumour sensitivity to these two drugs. Rather, there are inherent differences between cell types, which reflect differences in the drugs' cellular pharmacokinetics and modes of action. For both agents a growth-delay assay indicates cell-cycle delay as an important contributory factor in their modes of action. Finally, these studies indicate the importance of using a range of cell types when assaying the effectiveness of intercalating agents, and the use of spheroids provides a greater perspective as to the potential usefulness of any new agent.

Acknowledgements. This work was carried out at the Physics Department, Institute of Cancer Research, Sutton, Surrey whilst C. West was the recipient of a MRC student fellowship. We thank Elaine Mercer for typing the manuscript.

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Received June 20, 1986/Accepted April 15, 1987