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Activity and distribution studies of etoposide and mitozolomide in vivo and in vitro against human choriocarcinoma cell lines*

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Summary. The in vivo antitumor activity of etoposide and mitozolomide was assessed in nude mice bearing a xenograft (CC3) of human gestational choriocarcinoma. Both agents demonstrated, at best, marginal activity observed as a delay in tumour growth. This lack of sensitivity suggests that the CC3 xenograft is not a good model for selection of agents for clinical evaluation in gestational choriocarcinoma.

Plasma and tissue concentrations of etoposide and mitozolomide were measured in nude mice. Drug concentrations found in tumour tissue were 60% and 30% of plasma levels for mitozolomide and etoposide respectively.

Etoposide and mitozolomide activity was also evaluated in vitro with another choriocarcinoma cell line (JAR). Maximum cell-kill was achieved after exposure to etoposide 0.05–1 μg/ml for 3–24 h. In vitro response to etoposide demonstrates the importance of exposure time in determining cytotoxicity. In contrast, mitozolomide at concentrations from 1–100 μg/ml did not have a marked effect against JAR after exposure for 3–24 h.

Introduction

Etoposide (VP 16-213) has proven clinical effectiveness against a number of human tumours [16], including gestational choriocarcinoma [14]. We have evaluated the activity of etoposide in a human choriocarcinoma xenograft (CC3) implanted into nude mice in an attempt to assess the predictive value of this tumour model. Screening for antitumour activity in nude [22] or thymectomised [20] mice bearing human tumour xenografts is potentially useful in obtaining information on the activity of new drugs in specific tumour types. With this objective, we also investigated the activity of mitozolomide, an alkylating agent [8] with broad-spectrum antitumour activity [6, 10] which is currently undergoing phase II clinical evaluation.

In a separate experiment, we measured etoposide and mitozolomide concentrations in plasma and tissue in order to obtain pharmacokinetic information concerning the distribution of these agents and to assess any relationship with antitumour efficacy.

Data is presently unavailable on the sensitivity of choriocarcinoma cells in vitro to etoposide. This lack of information prompted us to investigate the in vitro activity of etoposide on another choriocarcinoma cell line (JAR) over a wide range of concentrations (0.001–5 μg/ml) and for exposure times up to 24 h.

Using the pharmacokinetic results in mice and an investigation of the stability of etoposide and mitozolomide in culture, the in vivo and in vitro effects of these drugs are related by the concept of total exposure (concentration and time; C × t) as a determinant of cytotoxicity.

Materials and methods

Drugs. Etoposide was obtained from Bristol-Myers Laboratories (Syracuse, USA) and mitozolomide was kindly supplied by Prof. M. F. G. Stevens (Dept. of Pharmaceutical Sciences, Cancer Research Campaign Laboratories, Aston University, UK).

Mitozolomide is unstable in aqueous solution and decomposes in a temperature- and pH-dependent manner to form cytotoxic products which also readily degrade [21]. In tissue culture medium at 37 °C, we found that mitozolomide decomposed by a first-order process with a half life of approximately 55 min. In contrast, etoposide was relatively stable and decomposed by approximately 21% in 72 h.

Cell culture. Human JAR choriocarcinoma cells [17] were grown in DMEM with 20 m*M* HEPES buffer (Flow Laboratories, UK) supplemented with 10% bovine calf serum (Flow Laboratories, UK), 100 units/ml penicillin (Gibco UK), 100 μg/ml streptomycin (Evans Medical Ltd., UK) and 2 m*M* L-glutamine (Flow Laboratories, UK). Cultures were maintained as monolayers at 37 °C and were passaged using 0.05% trypsin and 0.02% EDTA.

When used for drug activity testing, the cells were plated out in quadruplicate at 1.5×10^5 cells per 35-mm-diameter culture well (Costar, UK). Cultures were allowed to adhere for 24 h before use.

In vitro JAR cytotoxicity assay. The effect of etoposide and mitozolomide on JAR cell viability was examined by determining cell number of treated cells compared to cells incubated with drug vehicle alone. Etoposide was dissolved in Tween 80 and saline (1:10), and mitozolomide in DMSO and saline (1:10). All solutions were filtered through 0.2-μm filters (Amicon, UK) before being added to the cells to give a final drug concentration between 0.001 μg/ml and 100 μg/ml. Cells which had been incubat-

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ed at 37 °C with the drug for between 3 and 24 h were washed 3 times with 1 ml sterile saline, and reincubated for a total of 48 h before counting by haemocytometer. Cell viability was assessed by means of trypan blue.

Calculation of area under in vitro etoposide and mitozolomide exposure curves. Because of the instability of etoposide and mitozolomide, drug concentrations during cell incubations at 37 °C were corrected and total area under the C × t curves were calculated as

$$C \times t = \frac{Co}{Ke} - \frac{Ct}{Ke},$$

where *Co* is the initial drug concentration, *Ke* is the first-order rate constant for drug decomposition and *Ct* is the concentration of drug at the end of the incubation.

Studies in mice. Albino, nude (athymic) female mice (nu/nu × MF/1 albinos) weighing 20–25 g and aged 8–12 weeks were used in these experiments. Mitozolomide and etoposide were dissolved in the same vehicle as used in the in vitro studies to yield doses measured in mg/kg. For the antitumour efficacy studies, etoposide (20 mg/kg/day) and mitozolomide (8 or 16 mg/kg/day) were administered i.p. over 5 consecutive days. The etoposide schedule was repeated after a rest period of 10 days. A total dose of 40 or 80 mg/kg mitozolomide was amdinistered. The CC3 choriocarcinoma xenograft was originally obtained from a tumour biopsy of a patient with gestational choriocarcinoma prior to receiving chemotherapy. The in vivo sensitivity of this patient's tumour to chemotherapy could not be adequately assessed since her human chorionic gonadotrophin (hCG) concentration fell to the normal range following surgical excision. After implantation into nude mice the xenograft was maintained by serial s.c. passage. Up to this study tumour has been passaged 52 times with no significant change in morphology or growth rate (G. Boxer, personal communication). The CC3 tumour grows as an encapsulated nodule and does not metastasise. Histologically, it is poorly differentiated and contains both cytotrophoblastic and hCG-producing syncytiotrophoblastic elements. Extensive central necrosis is common in this model [18]. For the in vivo therapy experiments, 1 mm^3 tumour was implanted s.c. into the left flank of nude mice. Mice used as controls were implanted with the same volume of tumour in the same experiment and recieved drug vehicle only.

In each experiment, five mice were randomised for drug treatment and five for control. At the start of treatment all tumours had a volume of between 20 and 100 mm^3. Tumours were measured every 3–4 days in three dimensions by the same observer. The volume was calculated by the equation $a \times b \times c/2$ in which *a* represents tumour length, *b* tumour width and *c* tumour thickness expressed in cu.mm.

Because of the variety of tumour sizes at the initiation of treatment, tumour volumes were converted to values related to the initial tumour volume. The relative tumour volume was expressed by the formula Vt/Vo, where Vt is the tumour volume at any given time and Vo is the volume at the initiation of treatment. The effect of treatment was expressed numerically as the fractional volume reduction [20] (FVR).

$$FVR = \log_{10} \frac{\text{mean } Vc}{\text{mean } Vm}$$

where *Vc* and *Vm* are the relative tumour volume of the control and treated groups respectively at the time when tumour volume difference between control and treated mice was at a maximum.

On the same day as the tumours were measured, blood samples were taken from the tail vein and collected into 25-µl capillaries (Horwell, Hampstead, UK). These samples were diluted in 375 µl 0.7 *M* phosphate buffer and blood concentration of hCG was estimated by radioimmunoassay [11].

Preparation of plasma and tissue for HPLC analysis

For mitozolomide, blood samples were taken by cardiac puncture from mice under halothane anaesthesia at the following points: 0, 5, 10, 15, 30 min, 1, 2, 4 h after drug administration. Tissues (tumour, liver, kidney, lung, brain) were removed at the same time. Plasma and tissues were immediately frozen at −20 °C until required for analysis. Samples were estimated by high-pressure liquid chromatography (HPLC) as described previously [2].

For etoposide, blood and tissue samples were obtained by the procedure outlined above at the following times: 0, 15, 30 min, 1, 1.5, 2, 3 h after drug administration. HPLC analysis of etoposide was performed as described previously [1].

Pharmacokinetic calculations. No attempt was made to fit plasma and tissue C × t curves to a pharmacokinetic model. Area under the C × t curve (AUC) was estimated by the trapezoidal rule to the final sample time.

Results

Antitumour activity of etoposide and mitozolomide

The antitumour effect of etoposide and mitozolomide is shown in Fig. 1A–D. Both agents produced a significant tumour growth delay (Fig. 1A, C) without achieving complete remission. FVR values for etoposide and mitozolomide calculated from the data shown in Fig. 1A and C respectively were almost identical (0.55 and 0.58 respectively). Blood concentrations of hCG produced by the tumour did not show any appreciable changes between treated and control animals. However, both a reduction in tumour growth (FVR = 0.722) and a fall in hCG concentrations were apparent after a second course of etoposide.

Plasma and tissue distribution of etoposide and mitozolomide

Concentration vs time profiles for etoposide in plasma and tumour are shown in Fig. 2A. After i.p. administration of 20 mg/kg etoposide, the distribution in nude mice is similar to that described by Columbo et al. [3] in C57B1/66 mice. The highest concentrations were found in the liver (19.6 µg.h.ml^{-1}) and the lowest in the brain (0.555 µg.h.ml^{-1}). The distribution of mitozolomide in plasma and tumour (Fig. 2B) and in other tissues studied was similar to that observed in AKR mice [2]. Concentrations of drug in tumour were 30% and 60% of plasma levels for etoposide and mitozolomide respectively.

Effect of etoposide and mitozolomide against JAR viability in vitro

Figure 3A illustrates that JAR viability is significantly decreased as etoposide concentration is increased between

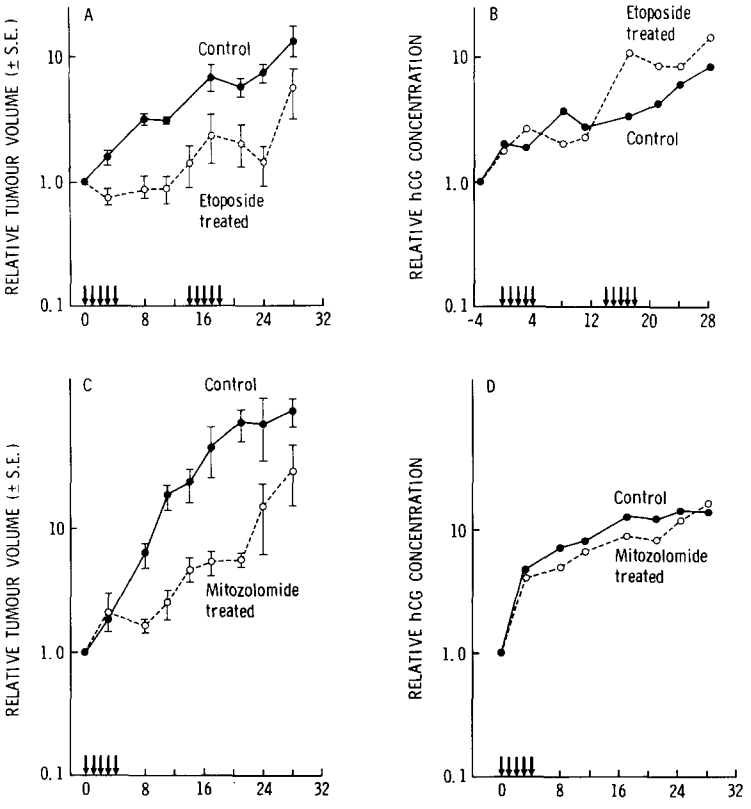

Fig. 1 A–D. Chemosensitivity profiles for the CC3 xenograft in the s.c. nude mouse assay. Drugs were administered i.p. at the times indicated *(arrows)*. Mitozolomide was given at a dose of 16 mg/kg/day and etoposide at 20 mg/kg/day

0.01 and 0.5 μg/ml. In vitro drug esposure as a function of C × t was calculated for each concentration and exposure time used. Etoposide was shown to decompose by 21% over 72 h (Ke = 0.003 min -1) and the C × t value was corrected for this loss. Cytotoxicity of etoposide is dependent on drug esposure. For example, an approximately 1-log cell kill was achieved using either 5 μg/ml over 3 h (15.0 μg.h.ml^{-1}) or 0.5 μg ml over 24 h (11.7 μg.h.ml^{-1}).

Mitozolomide (Fig. 3B) showed no significant activity in vitro at concentrations of 1–10 μg/ml after 3- to 24-h exposures. Increasing mitozolomide concentration to 100 μg/ml resulted in an increase in toxicity to JAR cells but there was less than a 1-log cell kill. Prolonging exposure time up to 24 h did not significantly increase cytotoxicity of mitozolomide. This observation was not unexpected because the decomposition half-life of mitozolomide in phosphate buffer [19] and in our studies in cell media was approximately 55 min. Therefore, after 6 h virtually no cytotoxic products would be present. On comparison with control wells where solvents alone were added, there was no effect on cell number.

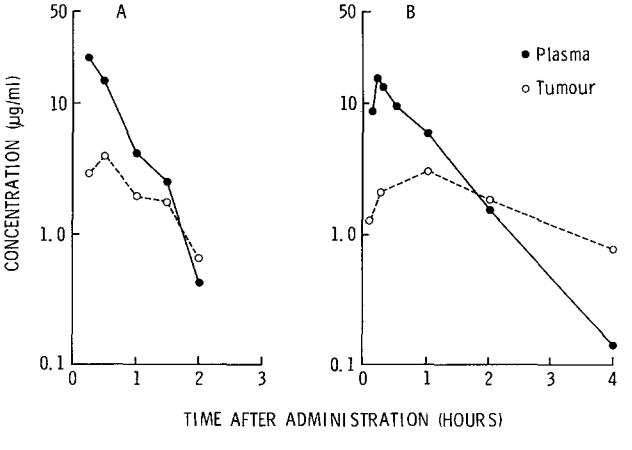

Fig. 2 A, B. Plasma and tumour concentrations of etoposide **(A)** and mitozolomide **(B)** in nude mice after administration of 20 mg/kg etoposide or 16 mg/kg mitozolomide

Discussion

In the present study we have evaluated the antitumour activity of a clinically effective drug (etoposide) and also a novel agent (mitozolomide), against the CC3 choriocarcinoma xenograft. Maximum tolerated doses of these agents were administered over a 5-day schedule and the relative tumour volume was used as the end-point. Tumour hCG production was also measured and compared to tumour growth.

Although etoposide shows a significant tumour growth delay, it is at best only marginally active (Fig. 1A). This does not correlate well with clinical observations where etoposide is used as a first-line drug in the treatment of choriocarcinoma [14]. In contrast, other authors have shown a good correlation between the activity of anticancer drugs against xenografts and clinical efficacy [20, 22]. The activity of mitozolomide against the CC3 xenograft (Fig. 1C) was comparable to that of etoposide. A similar growth delay was also observed at the lower total dose of 40 mg/kg mitozolomide (FVR = 0.26). The poor response of the CC3 choriocarcinoma to mitozolomide was unexpected, since mitozolomide had been shown to be highly effective against other human tumour xenografts [6]. In ad-

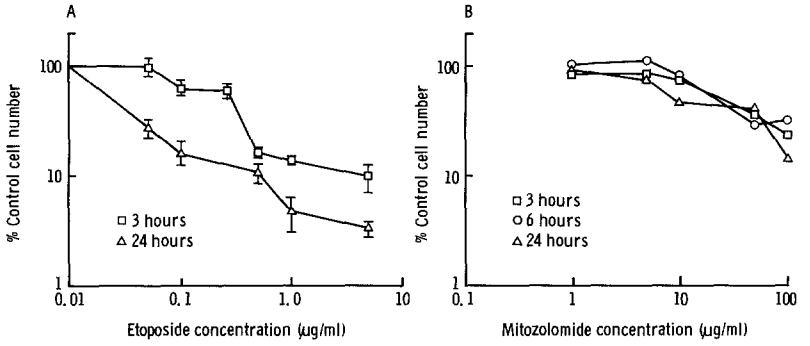

Fig. 3 A, B. Etoposide **(A)** and mitozolomide **(B)** cytotoxicity as a function of exposure time in vitro. JAR cells were exposed to drugs at the concentrations indicated for 3–72 h

dition to the limited responses reported here to etoposide and mitozolomide, CC3 showed only moderate growth delay when treated with methotrexate and the thymidylate synthetase inhibitor CB 3717 (M. Jones and K. R. Harrap, personal communication). The relative inactivity of both agents cannot be explained by different metabolic processes in man and mouse. Unchanged etoposide is the active cytotoxic species [23] and mitozolomide spontaneously decomposes to yield the active metabolite [8, 21]. It is possible that etoposide and mitozolomide fail to reach sufficient concentration in tumour tissue in order to elicit a cytotoxic response. This hypothesis was investigated by evaluating the pharmacokinetics of both agents in nude mice. The pharmacokinetics of etoposide [3] and mitozolomide [2, 9] have been previously studied in mice. In the present investigation, the plasma and tissue distribution of etoposide and mitozolomide in nude mice bearing a choriocarcinoma xenograft (Fig. 2A, B) was comparable to that found in those studies cited above. Both etoposide and mitozolomide were well distributed in tissues after i.p. administration, and appreciable concentrations of these drugs were found in tumour tissue. A dose of 20 mg/kg etoposide in the mouse is approximately equivalent to 62 mg/m^2 in man [7]. The plasma concentration of etoposide after infusion of 100 mg/m^2 rached 20 μg/ml [1], virtually identical to the level obtained in mice (Fig. 2A). Also, peak tumour concentration of etoposide was 3.9 μg/ml in mice, which is comparable to concentrations of 2–6 μg/ml etoposide observed in the myometrium of patients with gestational choriocarcinoma [5] after a therapeutic dose of this agent. In contrast, the AUC value in man (84 μg·h·ml^{-1}) [1] was over five times greater than that in mice (16 μg·h·ml^{-1}). Tumour concentrations of etoposide in man were 40%–50% of plasma levels [5], similar to the tumour:plasma ratios in mice (1:3) shown in Fig. 2A. Therefore the relative insensitivity of the CC3 xenograft to etoposide may be due, at least in part, to reduced exposure of the tumour to the drug compared with the greater potential drug contact time in patients. The peak plasma concentration of mitozolomide achieved in mice (10 μg/ml; Fig. 2B) was comparable to that achieved in man (6 μg/ml) [15] at a dose of 115 mg/m^2 (which will induce severe thrombocytopaenia on repeated dosing or in patients who have received prior therapy).

Unfortunately, attempts to grow CC3 in vitro have so far been unsuccessful, and in the absence of this cell line we employed a different choriocarcinoma cell line (JAR) derived from another patient. As was expected on the basis of the proposed mechanism of action of etoposide [23], JAR cell survival appeared to be greatly influenced by drug contact time. Drug concentrations of 0.5 μg/ml for 24 h produced a 1-log cell kill, whereas a comparable effect was realised after exposure for only 3 h when the concentration was raised to 5 μg/ml. The importance of contact time in determining the effect of etoposide has already been described for other cell types [4, 13]. The cytotoxicity against JAR cells approaches a plateau with increasing concentration at a fixed exposure time (Fig. 3A).

The inactivity of mitozolomide was unexpected. At in vitro concentrations comparable to those used in this study, mitozolomide had significant activity against a number of different human cell lines [6, 8]. Mitozolomide has activity comparable to that of CCNU [6], and in some cell lines concentrations of CCNU exceeding 10 μg/ml were required for activity [10]. However, exposure of CCNU in these studies was comparable to C × t values in the present study. Increasing the exposure time of mitozolomide would not be expected to increase toxicity in vitro since decomposition of the drug is virtually complete within 6 h.

The lack of correlation between cytotoxicity in JAR cells in vitro and antineoplastic activity against the CC3 xenograft in vivo is, of course, difficult to interpret. However, we are now able to grow the JAR xenograft in mice and investigations to evaluate the sensitivity of this tumour are under way.

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