Anti-Cancer Drugs 1997, **8,** pp. 756–762

The *ex vivo* chemosensitivity profile of choroidal melanoma

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Uveal melanoma has a high mortality rate and responds poorly to existing chemotherapy. We have therefore examined the sensitivity of uveal melanoma to cytotoxic agents using an *ex vivo* chemosensitivity assay to determine whether some agents which have not been used for this tumor might have activity. An ATP-based tumor chemosensitivity assay (ATP-TCA) was used to determine the effect of nine cytotoxic drugs at multiple dilutions in 28 primary uveal melanoma specimens. Evaluable assay results from up to 16 tumors with each drug showed variable sensitivity to alkylating agents (three of nine with mitomycin C, one of 15 with cisplatin and seven of 15 with treosulfan), cytosine arabinoside (seven of 16), paclitaxel (one of five) and doxorubicin (two of 16). No tumors were sensitive to temozolomide, or 5-fluorouracil, and only one of 14 to vincristine. The combination of treosulfan with cytosine arabinoside resulted in enhanced tumor cell inhibition in three of five tumors tested. Combinations containing paclitaxel combined with doxorubicin or cisplatin showed some activity, but none achieved 100% inhibition and the results were similar to those obtained with paclitaxel alone. Uveal melanoma shows considerable heterogeneity of sensitivity to cytotoxic drugs, with considerable resistance to most agents, matching clinical experience. The results suggest that cytosine arabinoside or gemcitabine plus treosulfan may be an active combination. Clinical trials will commence shortly. The use of the ATP-TCA provides a method of testing multiple agents and combinations in a way which would be otherwise impossible in rare tumors such as uveal melanoma.

Key words: ATP, chemosensitivity, choroid, eye, growth, melanoma.

This work was supported by the Guide Dogs for the Blind Association.

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Introduction

Uveal melanoma is fatal in approximately 43% of cases and is the commonest eye tumor. Death is due to metastasis, usually to the liver. Although local treatment is possible by local irradiation or enucleation, there is no effective treatment for metastatic disease. The median survival of those with liver metastasis is 5–7 months.[1,2] Although stage (tumor size) and microvascularity can define a high risk group,[3] no adjuvant therapy is yet available for routine use.

Previous attempts to treat metastatic uveal melanoma with chemotherapy have used similar regimens to skin melanoma, on the basis that the two tumors are derived from the same cell type, despite the many genetic and phenotypic differences which exist between them.[4] On this basis, occasional responses have been reported, but there is little trial-based data from which response rates can be obtained.[1] Systemic therapy is disappointing, with one large series reporting a response rate less than 1%.[2] Chemoembolization of the liver using cisplatin-based regimens was the most effective treatment, inducing responses in 36% of patients.[2] Others have also used chemoembolization with success, suggesting that at least some of these tumors are sensitive to platinum-based therapy.[5] One recent study of systemic treatment used cisplatin, dacarbazine, interferon-α and high-dose interleukin-2 in seven patients with metastatic uveal melanoma, but only one responded.[6]

We can find no previous studies of the chemosensitivity of uveal melanoma *ex vivo*, although some previous papers describe the radiosensitivity of cell lines derived from uveal melanomas.[7] Since it is difficult and costly to perform large numbers of clinical trials in rare tumors such as uveal melanoma, we have used a recently developed ATP-based

luminescence assay[8,9] to determine the sensitivity of primary uveal melanoma to a variety of chemotherapeutic agents.

Materials and methods

Melanomas

Material from 28 large primary intra-ocular melanomas (Table 1) was obtained under sterile conditions from fresh enucleation specimens removed consecutively at Moorfields Eye Hospital over a 1 year period. All except one were previously untreated: the exception had failed control by proton beam radiotherapy (Table 1). Enucleated eyes were examined externally for the presence of extrascleral extension or previous surgery and by transillumination using a fiber-optic light source to locate the tumor. The eye was then oriented in a steel eye-cup and sectioned posteriorly starting at the cornea to one side of the mid-line continuing to the same side of the optic disk. The larger calotte was placed immediately into 4% buffered formaldehyde for histopathology, while the smaller calotte without the optic disk was examined. Tumor material was scraped from the calotte and placed into 10 ml of Dulbecco's modified essential medium (DMEM) to which 100 U/ml penicillin and 100 mg/ml streptomycin had been added. The primary consideration is to obtain a histopathological diagnosis; in cases of doubt, the bulk of the tumour was fixed for diagnostic use. During the study period, four tumors were fixed without material being taken for assay.

ATP-based tumour chemosensitivity assay (ATP-TCA)

ATP-TCA was performed as previously described.[8] In the first 19 tumors, cells were dissociated by incubation overnight at 37°C with a commercially available tumor dissociation enzyme preparation (TDE; DCS Innovative Diagnostik Systeme, Hamburg, Germany). Since the evaluability rate in these tumors was relatively low (68%) and the tumors are soft with little collagen on histology, subsequent specimens were dissociated under the same conditions with 1.5 mg/ml collagenase type H (Sigma, Poole, UK). Following dissociation, the cells were washed in an antibiotic-containing serum-free complete assay medium (CAM; DCS Innovative Diagnostik Systeme) by centrifugation at 400 *g* for 10 min and their viability checked by Trypan blue exclusion. Viability ranged from 60 to 95% (mean 86%). Ficoll-Hypaque density centrifugation (Lymphoprep; Nycomed, Birmingham, UK) with two further washes was used in five cases to remove cell debris. However, this was found to be less of a problem following the switch to weak collagenase

Table 1. Clinical and pathological details of each tumor studied

TCA number	Site of tumor	Largest tumor diameter (mm)	Cell type (callender)	Mitotic count	Extrascleral extension	Previous treatment
96M002	choroidal	15	spindle	0.2	none	none
96M005	choroidal	16	epithelioid	0.9	yes	none
96M008	choroidal	19	spindle	1.7	none	none
96M009	choroidal	14	epithelioid	1.9	none	none
96M010	choroidal	12	epithelioid	0.2	none	none
96M011	choroidal	10	spindle	0.7	none	none
96M012	choroidal	15	mixed	ND	none	none
96M013	choroidal	10	epithelioid	0.3	none	none
97M015	choroidal	14	mixed	2.1	none	none
96M016	choroidal	18	spindle	0.3	none	none
96M018	choroidal	17	spindle	ND	none	none
96M019	choroidal	15	epithelioid	0.4	none	none
97M001	choroidal	17	epithelioid	0.1	none	biopsy
97M002	choroidal	12	mixed	0.9	yes	biopsy
97M003	choroidal/CB	14	mixed	0.2	none	none
97M004	choroidal/CB	10	epithelioid	1.7	none	none
97M006	choroidal	14	spindle	1	none	none
97M007	choroidal	12	spindle	0.5	none	biopsy
97M008	choroidal	18	spindle	0	none	proton
97M010	CB	12	epithelioid	0.5	none	none

CB, ciliary body. Mitotic count = mitoses/mm^2.

digestion and was not necessary in the last 12 tumors. The cells were adjusted to 200 000 viable cells/ml in CAM and 100 μl added to the wells of a 96-well polypropylene microplate (Costar, High Wycombe, UK) to which doubling dilutions of four drugs in triplicate wells (in 100 μl volumes) had been added while the cells were being prepared. Test drug concentrations (TDCs) are based on pharmacokinetic data adjusted to provide good discrimination between tumors (Table 2).[8] One row was reserved for six control wells with 100 μl CAM only (MO) and six wells to which 100 μl of a maximum inhibitor of cell survival (MI; DCS Innovative Diagnostik Systeme) was added.

The plate was incubated for 6–7 days at 37°C with high humidity in 5% CO_2 and the cells observed every 2–3 days by microscopy to check for infection or overgrowth. At the end of the incubation period, ATP was extracted from the cells by addition of a detergent-based extractant (TCER; DCS Innovative Diagnostik Systeme) and 50 μl from each well added to 3.5 ml polystyrene tubes (Sarstedt, Numbrecht, Germany) for estimation of ATP levels by luminescence assay. The tubes were loaded into a Berthold LB953 luminometer (EG&G Berthold, Wildbad, Germany) set to inject 55 μl of luciferin-luciferase reagent (DCS Innovative Diagnostik Systeme). Light output expressed as relative light units (RLU) was used to determine the mean percent inhibition of cell growth/survival in triplicate wells at each drug concentration according to the following equation: $1 - [(\text{Test} - \text{MI})/(\text{MO}-\text{MI})) \times 100$.

Data analysis

The results of each assay were analyzed individually in an Excel 5.0 spreadsheet (Microsoft) allowing graphical representation of the response (Figure 1) and collected in a database (Access 2.0; Microsoft). For comparison of responses (Table 3), a simple index was derived by summing the percent inhibition at each

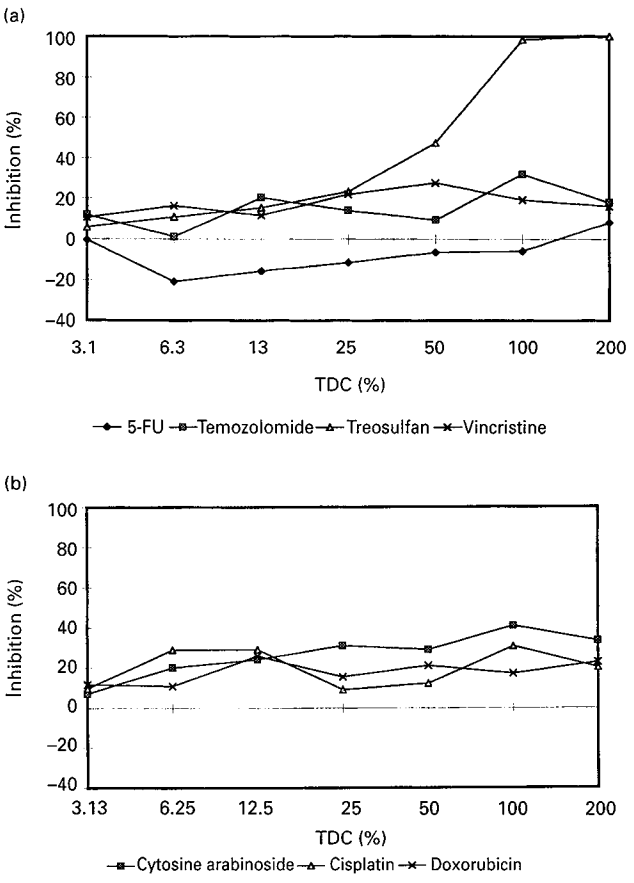

Figure 1. Typical results from one tumor. (a) Four drugs were tested in this microplate, but only one (treosulfan) shows activity with good inhibition at higher concentrations. (b) The three drugs tested in this plate are ineffective.

Table 2. Drug concentrations used in the assay and their clinically relevant doses (for combinations, each drug was added at the TDCs to the same wells)

Drug name	TDC (μg/ml)	Stock concentration (μg/ml)	Drug dose correlation
5-Fluorouracil	22.5	25	i.v. 500 mg/m^2
Cisplatin	3.8	1	i.v. 100 mg/m^2
Cytosine arabinoside	2.4	20	i.v. 100 mg/m^2
Doxorubicin	0.5	2	i.v. 60 mg/m^2
Mitomycin C	0.23	1	i.v. 10 mg/m^2
Paclitaxel	6.8	6	i.v. 275 mg/m^2
Temozolomide	10	10[a]	i.v. 150 mg/m^2
Treosulfan	3	50	oral 1 g/day
Vincristine	0.4	1	i.v. 1.5 mg/m^2

[a]Temozolomide increased to 20 mg/ml from 96M014.

level of TDC tested as Index = 700 − Sum[Inhibition$_{3.13 \ldots 200}$].[10] An arbitrary level of 50% inhibition (Index below 350) was used to assess relative *ex vivo* sensitivity or resistance (Table 4).

Table 3. Results of testing for each tumor expressed as a simple summary index of inhibition across the range of concentrations tested for single agents, and paclitaxel and combinations (low values indicate considerable inhibition, while higher values indicate resistance; values greater than 700 indicate growth greater than control wells, which is likely to be artefactual and simply reflects resistance)

TCA no.	Cytosine arabinoside	Cisplatin	5-Fluorouracil	Doxorubicin	Mitomycin C	Temozolomide	Treosulfan	Vincristine
96M002	413	NT	924	898	448	NT	NT	NT
96M005	170	753	1159	715	476	NT	306	563
96M008	92	NT	1423	374	275	NT	NT	NT
96M009	06	NT	NT	NT	NT	NT	284	NT
96M010	79	149	1115	429	157	NT	133	153
96M011	304	NT	NT	NT	NT	793	128	NT
96M012	NT	476	713	899	752	404	NT	413
96M013	NT	620	507	251	NT	NT	190	830
97M015	NT	405	642	NT	NT	789	233	422
96M016	453	530	1169	NT	332	792	275	659
96M018	346	786	NT	736	NT	NT	NT	NT
96M019	515	560	752	575	NT	593	398	576
97M001	NT	515	NT	731	NT	421	NT	554
97M002	454	401	581	719	424	526	509	430
97M003	436	573	795	629	584	661	376	469
97M004	685	625	528	465	398	619	538	671
97M006	640	681	NT	429	NT	874	509	664
97M007	751	736	NT	606	NT	914	720	603
97M008[a]	820	975	NT	306	NT	638	738	724
96M010	248	NT	NT	406	NT	419	379	NT

TCA no.	Paclitaxel	Cisplatin–paclitaxel	Doxorubicin–paclitaxel	Cisplatin–vincristine–temozolomide	Tresulfan–cytosine arabinoside
97M004	452	NT	NT	NT	257
97M006	220	344	334	740	249
97M007	393	419	356	1050	464
97M008[a]	406	283	284	356	610
97M010	371	NT	417	NT	206

[a]Tumor treated by proton beam ratiotherapy.

Table 4. Summary of results: treosulfan is the most active single agent tested and the combination with cytosine arabinoside is effective in three of five tumors tested; combinations including paclitaxel are effective, but the combination of cisplatin with vincristine and temozolomide (similar to DTIC) is ineffective in the small number of tumors tested

Drug	Sensitivity in ATP-TCA	Number assessed	Sensitivity (%)
5-Fluorouracil	0	12	0
Cisplatin	1	15	7
Cytosine arabinoside	7	16	44
Doxorubicin	2	16	13
Mitomycin C	3	9	33
Paclitaxel	1	5	20
Temozolomide	0	13	0
Treosulfan	7	15	47
Vincristine	1	14	7
Cisplatin–paclitaxel	2	3	67
Doxorubicin–paclitaxel	3	4	75
Cisplatin–vincristine–temozolomide	0	3	0
Treosulfan–cytosine arabinoside	3	5	60

Results

Evaluable results were obtained from 20 of 28 melanomas tested, giving an evaluability rate of 71%. The reasons for non-evaluability were low MO values indicating poor tumor cell survival ($n=7$) and low viable cell number before plating ($n=1$). No tumors became infected before or during culture. Evaluability of tumors dissociated using a weak collagenase digestion step was better (12 of 15, 80%). A typical result is shown in Figure 1 for two plates from one tumor, showing resistance to all seven drugs tested with the exception of treosulfan.

Collected data using the TCA index is shown in Table 3 for all nine agents and four combinations tested. It is readily apparent that primary uveal melanomas differ in their sensitivity to different agents, but most are at least weakly sensitive to alkylating agents and often to cytosine arabinoside. The inhibition due to cytosine arabinoside appears to be more intense in the earlier assays and may be influenced by cell stress as the result of the strong enzymatic tumor dissociation used in these cases. The summary data (Table 4) confirms the relative efficacy of alkylating agents, particularly treosulfan, in comparison with the other agents tested. Treosulfan appeared to be the best single agent. Since we have had some success with the combination of cisplatin-cytosine arabinoside[11] and more recently gemcitabine-cisplatin in ovarian tumors tested with the assay (Kurbacher *et al.*, unpublished), we decided to examine the combination of treosulfan with cytosine arabinoside in the assay. The results from one tumor tested with multiple dilutions of treosulfan and cytosine arabinoside are shown in Figure 2. There is clear evidence of modulation of treosulfan-induced inhibition by the addition of cytosine arabinoside, despite the fact that in this tumor cytosine arabinoside alone is ineffective.

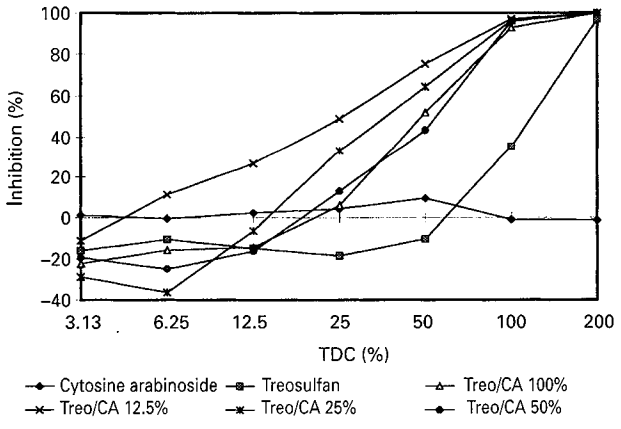

Figure 2. Combination of treosulfan with cytosine arabinoside in the assay. Cytosine arabinoside alone is ineffective at 3.13–200% of its TDC, while treosulfan is effective at higher concentrations. When cytosine arabinoside (CA) is added at concentrations from 12.5 to 100% the degree of inhibition achieved by treosulfan (Treo) is enhanced at lower concentrations. The negative inhibition shown at low concentrations for several of these curves suggests that the MO (no drug) control result was lower than it should be in this assay, possibly due to cooling or drying of the plate during the assay period: it does not represent growth in the presence of the drug.

The case treated with proton beam radiotherapy (Table 3) is interesting. This tumor was resistant to alkylating agents, but sensitive to doxorubicin and paclitaxel. The combination of treosulfan with cytosine arabinoside was ineffective, but the paclitaxel-doxrubicin combination was effective, albeit without 100% cell inhibition at 200% TDC of both agents. Although few tumors were tested with paclitaxel, this agent appears to be active in at least a proportion of uveal melanomas. However, combined addition of doxorubicin or cisplatin with paclitaxel usually produced little advantage over paclitaxel alone.

Discussion

Although this study shows that uveal melanoma is a chemoresistant tumor, there is variation between patients and this heterogeneity suggests that at least some patients could benefit from systemic chemotherapy. Treosulfan is the most active agent yet tested and its combination with a cytosine analog such as cytosine arabinoside or the less toxic gemcitabine may well prove useful in patients with metastatic disease. It would be possible to test this combination in the assay alongside one or two more conventional combinations using needle biopsies of metastatic lesions[9] before treatment was given.

The profile of chemosensitivity is of interest. Alkylating agents are clearly superior to metabolic inhibitors such as 5-fluorouracil and spindle inhibitors such as vincristine, which show no effect at all. A few tumors showed sensitivity to doxorubicin or vincristine, but many were resistant (Tables 3 and 4). These results are consistent with the expression of the *MDR1* multiple drug resistance gene by a high proportion of these tumors.[12] Paclitaxel shows some efficacy and, in combination with doxorubicin, might be effective clinically. The efficacy of this combination in some instances may in part be explained by the use of cremophor to maintain solubility of the paclitaxel in the i.v. preparation: this has been shown to block MDR1.[13] Cisplatin showed weak activity, but with a

consistent dose-response curve suggesting that dose intensification of platinum-based compounds should be explored. This corroborates the clinical studies suggesting that high local concentrations of carboplatin are effective.[2,5] We have previously shown that cisplatin and carboplatin are equipotent in the assay at clinically relevant concentrations for ovarian cancer.[8] Temozolomide is a derivative of DTIC which is active *in vitro*.[14] Although DTIC is used with some success in cutaneous melanoma,[15] it is of little value in uveal melanoma clinically.[1,2] The assay results appear to confirm this.

The chemoresistance of uveal melanoma may be explained by several factors: uveal melanoma is a slow-growing tumor with constitutive MDR1 expression,[12] known to be a major factor in anthracycline and etoposide resistance.[16,17] Slow growing cells are unlikely to respond to vincristine, which will also be affected by the MDR phenotype.[17] In the presence of an intact p53 pathway, DNA damage will lead to cell cycle arrest and repair of DNA damage instead of apoptosis.[18-20] The susceptibility to enhancement of the alkylating agent response by cytosine arabinoside[11] suggests that DNA repair is important in the resistance of uveal melanoma to alkylating agents. At low doses, cytosine analogs inhibit DNA polymerase, which is required to repair the double-stranded cross-links induced by drugs such as treosulfan.[21] Recently, a new derivative of cytosine arabinoside, gemcitabine, has been shown to have greater effects on solid tumors.[22] Previous experience with gemcitabine in modulating cisplatin activity in ovarian cancer both clinically and *in vitro* suggests that this should be used in preference to cytosine arabinoside in combinations.[23]

Clearly, primary tumors are different to metastases, and although previous studies of primary and metastatic tumor tissue in breast and ovarian cancer[8-10] suggest that there are no major differences, metastatic uveal melanoma may grow more rapidly than the primary tumor with a more less differentiated phenotype.[24] We cannot therefore be certain that primary uveal melanoma sensitivity will reflect the sensitivity of a metastatic tumor, but experience suggests that this likely, particularly as faster-growing cells usually show greater chemosensitivity.[25] Future studies will certainly include needle biopsies of liver lesions, as the assay is sensitive enough for this purpose.[9] Use of the ATP-TCA shows considerable promise as a guide to the choice of chemotherapy in individual patients,[9] as a means of developing new regimens,[11,26,27] as a way of assessing the impact of molecular alterations on chemosensitivity[20,28] and in this paper as a way of studying the chemosensitivity profile of rare tumors. The combination of treosulfan plus cytosine arabinoside or gemcitabine holds considerable promise and we hope to identify other active combinations using a similar approach in the future.

Acknowledgments

We wish to thank Dr P Ashman (Schering-Plough Ltd), Marylin Shedden (Medac Ltd) and Dr Alison Jeynes (Bristol-Myers Ltd) for their assistance in providing stock drugs used in the assay. We are grateful to M Neale, R Alexander, R Hart and R Patel for their assistance.

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(Received 12 July 1997; accepted 18 July 1997)