The chemosensitivity profile of retinoblastoma

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The Chemosensitivity Profile of Retinoblastoma

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Abstract

Retinoblastoma is a rare malignant tumour of the developing retina with an incidence of 1 in 20,000 live births in all human races. Chemotherapy is used in retinoblastoma as adjuvant therapy to prevent the growth of metastases and to treat metastatic disease once this has become clinically apparent. Current regimens are based on empirical drug combinations, and few clinical trials have been conducted because of the rarity of this tumour. Chemosensitivity testing offers a way of testing a large number of agents against tumours. The ATP-based chemosensitivity assay (ATP-TCA) has already helped to design new regimens for melanoma and breast and ovarian cancer. Primary retinoblastoma tumour material was obtained from 10 eyes, 7 of which contained sufficient viable cells for ATP-TCA. The results show very high sensitivity to single agents, particularly cisplatin, doxorubicin and vinca alkaloids. Of the anti-metabolites tested, 5-FU is relatively disappointing (although still active), and gemcitabine shows considerable activity consistent with a cytotoxic effect. The shape of the inhibition curves is interesting. There is a plateau effect with the topoisomerase inhibitors and vinblastine, which is not present with the cisplatin. One tumour was much more resistant than the others tested, particularly to vinblastine but also to the topoisomerase inhibitors, which failed to achieve complete kill at any concentration tested, consistent with a multidrug resistance phenotype. Of the combinations (VAC and VEC), the VAC regimen looks marginally more active in the more resistant of the two cases tested to date. These data confirm that retinoblastoma is a rapidly growing malignancy that is very susceptible to cytotoxic drugs of all types. Chemosensitivity testing provides a practical method of testing new regimens before clinical trials in retinoblastoma patients.

Introduction

Retinoblastoma is the most common primary intraocular tumour in children, with an incidence of 1 in 15,000-20,000 live births (Finger et al. 1999). More than 90% of cases are diagnosed before 5 years of age, and presentation of retinoblastoma in adults is rare (Biswas et al. 2000).

Successful treatment of retinoblastoma has traditionally depended on surgery and external beam radiation therapy, associated with significant short- and long-term morbidity (reviewed in Wilson et al. 2001). Recently, multiple studies have been published reporting initial experiences with an association of systemic chemotherapy and focal methods as a primary treatment for this type of cancer (Shields and Shields 1999; Friedman et al. 2000).

Chemotherapy is used in retinoblastoma as adjuvant therapy to control intraocular tumour growth with or without radiation, to prevent the growth of metastases and to treat metastatic disease once this has become clinically apparent (Gallie et al. 1996; Kingston et al. 1996; Gunduz et al. 1998). Current regimens are based on empirical drug combinations derived from neuroblastoma treatments, which have been found to work (Gobie et al. 1990; Kingston et al. 1996). Few clinical trials have been conducted because of the rarity of this tumour and the difficulty of funding such trials.

The current regimens in use tend to use the following four drugs:

- Carboplatin
- Etoposide or doxorubicin
- Vincristine

Carboplatin is a less toxic platinum compound than cisplatin but acts similarly by platination of DNA. The platinum adducts are usually repaired by mismatch repair, deficiency of which is associated with greater sensitivity to this agent (Brown et al. 1997). Etoposide is a podophyllin inhibitor of topoisomerase II, an enzyme responsible for unfolding DNA during transcription and replication. It is associated with an increased risk of leukaemia and is therefore rarely used as a first-line drug for other tumour types (Felix 1998). Doxorubicin is an anthracycline inhibitor of topoisomerase II which has recently been shown also to have some activity against topoisomerase I (Foglesong et al. 1992). Vincristine is a vinca alkaloid, one of a class of drugs which inhibit microtubule assembly and are particularly effective against rapidly cycling cell populations.

Metastasis is rare after adjuvant therapy, but potentially devastating. There is a need to design less toxic but equally or more effective regimens with a low risk of mutagenesis which might aid the development of second malignancies (Tucker et al. 1987). This is a particular problem for some alkylating agents (such as cyclophosphamide, which has been used in retinoblastoma) and for etoposide, which is still widely used, although at a dose not expected to cause problems (Shields et al. 1997). Although there is a feeling that more retinal tumours may develop in patients treated with chemotherapy (C.L. Shields, personal communication), there is as yet no evidence of an increase in second malignancies in retinoblastoma patients.

Chemosensitivity testing offers a way of testing a large number of agents against tumours. The ATP-based chemosensitivity assay (ATP-TCA) method does not require the generation of cell lines, unlike the single previously published study of chemosensitivity testing in retinoblastoma (Chan et al. 1989, 1991). ATP-TCA has already helped to design new regimens for melanoma and breast and ovarian cancer (Myatt et al. 1997; Kurbacher et al. 1998; Cree and Kurbacher 1997; Cree et al. 1999). It can also be used to examine the relationship between chemosensitivity and molecular mechanisms of resistance and sensitivity (Petty et al. 1994; Satherley et al. 2000).

Materials and Methods

Tumours

Material from ten untreated primary retinoblastomas [2 M:8 F, patient median age 5 months (range 2-36 months)] and one skin metastasis from a primary tumour which was not required for diagnosis was taken by a histopathologist under sterile conditions and transported to the laboratory in cell culture medium (DMEM, Sigma, Poole, UK) with antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin, Gibco BRL, Paisley, UK) at 4°C. Of these samples, only six primary tumours and the metastasis contained sufficient viable cells.

ATP-TCA

Cells were obtained from the tumours by a very gentle enzymatic dissociation, usually 75 µg/ml collagenase for 2 h. Viable tumour-derived cells were separated from dead cells and debris by density centrifugation (Histopaque 1077-1, Sigma), washed, counted and resuspended to 100,000 cells/ml. The cells were used to set up ATP-TCA plates according to Andreotti et al. (1995).

Briefly, cells from enzymatic digestion of solid tumour were placed in 96-well polypropylene microplates at 10,000 cells/well with each drug/combination at six doubling dilutions in triplicate from 200% TDC (test drug concentration) to 6.25% TDC. TDCs were 3 µg/ml for cisplatin (CDDP), 0.5 µg/ml for doxorubicin (DOXO), 16 µg/ml for etoposide (ETO), 45 µg/ml for 5-fluorouracil (5-FU), 12 µg/ml for gemcitabine (GEM) and 0.5 µg/ml for vinblastine (VINB).

The plates were then incubated at 37°C in 5% CO_2 for 6 days. The degree of cell inhibition at the end of this period was assessed by measurement of the remaining ATP in comparison with negative control (no drug, MO) and positive control (maximum inhibitor, MI) rows of 12 wells each. ATP was extracted from the cells and measured by light output in a microplate luminometer (Berthold Diagnostic Systems GmbH) after addition of luciferin-luciferase.

Statistics

The percentage inhibition for each drug concentration was calculated as 1- [(Test-MI)/(MO-MI)]*100 using at Excel 97 spreadsheet (Microsoft). For each drug-concentration curve, the area under the curve ($Index_{AUC}$), the sum of the inhibition at each concentration ($Index_{SUM}$), the 50% inhibitory concentration (IC_{50}) and the 90% inhibitory concentration (IC_{90}) were calculated as previously described (Cree 1998).

Results

The results show very high sensitivity to single agents, particularly cisplatin, doxorubicin and vinca alkaloids (Table 1). Of the anti-metabolites tested, 5-FU is relatively disappointing (although still active), and gemcitabine shows considerable activity consistent with a cytotoxic effect. The shape of the inhibition curves is interesting. There is a plateau effect with the topoisomerase inhibitors and vinblastine which is not present with the cisplatin (Fig. 1).

Table 1. Median values for AUCs, $Index_{SUM}$, IC_{90} and IC_{50} measured in seven samples. An $Index_{SUM}$ lower than 300 indicates strong sensitivity in the assay, if >95% inhibition is achieved

Drug	$Index_{AUC}$	IC_{90}	IC_{50}	$Index_{SUM}$
Cisplatin	18,409 (17,626-19,021)	18 (6-72)	4 (3-7)	55 (21-135)
Etoposide	17,939 (17,312-18,399)	48 (5-95)	4 (3-5)	51 (29-99)
Doxorubicin	16,963 (16,697-17,621)	17 (6-94)	4 (3-6)	77 (4-127)
Vinblastine	18,769 (11,739-19,041)	11 (6-293)	4 (3-9)	50 (14-256)
5-FU	14,153 (13,915-14,931)	188 (180-195)	12 (11-13)	218 (212-223)
Gemcitabine	18,386 (15,308-19,287)	20 (6-218)	4 (3-5)	50 (4-148)
VEC	18,806 (17,904-19,207)	6 (6-53)	3 (3-4)	21 (9-78)
VAC	19,033 (18,841-19,225)	6 (-)	3 (-)	17 (7-26)

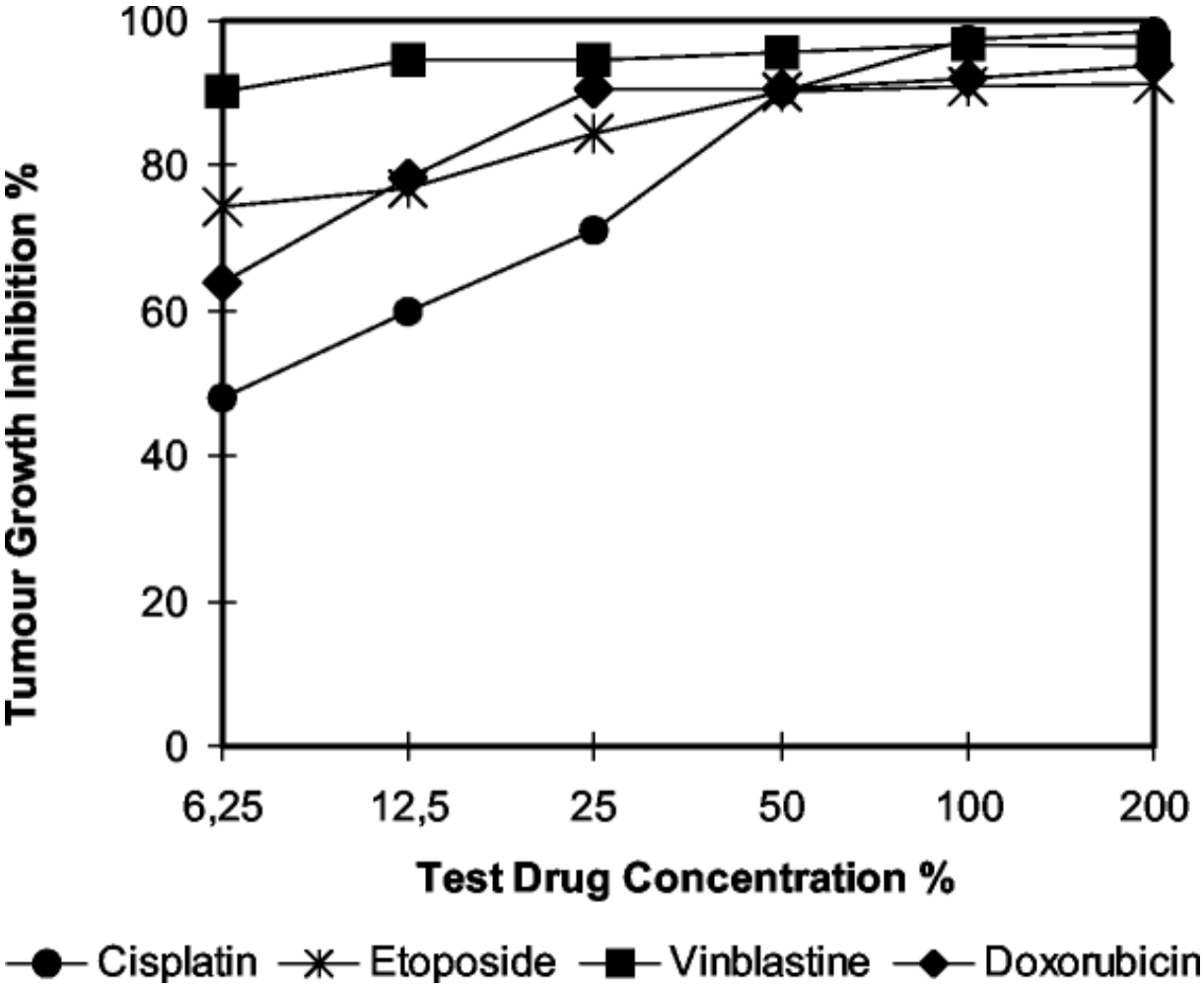

Fig. 1. Example concentration-inhibition curves showing a plateau effect with the topoisomerase inhibitors and vinblastine and not with cisplatin in a retinoblastoma

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One tumour (Fig. 2) is much more resistant than the others tested, particularly to vinblastine but also to the topoisomerase inhibitors, which fail to achieve complete kill at any concentration tested. This may represent overexpression of MDR1 by a proportion of the cells present, and it would be helpful to check this with immunohistochemistry.

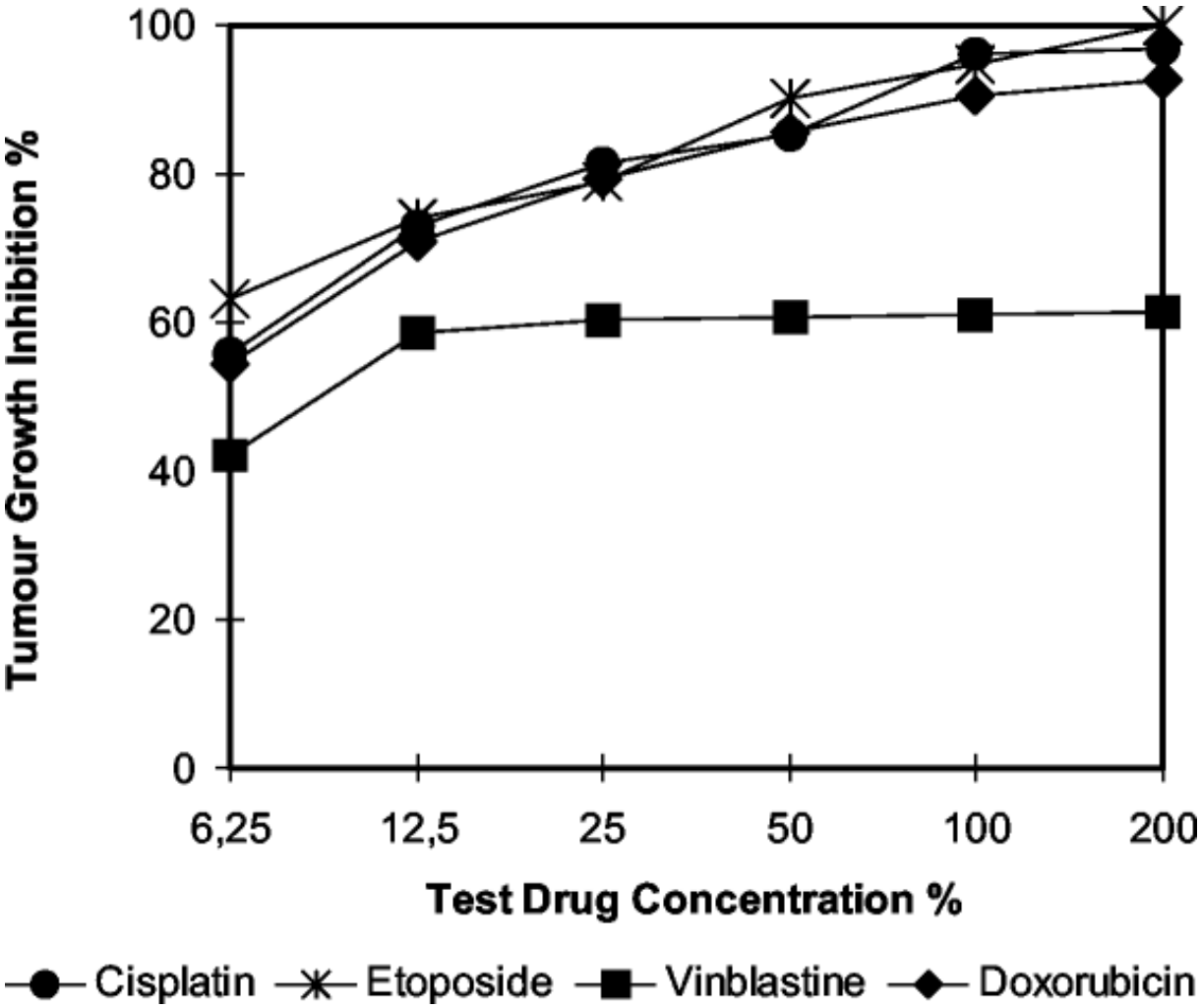

Fig. 2. Example concentration-inhibition curves in a resistant retinoblastoma

Of the combinations (VAC and VEC), the VAC regimen looks marginally more active in the more resistant of the two cases tested to date.

Discussion

These data confirm that retinoblastoma is a rapidly growing malignancy that is very susceptible to cytotoxic drugs of all types. Based on other tumour types, all these results show significant activity, although in one case none produced complete ATP inhibition consistent with 100% cell kill. This is probably academic in most patients. The best dose-response effect was seen with cisplatin, which probably indicates that platinum is the most effective drug here. The combination with doxorubicin is likely to be as active as the three-drug combination, and the availability of liposomal preparations (Doxil/Caelyx) should allow high concentrations to be achieved with a lower risk from MDR+ tumours. Addition of an MDR inhibitor in such patients may be more rational than the use of the current three-drug regimen incorporating vincristine.

The major risk to survival in these patients is second malignancy. Etoposide use is associated with haematogenous malignancy and could probably be avoided. The evidence of this study suggests that doxorubicin is equally effective and it does carry lower risk. However, retinoblastoma patients may not behave in the same way as those with other malignancies in this regard, given the propensity for DNA damage to kill rapidly growing cells with Rb mutations.

Drug resistance can occur, and it may arise in several different ways. There is relatively little information on the relative importance of these mechanisms in retinoblastoma, although as a tumour with a relatively simple molecular pathogenesis, it may have much to teach oncology in general. The MDR1/PgP mechanism has been studied in some detail by the Toronto group. Up to 15% of retinoblastomas express the MDR1 drug efflux protein, and there is evidence that resistant cases express MRP (Chan et al. 1997). Cyclosporin, an inhibitor of MDR1, can augment the control of retinoblastoma (Chan et al. 1996), and new inhibitors are in development. Other mechanisms are less well studied: LRP and MRP are alternative drug efflux pump molecules similar to PgP in their function; resistance to DNA-damaging agents could be influenced by anti-apoptotic mechanisms. In retinoblastoma, p53 inactivation and $p21^{Waf1}$ expression have recently been implicated in resistance to apoptosis, as could BCL-2 expression, which also occurs in retinoblastoma (Divan et al. 2001).

Although chemotherapy for retinoblastoma is already very successful, several newer cytotoxic drugs are available with improved side effect profiles and anti-neoplastic activity, including different mechanisms of action. While it would be difficult to justify clinical trials of the large number of different options in retinoblastoma, it would be sensible to explore these options pre-clinically and then conduct a trial against existing practice.

In this study we have shown that ATP-TCA can be used to study the chemosensitivity profile of retinoblastoma samples despite the small numbers of cells available. The results show that this is a very chemosensitive tumour, in keeping with its high growth fraction and the observed clinical efficacy of the drugs tested. Similar data have been produced by Gertjan Kaspers' group in Amsterdam with the MTT assay (Kaspers and Veerman, *vide infra*). This suggests that chemosensitivity testing of retinoblastoma is feasible and that it can be used to improve understanding of the sensitivity to/resistance of retinoblastoma to chemotherapy and to develop new drug combinations.

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