The effect of tricyclic antidepressants on cutaneous melanoma cell lines and primary cell cultures

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The tricyclic antidepressants have previously been shown to exert activity against glioma cells *in vitro*. Initial studies in cell lines suggested that this might extend to melanoma cells. We have therefore conducted a study in primary cell cultures from metastatic cutaneous melanoma deposits using a well established ATP-based tumour chemosensitivity assay to confirm and extend these findings. Two cell lines and eight primary cell cultures from metastatic melanoma deposits were exposed to three tricyclic drugs, amitriptyline, nortriptyline and clomipramine, at concentrations ranging from 200 to 6.25 μmol/l in the ATP-based tumour chemosensitivity assay. All three drugs showed activity, although nortriptyline was more active than clomipramine or amitriptyline in both cell lines and primary cell cultures, with an IC50 of 9, 27 and 33 μmol/l, respectively. Tricyclic agents show activity against melanoma *in vitro*. This could be related to the lysosomal effects based on their cationic amphiphilic properties, or effects at the mitochondrial membrane. *Anti-Cancer Drugs* 23:65–69 © 2011 Wolters Kluwer Health | Lippincott Williams & Wilkins.

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Background

Metastatic melanoma has a poor prognosis, with a median survival rate of 6 months and a 5-year survival rate of less than 5% [1]. Chemotherapy is largely ineffective and long-term remissions are rare. In the last few years, it has become clear that melanomas differ in their genetics and in many patients, it is hoped that drugs targeting activating mutations within growth receptor pathways will have a beneficial effect, and have less toxicity than chemotherapy. Recent results with BRAF inhibitors are particularly encouraging [2–4]. Nevertheless, alternative strategies are urgently required.

Previous research in our laboratories has shown that some tricyclic antidepressants have the ability to induce apoptosis in malignant glioma and neuroblastoma cells *in vitro* [5–7]. Tricyclic antidepressants have been used to inhibit the serotonin and norepinephrine transporters competitively for over 40 years. The lipophilic compounds, which were first shown to block oxygen consumption in yeast and human fibroblasts [8,9], are thought to exert their effect by means of the inhibition of complex III of the mitochondria [5,10]. By blocking the mitochondrial electron transport chain, the tricyclics are thought to initiate an increase in superoxide production and hence hydrogen peroxide production, and decrease in membrane production, possibly mitochondrial permeability transition pore formation. There is an associated release of cytochrome C, pro-caspase 9, caspase 3 and endonuclease G resulting in DNA degradation and apoptotic death by means of the intrinsic pathway.

Initial studies performed by one of us (V.A-M.) using cell lines suggested that melanoma cells might also be susceptible to tricyclics. We have therefore performed a more comprehensive study of both melanoma cell lines and primary cell cultures to determine the potential of these agents using a highly standardized ATP-based tumour chemosensitivity assay (ATP-TCA) previously used to show the effects of drugs in melanoma [11,12] and to design active combinations [13].

Materials and methods

Cell lines

Cell lines (SK-MEL28 and SK-MEL2) were obtained from Cancer Research UK (Sutton, UK) and grown in RPMI1640 cell culture medium (Sigma-Aldrich, Poole, UK; R7638)+10% foetal bovine serum (Sigma-Aldrich, F2442) in 75 ml flasks (Corning Life Sciences, High Wycombe, UK) for four to six passages in the ATP-TCA before use, as described below. All cell lines were checked regularly for mycoplasma contamination using aliquots of early passages frozen to allow comparative experiments to be performed.

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Melanoma samples

Samples were obtained as metastatic melanoma deposits in lymph node or skin surgical resection specimens from eight chemonaive melanoma patients (Table 1). In all cases, the material used for sensitivity testing was surplus to diagnostic requirements and was sent to the laboratory unfixed in Dulbecco's modified Eagle's medium+antibiotics as a transport medium, cooled on ice. Written informed consent was obtained from each patient and the study had full ethics committee approval.

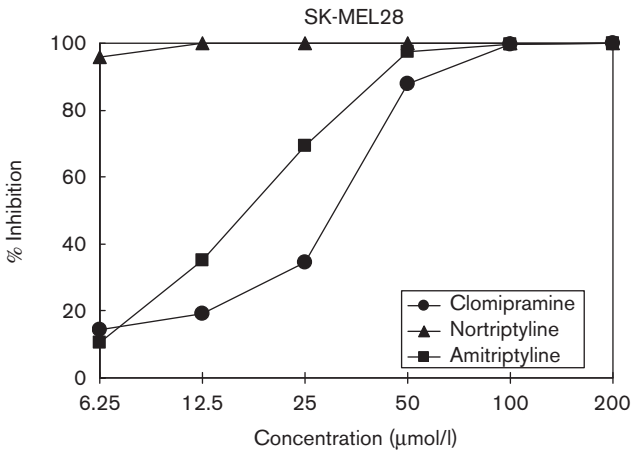
ATP-based tumour chemosensitivity assay

The ATP-TCA is a sensitive cell viability assay using a luminescence endpoint and was performed as previously published [14,15]. The tumour sample was minced and digested overnight with collagenase H (Sigma-Aldrich). Following density centrifugation over Ficoll-Hypaque (Sigma-Aldrich) at 1500 × g for 35 min to remove necrotic material and red blood cells, the tumour cells were washed twice in Dulbecco's modified Eagle's medium with 1% pencillin–streptomycin (Sigma-Aldrich) and plated at 20 000 cells per well with six doubling dilutions of each drug in triplicate in 96-well polypropylene plates (Corning Life Sciences) with a proprietary serum-free medium (Complete Assay Medium, available from DCS Innovative Diagnostik Systeme GmbH, Hamburg, Germany). Cell lines were used directly following trypsin–ethylenediamine tetraacetic acid detachment (Sigma-Aldrich, 4174), washed and resuspended in complete assay medium with antibiotics at 2000 cells per well. All three tricyclic antidepressant drugs (nortyptyline, amitriptyline and clomipramine) were used with concentrations ranging from 6.25 to 200 µmol/l. One row of each plate contained cells with medium only (MO), whereas another row contained cells to which a maximum inhibitor (MI) was added. The plates were incubated at 37°C for 6 days in a humidified incubator with 5% CO_2. At the end of this period, cellular ATP was extracted by the addition of tumour cell extraction reagent (DCS) and measured in white 96-well polystyrene microplates by the addition of luciferin–luciferase reagent. Luminescence was read in a microplate luminometer (MPLX, Berthold Diagnostic Systems, Pforzheim, Germany) and the results were transferred to an Excel spreadsheet for analysis. The coefficient of variation on MO wells for acceptable assays was defined as $< 25\%$ for primary cell cultures, and $< 10\%$ for cell lines.

Table 1 Patients and samples

Case number	Age	Sex	Sample
1	63	Male	Paraspinal muscle deposit
2	69	Female	Thumb skin (primary acral lentiginous)
3	80	Female	Lymph node metastasis
4	83	Male	Skin metastasis
5	42	Female	Lymph node metastasis
6	84	Female	Skin deposits
7	72	Male	Left inguinal nodes
8	79	Female	Lymph node metastasis

Fig. 1



Single agent results for the SK-MEL28 melanoma cell line, showing greater activity of nortriptyline than amitriptyline or clomipramine.

Data analysis

Luminescence values were converted to percentage inhibition values as % inhibition = [1 – (Test – MI)/(MO – MI)]%. For each experiment, a concentration–inhibition curve was constructed, from which IC50 and IC90 values were calculated according to the trapezoidal rule. An index of activity was derived as IndexSUM = Sum(Inh200…Inh6.25) as published previously [14].

Results

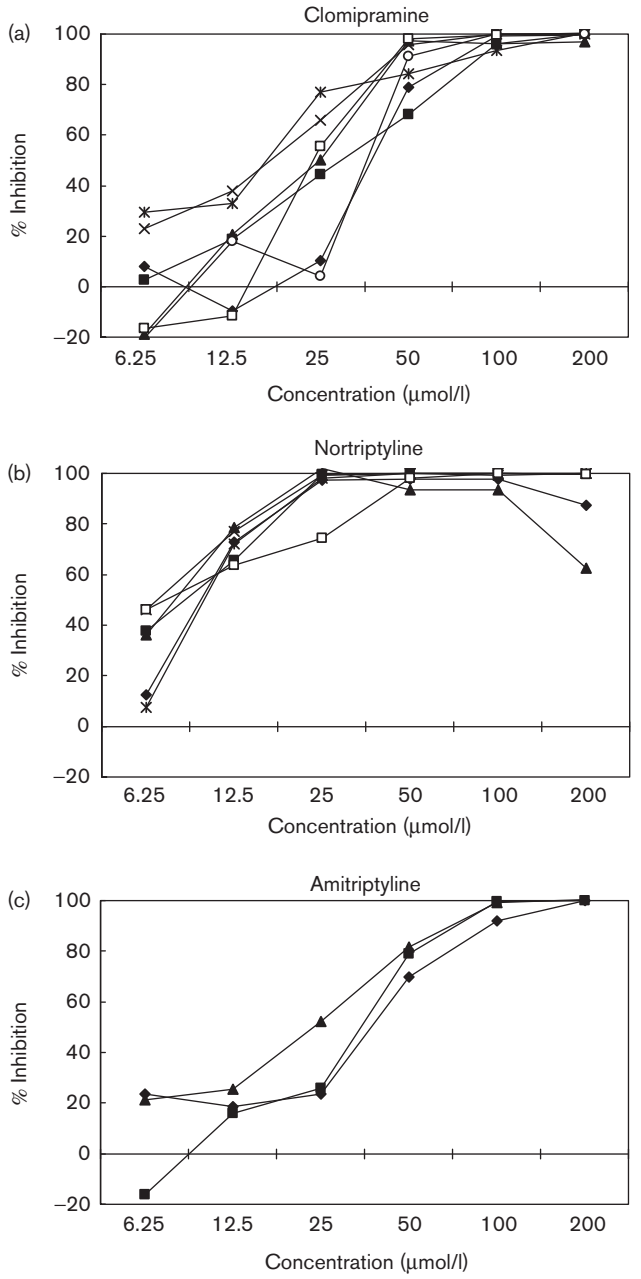
All three agents showed increasing inhibition with increasing concentration in both cell lines (Fig. 1) and primary cell cultures (Fig. 2). In the two cell lines tested, nortriptyline proved to be the most active agent, with lower IC50 and IC90 values in two different passages of SKMEL28, conducted 2 months apart, and in SKMEL2 cells which seemed more resistant and were slow growing (Table 2). As shown in Fig. 1, amitriptyline was slightly more active than clomipramine, but still considerably less active than nortriptyline.

The same order held true for the primary cell cultures (Fig. 2), although these were less sensitive to nortriptyline (Table 2). In tumour-derived cells and cell lines, the inhibition showed concentration dependency with some variation between individual tumours indicating a degree of heterogeneity of activity.

Discussion

This study shows considerable activity of tricyclic antidepressant drugs against cutaneous melanoma cell lines and primary cell cultures. All three drugs seem less active in primary cell culture, but not as much as that

Fig. 2



Melanoma primary cell cultures for (a) clomipramine, (b) nortiptyline and (c) amitriptyline showing considerable heterogeneity of sensitivity, although overall higher inhibition for nortriptyline than clomipramine or amitriptyline.

observed with cytotoxic agents in ovarian cancer [16] and melanoma (Fernando *et al.*, unpublished), in which the difference can be considerably greater, and is thought to be a result of the higher growth fraction present in cell cultures in serum-containing media than that present in serum-free culture, or indeed within the tumour.

The order of activity is interesting as all three drugs were used at the same concentration and suggests a structure–activity relationship. Although amitriptyline is a sedative tricyclic, related to its preferential activity on serotonin re-uptake, nortriptyline is considered to be stimulating, inhibiting the reuptake of norepinephrine. In comparison with clomipramine, both amitriptyline and nortriptyline have replacement of a nitrogen in the dibenzocycloheptadiene group by a carbon atom [10]. However, this does not explain the differential activity of nortriptyline. In glioma cells, as in this study, both nortriptyline and norclomipramine are more active than their counterparts, clomipramine and amitryptiline which lack a methyl group [10]. Inhibition of complex III of the mitochondria [5,10] has been postulated as a mechanism of action, and it may be that these drugs are more active against this mitochondrial target, although no structure–activity relationship has yet been demonstrated in isolated mitochondria.

The activity of tricyclic antidepressants is thought to be largely dependent on expression of the norepinephrine transporter (SLC6A2), which is known to be expressed in melanoma as RNA by expression profiling [17] (GEO database – GDS1375), and by melanocyte precursor cells [18]. Off target effects have been reported based on their cationic amphiphilic properties, resulting in their concentration within lysosomes [19–21], which could activate lysosomal cell death pathways [22]. It seems possible that similar effects could be responsible for the observed effects of tricyclics on mitochondrial function in gliomas and other tumours [5]. Daniel [20] suggests that 'slow accumulation of psychotropic drugs in acidic subcellular compartments … may partly explain the therapeutic latency of these drugs', which may of course result in pleiotropic effects on other membrane-bound organelles. There is evidence indicating that tricyclics have activity against other tumours, notably gliomas [5,10,23], but also lymphomas and myeloma [24,25], lung, liver, colon, squamous and cervical cancer cell lines [26–28]. Equally, if these drugs are concentrated in lysosome or other organelles important to apoptosis, then they may alter the apoptotic potential of the cells, which we have recently shown to be an important determinant of response to chemotherapy in melanoma [29]. We have not looked at cell death pathways in detail as this was not possible in the current study, using the ATP-TCA, but further studies in this area would be valuable.

Combination studies with chemotherapy would be interesting, but should therefore use lower concentrations of tricyclic agents that those used here. However, it should be noted that the concentrations used in this study are within the therapeutic range: the concentration of clomipramine used equates to around 150 mg daily intake, the upper limit of the recommended dose, although the maximum is 250 mg. For nortriptyline, the maximum recommended is 150 mg daily, and for amitriptyline, the maximum is similarly 150–200 mg

Table 2 Activity of tricyclic antidepressants against (a) melanoma cell lines and (b) primary cell cultures (μmol/l)

	Clomipramine			Nortriptyline			Amitriptyline		
	IC50	IC90	IndexSUM	IC50	IC90	IndexSUM	IC50	IC90	IndexSUM
Cell line									
SKMEL28	59	32	244	6	3	4	43	18	188
SKMEL28	42	81	313	3	6	3	27	47	208
SKMEL2	92	178	420	4	7	18	35	83	260
Primary cell culture									
TORC07–0081	39	77	313	NA	NA	NA	39	96	273
TORC07–0091	31	89	270	NA	NA	NA	NA	NA	NA
TORC08–0009	25	46	257	10	21	134	NA	NA	NA
TORC08–0052	18	45	178	9	21	97	NA	NA	NA
TORC08–0065	17	81	183	8	19	133	NA	NA	NA
TORC08–0084	38	50	307	7	20	77	36	77	296
TORC09–0033	24	45	275	10	21	123	24	74	220
TORC10–0065	NA	NA	NA	8	42	118	NA	NA	NA
Mean	27	62	255	9	24	114	33	82	263
Median	25	50	270	9	21	121	36	77	273
SD	9	19	55	1	9	22	8	12	39

NA, not available (because of insufficient cell numbers for testing).

(http://*www.bnf.org/bnf/current/*). Clearly many patients take tricyclics on a regular basis for their psychological effects and there is some evidence that they may have a preventive effect against both colorectal cancer and glioma [30].

The project was constrained by the small number of melanomas available for study, which is the result of changes to clinical practice that make it much more difficult to obtain material from metastatic melanoma deposits. However, in this instance, the cell line data do seem to be similar to those obtained from tumours, perhaps suggesting that mechanistic studies may be feasible and relevant using these cell lines as a surrogate for primary cell cultures.

In conclusion, although tricyclic agents show activity against melanoma cells *in vitro*, their mechanism of action is debatable. However, in our view, further research in xenograft models would be warranted to confirm these findings.

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Conflicts of interest

There are no conflicts of interest.

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