Pergamon

Biochemical Pharmacology, Vol. 48, No. 4, pp. 743–752, 1994.

0006-2952/94 $7.00 + 0.00

0006-2952(94)E0187-P

CATECHOL-*O*-METHYLTRANSFERASE AS A TARGET FOR MELANOMA DESTRUCTION?

NICO P. M. SMIT,*†‡ AMY J. M. LATTER,§ SANDRA NAISH-BYFIELD,‖ WIETE WESTERHOF,* STAN PAVEL† and PATRICK A. RILEY§

*Department of Dermatology, Academic Medical Center, University of Amsterdam, 1105 AZ Amsterdam, The Netherlands; §Department of Chemical Pathology, University College and Middlesex School of Medicine, University College London, London W1P 6DB, U.K.; ‖Department of Biology and Biochemistry, Brunel University, Uxbridge, Middlesex UB8 3PH, U.K.; and †Department of Dermatology, University Hospital, 2300 RC Leiden, The Netherlands

(*Received* 8 *October* 1993; *accepted* 15 *April* 1994)

Abstract—Catechols may interfere in melanogenesis by causing increased levels of toxic quinones. Several catechols and known inhibitors of the enzyme catechol-*O*-methyltransferase (COMT) were therefore tested for their toxicity towards a pigmented melanoma cell line, UCLA-SO-(M14). The inhibition of thymidine incorporation as a result of exposure to the compounds was measured. All agents were compared to 4-hydroxyanisole (4HA), a depigmenting agent extensively studied as an antimelanoma drug. The compounds were also tested on the epithelial cell line, CNCM-I-(221) in the presence and absence of tyrosinase. All the compounds were more effective than 4HA towards the M14-cells at either 10^{-4} M or 10^{-5} M. The toxicity of 4HA towards the 221-cells was shown to be completely dependent on the presence of tyrosinase. Effects of the test agents on the 221-cells were also observed in the absence of tyrosinase. Although some of them were shown to be good substrates for tyrosinase only small changes in toxicity were observed as a result of the presence of the enzyme in comparison with 4HA. No direct correlation of the toxicity of the agents and COMT inhibition was observed. The possible mode of action of the compounds through inhibition of COMT and interference in melanogenesis is discussed together with other possibilities and factors involved.

Key words: catechol-*O*-methyltransferase; melanoma cytotoxicity; melanogenesis; catechols; 4-hydroxyanisole

Melanogenesis is a process specific for melanocytes and melanoma cells resulting in the formation of the pigment melanin inside a specialized compartment, the melanosome. Melanin is a polymer which contains a high proportion of 5,6-dihydroxyindole-2-carboxylic acid and its derivatives. The initial step in the formation of the indolic precursors of melanin is the oxidation of the amino acid L-tyrosine by the enzyme tyrosinase. Several intermediates of melanin metabolism have been shown to be potentially cytotoxic [1–3] by virtue of the formation of reactive quinone intermediate products (dopaquinone), indolequinone and indole-2-carboxylic acid-5,6-quinone (see Fig. 1). The possibility of amplifying the generation of toxic intermediates by making use of tyrosine analogues has long been viewed as a rational approach for melanoma chemotherapy [see Refs. 4,5 for reviews].

One of the natural protective mechanisms that prevents the accumulation of the indole quinones is the O-methylation of 5,6-dihydroxyindole and 5,6-dihydroxyindole-2-carboxylic acid (Fig. 1). This O-methylation may play a significant role in the detoxification of melanin precursors after leakage from the melanosomes since the O-methylated indoles comprise a large part of the melanogenic indoles that can be detected in urine and are also detected in the culture medium of melanoma cell cultures [6, 7]. The O-methylation of 5,6-dihydroxyindole and 5,6-dihydroxyindole-2-carboxylic acid is catalysed by the enzyme catechol-*O*-methyltransferase (COMT,¶ EC 2.1.1.6) which has been shown to be present in melanocyte and melanoma cell extracts [8]. Inhibition of COMT could be an alternative approach to increasing the concentration of the intracellular quinone intermediates as a tool for melanoma destruction.

In a preliminary study we therefore tested the cytotoxic properties of known or potential COMT inhibitors such as Ro-41–0960: 3,4-dihydroxy-5-nitrophenyl-2-fluorophenyl ketone [9]; OR-462: 3-(3,4-dihydroxy)-5- nitrobenzylidene-2,4-pentanedione; OR-486: 3,5-dinitropyrocatechol [10]; tropolone; pyrogallol [11]; 3,4-dihydroxycinnamic acid; norepinephrine; 2,3-dihydroxynaphthalene; quercetin: myricetin; L-3,4-dihydroxyphenylalanine; and 3,4-dihydroxytryptamine. The effect of each of these agents was compared with that of the tyrosine analogue 4HA (4-methoxyphenol), a compound extensively studied as an antimelanoma agent [12]. The compounds were tested for the ability to inhibit [^{3}H]-thymidine incorporation in human epithelial cells and melanoma cells. They were also screened for their capacity to inhibit COMT enzyme activity

‡ Corresponding author. Tel. (71) 263031; FAX (71) 248206.

¶ Abbreviations: COMT, catechol-*O*-methyltransferase; 4HA, 4-hydroxyanisole; L-dopa, L-3,4-dihydroxyphenylalanine; PBS, phosphate-buffered saline; LDH, lactate dehydrogenase; GDH, glutamate dehydrogenase; 3-OMD, 3-*O*-methyldopa.

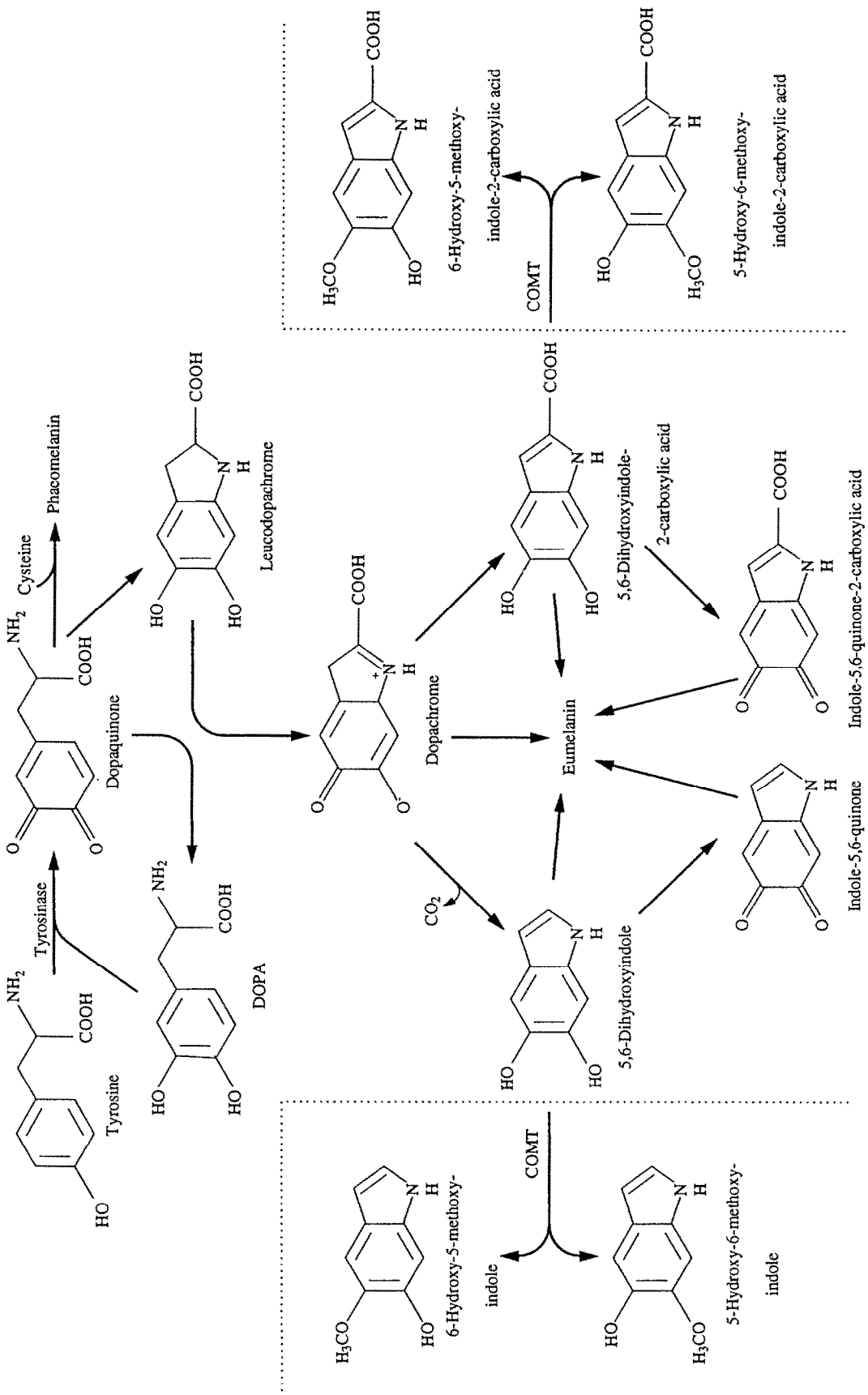


Fig. 1. The biosynthesis of melanin. The methylation products of the indolic intermediates through COMT activity are indicated within the dashed lines.

and tested as potential substrates for tyrosinase by combined measurement of O_2-uptake and scanning spectrophotometry. Our results indicate that COMT inhibition can be considered a realistic potential target for melanoma chemotherapy.

MATERIALS AND METHODS

Cell cultures. UCLA-SO-M14 melanoma cells [13] were cultured at 37° in a humidified 5% CO_2 atmosphere in Dulbecco's modified Eagle's medium containing 2 mM L-glutamine, 10% FBS, 0.12% $NaHCO_3$, 1000 U/mL penicillin and 1000 μg/mL streptomycin.

CNCM-I-221 epithelial cells were routinely propagated at 37° under 2% CO_2 in minimal essential medium with Earle's salts (Imperial Labs Ltd, Andover, U.K.) containing 0.03% $NaHCO_3$ and FBS, glutamine, penicillin and streptomycin as above. Normal human melanocytes were isolated as previously described [14] and cultured in Ham's F-10 medium containing 16 nM 12-*O*-tetradecanoyl phorbol 13-acetate, 0.1 mM isobutylmethylxanthine and 1% Ultroser-G.

Compounds. The chemical structures of 13 compounds tested are shown in Fig. 2. Compound **1**, 4HA was purchased from Koch-Light (Haverhill, U.K.) and recrystallized prior to use. Compound **2**, Ro-41–0960, was obtained from Research Biochemicals Inc. (Natick, MA, U.S.A.). Compounds **3** and **4**, OR-462 and OR-486, were a kind gift of Dr I. Ulmanen, Orion Pharmaceutica, Espoo, Finland. Compounds **5**, **6**, **7** and **9**, tropolone, pyrogallol, 3,4-dihydroxycinnamic acid and 2,3-dihydroxynaphthalene, were from Aldrich, Steinheim, Germany. Compound **8**, L-norepinephrine, was from Serva, Heidelberg, Germany. Quercetin (**10**) and L-3,4-dihydroxyphenylalanine (L-dopa, **12**) were from Merck, Darmstadt, Germany. Compound **11**, myricetin, was from Fluka, Buchs, Switzerland and Compound **13**, 3,4-dihydroxytryptamine, was from the Sigma Chemical Co., St Louis, MO, U.S.A.

Cytotoxicity tests. The compounds were tested as described previously [15]. The cells were harvested by trypsinization and resuspended at 5×10^4 cells/well. One mililitre was inoculated in each of the central eight wells of a 24-well dish with the outer wells filled with 1 mL serum-free medium to prevent dehydration. After 48 hr the cells were washed with PBS and subsequently exposed to the agents in PBS at 10^{-4} M and 10^{-5} M, for 30 min at 37°. The cells were incubated with the agents in four wells for each concentration and in case of the CNCM-1-221 cells the agents were applied in the absence as well as in the presence of (343 U) mushroom tyrosinase (Sigma) (16 wells/test agent). Effects of the compounds were compared to control cultures incubated without and with tyrosinase in PBS, respectively. At the chosen tyrosinase concentration phenolic compounds like 4HA are fully oxidized [15]. The tyrosinase-containing M14-melanoma cells were incubated in quadruplicate wells in the absence of tyrosinase only, with the compounds at both concentrations (8 wells/test agent). At the end of the exposure the cells were washed twice with PBS followed by a 30 min incubation with growth medium containing 1 μCi [methyl-^{3}H]thymidine; radioactive concentration 37 MBq/mL (Amersham Intl plc, Amersham, U.K.). Cells were then washed 5× with PBS, extracted with 1 mL cold 5% trichloroacetic acid, washed 2× with PBS and dried under a stream of hot air. The cells were digested overnight in 250 μL 1 N NaOH at 37° and 100 μL aliquots were transferred to 4 mL Ecoscint A and counted in a Beckman scintillation counter.

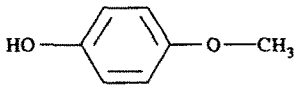
COMT was measured as previously described with M14-melanoma crude cell extracts as the source of the enzyme [8]. All compounds were tested for COMT-inhibitory action in the assay at 10^{-4} and 10^{-5} M. After 1 hr of incubation the reaction was stopped with 0.4 M perchloric acid. Protein was removed by centrifugation in an Eppendorf centrifuge and 20 μL supernatant was analysed by HPLC separation. The substrate (5,6-dihydroxyindole-2-carboxylic acid) and the product peaks (5-hydroxy-6-methoxyindole-2-carboxylic acid and 6-hydroxy-5-methoxyindole-2-carboxylic acid) were detected with a Spectrovision fluorimetric detector with emission and exitation wavelengths set at 315 and 360 nm, respectively.

Testing of compounds as substrates for tyrosinase. The compounds were tested as substrates for tyrosinase in order to investigate whether a possible cytotoxic effect of the agent could be ascribed to the formation of quinone products of the different catechols used. Compounds were tested at 500 μM in PBS and the effect of tyrosinase (343 U/mL) was studied by scanning spectrometry using a Hewlett Packard, UV–Visible Diode-Array, spectrophotometer which measured the spectral range from 190 to 600 nm with a scanning cycle time of 30 sec. Simultaneously, O_2-uptake from the incubation mixture was measured with a Clark-type electrode (Yellow Springs Co.).

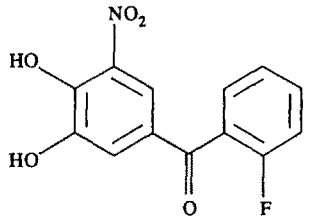
Density gradient fractionation of melanocytes. Cultured human melanocytes were harvested and fractionated on a Nycodenz gradient as previously described [16]. Marker enzymes for the cytosol (lactate dehydrogenase), melanosomes (tyrosinase) and mitochondria (glutamate dehydrogenase) were measured in the gradient fractions [16] and compared to the localization of COMT.

RESULTS

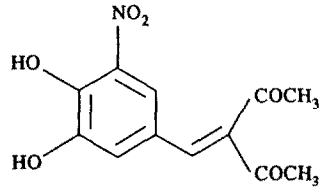
The cytotoxicity of the compounds was tested and compared to 4HA as the reference compound. In Fig. 3 the relative toxicity of the compounds towards the M14-melanoma cells is illustrated by the percentage of inhibition of thymidine incorporation. At 10^{-4} M final concentration a 24% inhibition was obtained with 4HA. At this concentration, nine of the test compounds showed higher inhibition of thymidine incorporation. At the lower drug concentration (10^{-5} M) 10 of the 12 compounds were more effective than 4HA. No inhibition of thymidine incorporation was found after exposure of cells to Ro-410960 and OR-462 at 10^{-5} M (2.0% and 0.7% increase, respectively was found in comparison to the controls). Compounds **4**, **5** and **12** (OR-486,



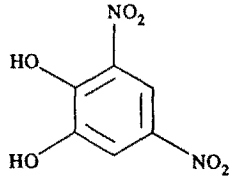
1; 4-Hydroxyanisole (4HA)



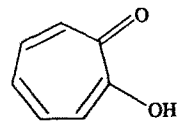
2; Ro-41-0960



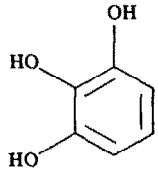
3; OR-462



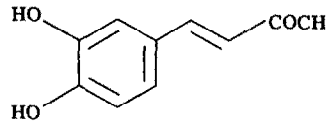
4; OR-486



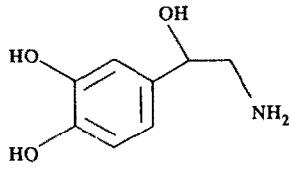
5; Tropolone



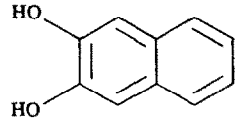
6; Pyrogallol



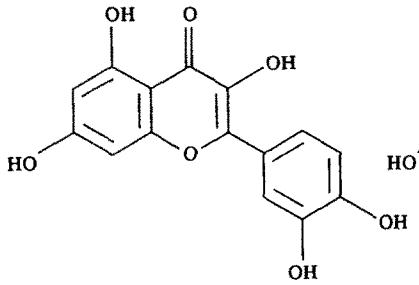
7; 3,4-Dihydroxycinnamic acid



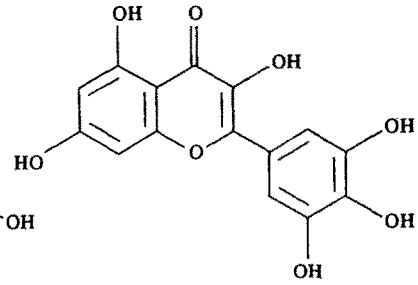
8; L-Norepinephrin



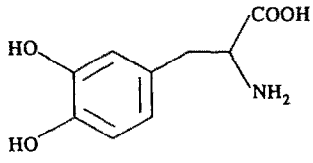
9; 2,3-Dihydroxynaphthalene



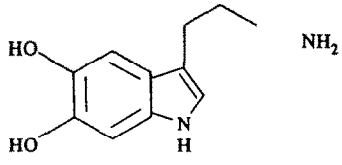
10; Quercetin



11; Myricetin



12; 3,4-Dihydroxyphenylalanine



13; 5,6-Dihydroxytryptamine

Fig. 2. Structural formulae of 4HA and the 12 test compounds.

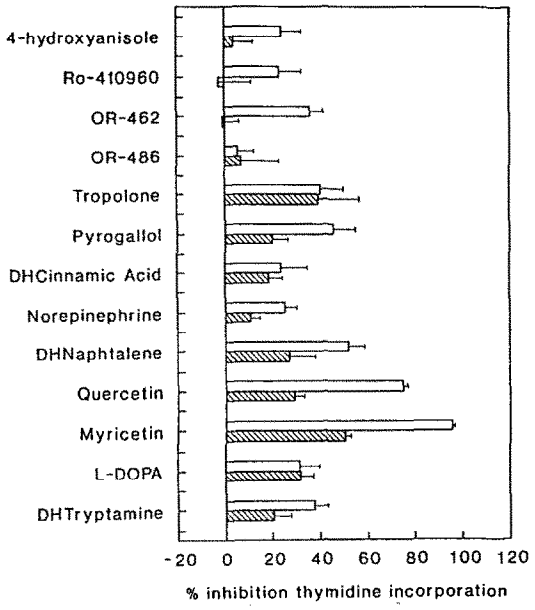


Fig. 3. Inhibition of thymidine incorporation of M14-melanoma cells as a result of exposure to the compounds at 10^{-4} M (▨) and 10^{-5} M (□) as described in Materials and Methods.

tropolone and L-dopa, respectively) were apparently equally effective at 10^{-4} M and 10^{-5} M.

The toxicity of the catechols was also tested on the epithelial CNCM-I-221 cells in the presence and absence of tyrosinase and compared with 4HA (see Table 1, columns 2 and 3 for the compounds at 10^{-4} M without (−) and with (+) tyrosinase, respectively). In contrast to the M14-cells, the 221-cells showed higher values of thymidine incorporation after incubation with 4HA at 10^{-4} M in the absence of tyrosinase (+18%). In the presence of tyrosinase a decrease of >99% was found as compared to the controls. The compounds **2** to **13** all exhibited reduced values of thymidine incorporation in comparison to the increase obtained with 4HA at 10^{-4} M in the absence of tyrosinase (column 2). For the M14 cells this was also the case for most of the compounds (9/12) with only compound **4** showing less inhibition of thymidine incorporation than 4HA at this concentration. With the 221-cells the compounds **6**, **9**, **12** and **13** showed no inhibitory effect in the thymidine incorporation assay at 10^{-4} M. The compounds **2**, **3**, **4**, **5**, **7** and **8** exhibited moderate direct inhibitory effects (inhibition > 12.8 and <28.8%) in the absence of tyrosinase and the compounds quercetin (**10**) and especially myricetin (**11**) showed relatively strong tyrosinase-independent toxicity towards the 221-cells with 49.8 and 95.3% inhibition, respectively. In the presence of tyrosinase (column 3) none of the compounds showed the complete inhibition of thymidine incorporation observed for 4HA. For compounds **2**, **5**, **6**, **7**, **9** and **12** the relative toxicities increased in the presence of tyrosinase. For the compounds **6**, **9** and **12** this toxicity was only found in the presence of tyrosinase. In some cases, however, tyrosinase seemed to protect the cells since a reduction of the inhibitory effects of the compounds (**3**, **4**, **8** and **10**) was observed.

Table 1. Inhibition of thymidine incorporation in CNCM-I-221 cells in the presence and absence of tyrosinase

	CNCM-I-221 cells							
	% Inhibition [^{3}H]thymidine incorporation +/− (SD)*							
	10^{-4} M				10^{-5} M			
	Tyrosinase							
Compounds†	−		+		−		+	
(1) 4-hydroxyanisole	−18.0	(6.8)	99.7	(0.03)	−5.7	(6.1)	26.0	(3.1)
(2) Ro-41-0960	15.5	(9.1)	23.6	(7.3)	−1.9	(8.2)	13.5	(5.2)
(3) OR-462	19.5	(5.8)	−0.3	(8.0)	12.5	(2.5)	1.4	(7.3)
(4) OR-486	28.8	(3.0)	10.2	(7.3)	33.3	(4.2)	14.8	(6.3)
(5) Tropolone	12.8	(5.6)	17.6	(3.8)	21.1	(3.4)	5.7	(4.0)
(6) Pyrogallol	−0.1	(4.0)	18.8	(4.2)	−8.7	(8.0)	9.4	(7.4)
(7) DHCinnamic acid	14.4	(4.8)	18.4	(7.2)	11.6	(3.5)	3.6	(10.2)
(8) Norepinephrine	27.6	(5.7)	10.8	(4.8)	35.9	(6.6)	8.8	(2.0)
(9) DHNaphthalene	−1.8	(18.3)	21.8	(5.2)	19.0	(8.4)	15.4	(2.5)
(10) Quercetin	49.8	(2.6)	42.6	(7.8)	31.0	(7.3)	22.7	(6.0)
(11) Myricetin	95.3	(0.5)	92.6	(1.1)	18.4	(4.2)	24.3	(2.9)
(12) L-dopa	−5.9	(3.7)	13.4	(6.2)	10.3	(4.5)	11.0	(2.2)
(13) DHTryptamine	−9.3	(1.9)	−7.7	(3.1)	−16.9	(4.5)	−4.7	(4.1)

* All values are related to controls (100%) without (−) and with (+) tyrosinase. Standard deviations (%) (SD in brackets) were estimated from the measurements (cpm) in four different wells (see Materials and Methods section).

† Structural formulae of the compounds are shown in Fig. 2.

Table 2. Relative toxicity of the compounds towards UCLA-SO-M14 and inhibition of COMT activity from M14-cell extract

	M14—Melanoma				
Compounds	Relative toxicity* 10^{-4} M	% Inhibition COMT† 10^{-4} M		10^{-5} M	
(1) 4-hydroxyanisole	1.00	0	(6.9)	0	(1.4)
(2) Ro-41-0960	0.95	100	(0.0)	91.4	(0.6)
(3) OR-462	1.48	92.7	(0.0)	62.9	(3.6)
(4) OR-486	0.22	100	(0.0)	93.8	(0.4)
(5) Tropolone	1.67	17.4	(1.8)	0	(8.4)
(6) Pyrogallol	1.88	41.4	(2.5)	7.6	(5.8)
(7) DHCinnamic acid	0.98	64.4	(3.6)	0	(3.3)
(8) Norepinephrine	1.04	11.3	(3.6)	0	(4.7)
(9) DHNaphthalene	2.14	43.3	(5.1)	4.4	(1.1)
(10) Quercetin	3.09	29.3	(5.6)	0	(1.6)
(11) Myricetin	3.93	16.4	(3.3)	0	(0.7)
(12) L-dopa	1.30	0	(8.4)	0	(0.7)
(13) DHTryptamine	1.55	0	(9.4)	0	(7.6)

* All activities are compared to 4HA (24% inhibition of thymidine incorporation => relative toxicity = 1.00)

† The range of duplicate measurements of COMT activity is given in brackets and is related to the 100% control values.

Table 3. Testing of the compounds as tyrosinase substrates

Compounds	Optimal changes in Absorbance* Wavelength γ (nm) Decrease	Increase	O_2-uptake nmol/min +/− (range)†		Tyrosinase substrate
(1) 4-hydroxyanisole	—	260/420	34.8	(2.7)	yes
(2) Ro-41-0960	—	—	0.3	(0.3)	no
(3) OR-462	—	—	1.2	(0.1)	no
(4) OR-486	—	—	0.9	(0.4)	no
(5) Tropolone	—	—	0.0	(0.0)	no
(6) Pyrogallol	326	444	427.0	(29.9)	yes
(7) DHCinnamic acid	288/314	248/404	182.2	(14.1)	yes
(8) Norepinephrine	—	306/482	29.1	(0.5)	yes
(9) DHNaphthalene	—	—	0.8	(0.0)	no
(10) Quercetin	—	—	0.9	(0.7)	no
(11) Myricetin	—	—	3.7	(1.0)	no
(12) L-dopa	—	306/474	81.6	(4.1)	yes
(13) DHTryptamine	—	322	28.8	(0.5)	possible

* Results of scanning spectrometry of the compounds in the presence of tyrosinase.

† O_2-uptake was performed in duplicate and the range of measurements is given in brackets.

At the lower concentration (10^{-5} M, columns 4 and 5) the tyrosinase dependency is once again most obvious for 4HA. Also for compounds **2** and **6** the inhibition of thymidine incorporation was clearly higher in the presence of tyrosinase. Increased thymidine incorporation in the presence of tyrosinase as observed for compounds **3**, **4**, **8** and **10** at 10^{-4} M was found at 10^{-5} M for these compounds as well. At this concentration a positive effect of tyrosinase was obtained also for compounds **5**, **7**, **9** and **10**. In Table 2 the toxicities as obtained with the M14-cells at 10^{-4} M (Fig. 3) are all expressed as relative toxicities with the 24% inhibition of the M14-cells as the reference value (relative toxicity = 1.00). The values for COMT inhibition in the M14-cell extract with the compounds at 10^{-4} and 10^{-5} M concentrations do not show a direct correlation with their respective cytotoxicities. The possible tyrosinase dependence of the toxicity of the compounds was examined by spectrophotometric scanning of the compounds in the presence of excess tyrosinase and by measuring the O_2-uptake simultaneously (see

Table 3). The tyrosinase catalysed oxidation of 4HA results in a typical shift in the spectrum with increased absorbance at 260 nm and 420 nm of the corresponding quinone product. For compounds **6**, **7**, **8**, **9**, **12** and **13** changes in the spectra were observed as a result of tyrosinase activity that could be indicative for formation of the oxidation products. The O_2-uptake measurements confirmed the oxidation of these compounds. For compound **11** minimal O_2-uptake was measured with no detectable change in the absorption spectrum.

In Fig. 4 the distribution of enzyme activities is illustrated for LDH, COMT, tyrosinase and GDH, with the cytosolic LDH in fractions 18 to 20 representing the top fractions of the gradient. It is obvious that COMT is also present as a soluble enzyme in the cytosol (fractions 18 to 20) of melanocytes. A substantial part of the activity is found lower in the gradient, mainly in fractions 14 to 17, and is therefore considered to be due to the membrane-bound form of the enzyme. The melanosomal enzyme tyrosinase showed a broad peak with optimal activity in the fractions 15 and 16. The mitochondria formed a visible narrow band in the centrifuge tube which was confirmed by the concentration of the mitochondrial GDH activity in fractions 11 and 12.

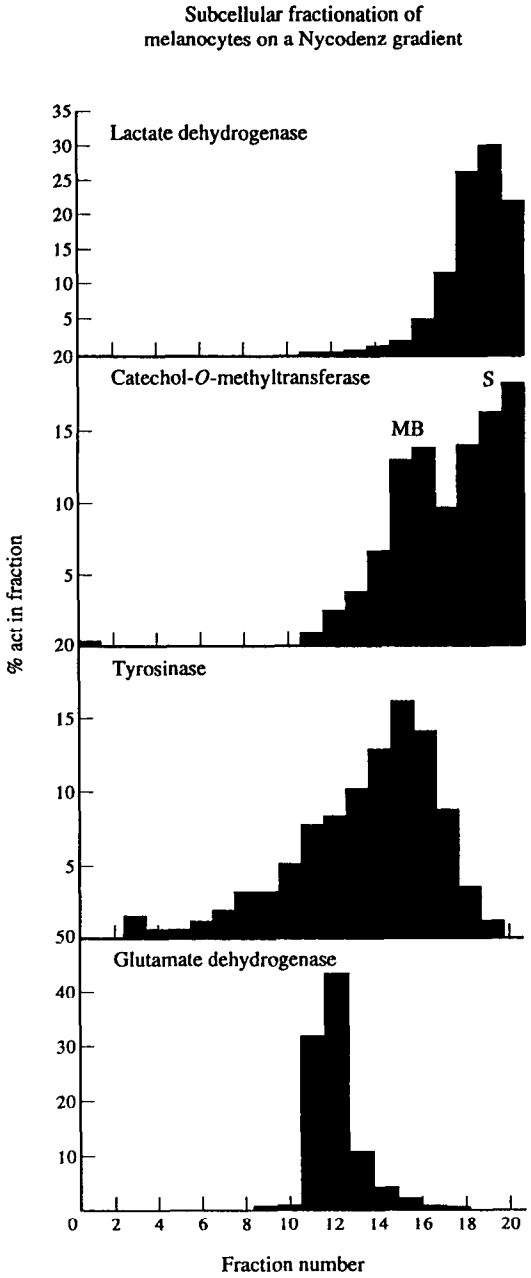


Fig. 4. Distribution of enzyme activities after subcellular fractionation of melanocytes on a Nycodenz gradient (fraction 20 located on top of the gradient). Lactate dehydrogenase is used as marker enzyme for the cytosol, tyrosinase for melanosomes and glutamate dehydrogenase for mitochondria.

DISCUSSION

The results of the estimation of the relative toxicities of the catecholic compounds and *p*-methoxyphenol (4HA) towards the M14 melanoma and the 221-epithelial cell lines indicate a higher sensitivity of the former for these agents. Furthermore, the toxicity of 4HA towards the 221-cells is fully ascribable to tyrosinase action and was almost complete at 10^{-4} M concentration. The effects of the catechols on thymidine incorporation of the 221-cells as expressed by their relative toxicities were much lower for most of the compounds compared to the toxicity of 4HA in the presence of tyrosinase. Except for the two flavonoids, quercetin and myricetin (**10** and **11**), which both exhibited high toxicity in the presence and absence of tyrosinase, all other catechols showed absent or only moderate inhibition of thymidine incorporation in comparison to 4HA + tyrosinase. Although 7 of the 12 catechols were found to be possible substrates for tyrosinase, only in case of pyrogallol (**6**) was increased inhibition of thymidine incorporation observed in the presence of tyrosinase at both concentrations. In most cases, addition of tyrosinase was slightly beneficial or had no effect. Possibly the presence of the tyrosinase protein in the incubation mixture serves as a scavenger for (auto)oxidative products. Thirty substituted phenols including 4HA were previously described as exhibiting cytotoxicity towards the epithelial CNCM-I-221-cells in the presence of tyrosinase due to the formation of the quinone products [15]. In a subsequent study, cytotoxicity of selected phenols towards melanoma cell lines was found, some of which may be mediated by intracellular tyrosinase activity [17]. The selectivity of phenol derivatives like 4HA and others for (malignant) melanocytes has been the topic of discussion ever since these compounds were studied in relation to melanoma therapy [12, 18–20]. A correlation between tyrosinase levels and IC_{90} values in 15 melanoma lines was described for (*p*-hydroxyphenyl)ornithine but not for 3,4-dihydroxybenzylamine [20]. This dihydroxy compound was shown to be equally potent but less selective for melanoma tumour cells than two other mono *p*-

hydroxyphenyl derivatives and L-dopa methyl ester. Although it was shown to be a substrate for tyrosinase a different mechanism for 3,4-dihydroxybenzylamine was suggested in this study [20]. In this regard it should be noted that in case of the melanin precursor L-dopa and its derivative, the L-dopa methyl ester, inhibition of growth of non-melanoma tumours was observed. The degree of inhibition in non-melanocytic cells was always less than that observed for tyrosinase positive cells [21, 22]. The toxicities estimated in this study for the COMT-inhibitors and catechols towards the M14-melanoma line are relatively high especially since the potency of the compounds **2**, **3** and **5** (which were not substrates for tyrosinase) was stronger or equal to 4HA. None of the compounds that acted as a substrate for tyrosinase showed tyrosinase-dependent toxicity comparable to that of 4HA towards the epithelial CNCM-I-221-cells. Different mechanisms for toxicity of the compounds in this study need therefore to be considered. Although no direct correlation with COMT inhibition and toxicity of the compounds was observed this may still play a role in their cytotoxic action. Many factors are involved in the overall cytotoxicity observed for the compounds. In general, the accessibility of the compound to the target enzyme(s) will depend on the localization of the enzyme, the lipophilicity of the agent, and its transport through membranes. If the compounds act principally via COMT inhibition the toxicity would also depend on the rate of formation of indole quinones via melanin metabolism and thus indirectly on tyrosinase activity. Hence the stability of the catechols, their rate of oxidation by tyrosinase and their rate of decomposition could be of great importance in determining their cytotoxicity. In the case of L-dopa no correlation of toxicity with tyrosinase activity in 6 melanoma lines was found but the toxicity has been ascribed to the formation of a stable toxic product (possibly melanin) outside the cell [23]. Radical scavenger enzymes such as peroxidase, superoxide dismutase and catalase were shown to reduce the toxicity of L-dopa and dihydric phenols (dopamine, hydroquinone and terbutylcatechol [23, 24]) which are substrates for tyrosinase. On the contrary, phenols that were not substrates for tyrosinase, e.g. resorcinol, butylated hydroxyanisole and hydroquinone dimethyl ether, did not exhibit reduced toxicity [24]. It was concluded that tyrosinase does not play a role in the *in vitro* toxicity of the catechols tested which is probably due to products of catechol decomposition acting outside the cells [24]. On the other hand, the observed cytotoxic effects of L-dopa and dopa methyl ester towards melanoma cells [21, 22] could be a result of intracellular damage since it was shown that L-dopa [25] and 2-[^{18}F]fluorodopa [26] are selectively incorporated into melanoma cells. The use of L-dopa as an antimelanoma agent is, however, limited because of toxic side-effects on the neural and cardiovascular systems [27]. *In vivo* a large part of L-dopa seems to be metabolized by COMT to the O-methylated product, 3-*O*-methyldopa, in plasma [28, 29]. This is also a problem for using L-dopa as a therapeutic drug for the treatment of neurological disorders such as Parkinson's disease [30]. COMT inhibitors have been studied in relation to Parkinson's disease in order to investigate whether they could be used to increase the bioavailability of L-dopa and its transport into the brain. In this regard the characteristics of most of the inhibitors tested in this study have been discussed in the review by Guldberg and Marsden [11] or were described by others (tropolone [31], dihyroxycinnamic acid [32], norepinephrine [33], quercetin, myricetin [34] and dihydroxytryptamine [35]). Tropolone has also been reported as a strong inhibitor of tyrosinase [36]. 2,3-Dihydroxynaphthalene was included since a strong inhibition of COMT from bovine liver and a high toxicity towards M14 melanoma cells was found for this compound in earlier studies (unpublished results). The different characteristics of the COMT inhibitors may account for some of the effects of the compounds towards the M14 and 221-cells observed in this study.

Recently, the compounds Ro-41-0960 and OR-462 and OR-486 were described as new COMT-inhibitors that were especially designed as stable drugs with favourable pharmacological and toxicological properties by two different groups [9, 10]. These compounds were effective in preventing 3-OMD formation after L-dopa administration [9, 10] and OR-462 proved useful for clinical application in different model systems [37, 38]. OR-462 seems promising for further studies as an antimelanoma agent since it was more effective than 4HA and OR-486 at 10^{-4} M. Apart from 4HA and L-dopa very little is known about the effects on malignant melanoma of the other compounds tested in this study. Only the 3,7-dihydroxyderivative of tropolone (BMY-28438) was recently described as an antitumor antibiotic with specific activity against B-16 melanoma in mice whereas no activity was found for tropolone itself [39]. Furthermore the effects of flavonoids on artificially induced B16-melanoma metastases revealed that treatment with quercetin resulted in increased numbers of metastases [40]. Since high toxicity was found for quercetin and myricetin towards the M14 as well as the 221-cells it needs to be investigated further whether these compounds may be selectively toxic towards the melanoma cells at lower concentrations.

Not all the results of this study can be interpreted satisfactorily at present. Some of the compounds are equally toxic at 10^{-4} M and 10^{-5} M which may be indicative of a maximal possible uptake of these compounds. Furthermore, many different modes of action of these compounds after formation of quinone products have been suggested. DNA polymerase, ribonucleotide reductase and thymidilate synthase are all regarded as possible targets for quinone products of phenol derivatives [41–43]. Indirect action like the inhibition of mitochondrial metabolism as shown for 4HA [44] may also be considered for some of the compounds. For 4HA it has been demonstrated that it can be metabolized *in vivo* to 3,4-dihydroxyanisole and its methylated products [45]. Also for 2-[^{18}F]fluorodopa it has been shown that a predominant part (>71%) of the acid-soluble radioactivity in (B16 melanoma) tumours was present as the O-methylated metabolite [26]. These are indications that COMT may be very

important as a detoxifying enzyme for phenolic or catecholic anti-melanoma drugs. The localization of soluble COMT in the cytosol of melanocytes seems to agree with a role as a detoxifying enzyme [46]. The exact localization of MB-COMT should further elucidate whether this part of the enzyme activity is present on membranes which are optimal for detoxification of free catechols (possibly on the plasma membrane or endoplasmic reticulum). Inhibition of COMT with newly developed stable drugs could prove to be useful as a direct tool for selective induction of cytotoxicity in melanoma cells but may also serve as a method to prevent the early detoxification of other antimelanoma agents.

Acknowledgements—This work was supported by grants from the Dutch Cancer Society (grant No. IKA 87–19) and from the CEC Medical and Health Research Programme.

REFERENCES

1. Hochstein P and Cohen G, The cytotoxicity of melanin precursors. *Ann NY Acad Sci* **100**: 878–886, 1963.
2. Graham DG, Tiffany SM and Vogel SF, The toxicity of melanin precursors. *J Invest Dermatol* **70**: 113–116, 1978.
3. Pawelek JM, Factors regulating growth and pigmentation of melanoma cells. *J Invest Dermatol* **66**: 201–209, 1976.
4. Wick MM, An experimental approach to the chemotherapy of melanoma. *J Invest Dermatol* **74**: 63–65, 1980.
5. Riley PA, Melanogenesis: a realistic target for antimelanoma therapy? *Eur J Cancer* **27**: 1172–1177, 1991.
6. Pavel S and Van der Slik W, Analysis of eumelanin-related indolic compounds in urine by high-performance liquid chromatography with fluorimetric detection. *J Chromatogr* **375**: 392–398, 1986.
7. Pavel S, Muskiet FAJ, De Ley L, The TH and Van der Slik W, Identification of three indolic compounds in a pigmented-melanoma cell-culture supernatant by gas chromatography-mass spectrometry. *J Cancer Res Clin Oncol* **105**: 275–279, 1983.
8. Smit NPM, Pavel S, Kammeyer A and Westerhof W, Determination of catechol-*O*-methyltransferase activity in relation to melanin metabolism using high-performance liquid chromatography with fluorimetric detection. *Anal Biochem* **190**: 286–291, 1990.
9. Borgulya J, Bruderer H, Bernauer K, Zürcher G and Da Prada M, Catechol-*O*-methyltransferase-inhibiting pyrocatechol derivatives: Synthesis and structure–activity studies. *Helv Chim Acta* **72**: 952–968, 1989.
10. Nissinen E, Linden I, Schultz E, Kaakkola S, Männistö PT and Pohto P, Inhibition of catechol-*O*-methyltransferase activity by two novel disubstituted catechols in the rat. *Eur J Pharmacol* **153**: 263–269, 1988.
11. Guldberg HC and Marsden CA, Catechol-*O*-methyltransferase: pharmacological aspects and physiological role. *Pharmacol Rev* **27**: 135–206, 1975.
12. Riley PA (Ed.), *Hydroxyanisole: Recent Advances in Antimelanoma Therapy*. IRL Press, Oxford, 1984.
13. Katano M, Saxton RE, Cochran AJ and Irie RF, Establishment of an ascitic human melanoma cell line that metastasizes to lung and liver in nude mice. *J Cancer Res Clin Oncol* **108**: 197–203, 1984.
14. Smit NPM, Westerhof W, Asghar SS, Pavel S and Siddiqui AH, Large scale cultivation of human melanocytes using collagen-coated beads (Cytodex 3). *J Invest Dermatol* **92**: 18–21, 1989.
15. Naish-Byfield S, Cooksey CJ, Latter AM, Johnson CI and Riley PA, *In vitro* assessment of the structure–activity relationship of tyrosinase-dependent cytotoxicity of a series of substituted phenols. *Melanoma Res* **1**: 273–287, 1991.
16. Smit NPM, van Roermund CWT, Aerts HMFG, Heikoop JC, Van den Berg M, Pavel S and Wanders RA, Subcellular fractionation of cultured normal human melanocytes: New insights into the relationship of melanosomes with lysosomes and peroxisomes. *Biochim Biophys Acta* **1181**: 1–6, 1993.
17. Smit NPM, Peters K, Menko W, Westerhof W, Pavel S and Riley PA, Cytotoxicity of a selected series of substituted phenols towards cultured melanoma cells. *Melanoma Res* **2**: 295–304, 1992.
18. Ito S, Kato T, Ishikawa K, Kasuga T and Jimbow K, Mechanism of selective toxicity of 4-*S*-cysteinylphenol and 4-*S*-cysteaminylphenol to melanocytes. *Biochem Pharmacol* **36**: 2007–2011, 1987.
19. Ito Y and Jimbow K, Selective cytotoxicity of 4-*S*-cysteaminylphenol on follicular melanocytes of the black mouse: Rational basis for its application to melanoma chemotherapy. *Cancer Res* **47**: 3278–3284, 1987.
20. Kern DH, Shoemaker RH, Hildebrand-Zanki SU and Driscoll JS, Structure–activity relationships defining the cytotoxicity of catechol analogues against human malignant melanoma. *Cancer Res* **48**: 5178–5182, 1988.
21. Wick MM, Levodopa and dopamine analogs: Melanin precursors as antitumor agents in experimental human and murine leukemia. *Cancer Treat Rep* **63**: 991–997, 1979.
22. Wick MM, L-DOPA methyl ester: Prolongation of survival of neuroblastoma tumor bearing mice following treatment. *Science* **199**: 775–776, 1978.
23. Parsons PG, Modification of DOPA toxicity in human tumour cells. *Biochem Pharmacol* **34**: 1801–1807, 1985.
24. Picardo M, Passi S, Nazzarro-Porro M, Breathnach A, Zompetta C, Faggioni A and Riley P, Mechanism of antitumoral activity of catechols in culture. *Biochem Pharmacol* **36**: 417–425, 1987.
25. Wick MM, Byers L and Frei III E, Selective toxicity of L-DOPA for melanoma cells. *Science* **197**: 468–469, 1977.
26. Ishiwata K, Kubota K, Kubota R, Iwata R, Takahashi T and Ido T, Selective 2-[^{18}F]fluorodopa uptake for melanogenesis in murine metastatic melanomas. *J Nucl Med* **32**: 95–101, 1991.
27. Wick MM, The chemotherapy of malignant melanoma. *J Invest Dermatol* **80**: 61S–62S, 1983.
28. Sharpless NS, Muenter MD, Tyce GM and Owen CA, 3-Methoxy-4-hydroxy-phenylalanine (3-*O*-methyldopa) in plasma during oral L-DOPA therapy of patients with Parkinson's disease. *Clin Chim Acta* **37**: 359–369, 1972.
29. Cumming P, Boyes BE, Martin WRW, Adam M, Ruth TJ and McGeer EG, Altered metabolism of [^{18}F]-6-fluorodopa in the hooded rat following inhibition of catechol-*O*-methyltransferase with U-0521. *Biochem Pharmacol* **36**: 2527–2531, 1987.
30. Reches A and Fahn S, 3-*O*-Methyldopa blocks DOPA metabolism in rat corpus striatum. *Ann Neurol* **12**: 267–271, 1982.
31. Belleau B and Burba J, Occupancy of adrenergic receptors and inhibition of catechol-*O*-methyl transferase by tropolones. *J Med Chem* **6**: 755–759, 1963.
32. Masri MS, Booth AN and DeEds, F, O-methylation *in vitro* of dihydroxy-and trihydroxy-phenolic compounds by liver slices. *Biochim Biophys Acta* **65**: 495–505, 1962.
33. Thakker DR and Creveling CR, In: *Conjugation*

Reactions in Drug Metabolism (Ed. Mulder GJ), pp. 193–232. Taylor and Francis Ltd, 1990.
34. Gugler R and Dengler HJ, Inhibition of human liver catechol-*O*-methyltransferase by flavonoids. *Naunyn-Schmiedebergs Arch Pharmacol* **276**: 223–233, 1973.
35. Borchardt RT and Bhatia P, Catechol-*O*-methyltransferase. 12. Affinity labeling the active site with the oxidation products of 5,6-dihydroxyindole. *J Med Chem* **25**: 263–271, 1982.
36. Andrawis A and Kahn V, Inactivation of mushroom tyrosinase by hydrogen peroxide. *Phytochemistry* **24**: 397–405, 1985.
37. Lindén I, Nissinen E, Etemadzadeh E, Kaakkola S, Männistö P and Pohto P, Favorable effect of catechol-*O*-methyltransferase inhibition by OR-462 in experimental models of Parkinson's disease. *J Pharmacol Exp Ther* **247**: 289–293, 1988.
38. Cedarbaum JM, Léger G, Reches A and Guttman M, Effect of Nitecapone (OR-462) on the pharmacokinetics of levodopa and 3-*O*-methyldopa formation in cynomolgus monkeys. *Clin Neuropharmacol* **13**: 544–552, 1990.
39. Sugawara K, Ohbayashi M, Shimizu K, Hatori M, Kamei H, Konishi M, Oki T and Kawaguchi H, BMY-28438 (3,7-dihydroxytropolone), a new antitumor antibiotic active against B16 melanoma I. production, isolation, structure and biological activity. *J Antibiotics* **41**: 862–868, 1988.
40. Zbytniewski Z, Palgan K, Chesy M and Drewa G, Effect of some flavonoids on the artificial lung metastasis on B16 melanoma in C57BL/6 mice. Abstract *3rd ESPCR meeting Amsterdam, 8–11 September, 1991*.
41. Wick MM, Levodopa and dopamine analogs: DNA polymerase inhibitors and antitumor agents in human melanoma. *Clin Res* **27**: 246A, 1979.
42. Lassman G, Liermann B, Arnold W and Schwabe K, Ribonucleotide reductase in melanoma tissue: EPR detection in human amelanotic melanoma and quenching of the tyrosine radical by 4-hydroxyanisole. *J Cancer Res Clin Oncol* **117**: 1–5, 1991.
43. Prezioso JA, Wang N and Bloomer WD, Thymidilate synthase as a target enzyme for the melanoma-specific toxicity of 4-*S*-cysteaminylphenol and *N*-acetyl-4-*S*-cysteaminylphenol. *Cancer Chemother Pharmacol* **30**: 394–400, 1992.
44. Passi S, Picardo M and Nazzaro-Porro M, Effect of *para*-hydroxyanisole on tyrosinase and mitochondrial oxidoreductase. In: *Hydroxyanisole: Recent Advances in Antimelanoma Therapy* (Ed. Riley PA), pp. 57–70. IRL Press, Oxford, 1984.
45. Pavel S, Holden JL and Riley PA, The metabolism of 4-hydroxyanisole: Identification of major urinary excretory products. *Pigment Cell Res* **2**: 421–426, 1989.
46. Shibata T, Pavel S, Smit NPM and Mishima Y, Differences in subcellular distribution of catechol-*O*-methyltransferase and tyrosinase in malignant melanoma. *J Invest Dermatol* **100**: 222S–225S, 1993.