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Indirect oxidation of the antitumor agent procarbazine by tyrosinase—Possible application in designing anti-melanoma prodrugs

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Abstract—The interaction of tyrosinase with the anticancer drug procarbazine has been investigated. In the presence of the enzyme alone no oxidation of this dialkylhydrazine above the background level was observed. However, when phenolic substrates (4-*tert*-butylcatechol or *N*-acetyl-L-tyrosine) were included in the reaction mixture, procarbazine was rapidly degraded. Oxygen consumption measurements showed that in a mixture both the phenolic substrate and the drug were oxidized. The major product of procarbazine degradation was isolated and identified as azoprocarbazine, the first active metabolite of this drug detected in previous in vivo and in vitro studies. This indirect oxidation of the hydrazine group in this anticancer agent indicates possible application of a hydrazine linker in construction of tyrosinase-activated anti-melanoma prodrugs.

Malignant melanoma is the most aggressive skin tumor resulting from neoplastic transformation of melanocytes, which shows a steadily increasing incidence.[1] Despite over 40 years of development of chemotherapy there is currently no effective drug against this cancer and new therapeutic agents are urgently needed. In the early stages of the disease surgical intervention is usually sufficient and gives good prognosis, but in cases of metastasis there is little hope of survival. Dacarbazine (DCIT) is the primary agent applied in chemotherapy, frequently combined with other cytostatic agents (tamoxifen, cisplatin, nitrozoureas, and vinca alkaloids), with the CVD and Dartmouth regimens appearing to be the most effective.[1] However, positive responses are only observed in about 30–40% of cases and complete remissions are rare.[2]

In mammals the metabolic pathway unique to melanocytes is melanogenesis, which offers the possibility of developing a targeted chemotherapy specific to this tumor. Tyrosinase (EC 1.14.18.1) is the key enzyme in this pathway catalyzing the two initial and rate limiting steps: hydroxylation of L-tyrosine to L-Dopa and its subsequent oxidation to dopaquinone, which undergoes a series of non-enzymatic and enzymatic reactions leading to melanins.[3] Tyrosinase can also convert other monophenols and *o*-diphenols to *o*-quinones, which are inherently cytotoxic. Utilization of tyrosinase in the treatment of melanoma was therefore considered long time ago. The first tyrosinase's substrate displaying promising cytotoxic effects against melanoma was 4-hydroxyanisole.[4,5] Later examples include 2,4-dihydroxyphenylalanine[6] and 4-*S*-cysteaminylphenol and its derivatives.[7–10] In the past decades numerous analogues of these compounds have been synthesized and tested (reviewed in Ref. 11). However, only 4-hydroxyanisole reached clinical trials and was withdrawn because of significant renal and hepatotoxicity,[12] later attributed to its epoxidation by liver cytochromes P450, leading to the formation of *p*-quinone.[13]

The possibility of selectively releasing cytotoxic agents in melanocytes from tyrosinase-activated prodrugs has recently also drawn substantial attention.[14–17] This concept has been named Melanocyte-directed Enzyme Prodrug Therapy.[14] Initially, the cytotoxic agents were attached to the primary amino group of tyrosinase substrates (dopamine, tyrosine, and related compounds) by carbamate or urea linkers.[14–16] However, recent studies on such dopamine derivatives have shown that the con-

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version of the amino group to amide, carbamate, or urea derivatives makes the nitrogen atom insufficiently nucleophilic for the cyclization of the corresponding *o*-quinones to dihydroindoles and therefore the release of the active group is unlikely.[18] In the meantime, new derivatives have been developed, where the effector part has been connected to the aromatic amino group of 4-aminophenol or 6-aminodopamine via a urea or thiourea linker.[17]

Tyrosinase is also capable of oxidizing compounds other than phenols and *o*-diphenols. Aromatic amines, *o*-aminophenols, and *o*-diamines were described as substrates for the enzyme from *Neurospora crassa*[19] and *Agaricus bisporus*,[20] although the reaction rates were orders of magnitude smaller than for the corresponding phenols or catechols. Phenylhydrazine can also be oxidized by this enzyme[21] and the reaction leads to its irreversible inactivation.[22]

Hydrazine derivatives are commonly found in nature, either as natural compounds, industrial chemicals, or pharmaceuticals. Drugs containing a hydrazine group include the anti-parkinsonians carbidopa and benserazide, the primary antituberculosis agent isoniazid, older generation antidepressants iproniazid, nialamide, phenelzine, and isocarboxazid, the antihypertensive hydralazine, and the antitumor agent procarbazine.[23] Most of these compounds are actually prodrugs, which are converted to their active forms in the organism by oxidative enzymes, primarily cytochrome P450 monooxygenases and peroxidases. However, participation of tyrosinase in the oxidation of any of these compounds has not been taken into consideration.

We have recently shown that amino acid phenylhydrazides are oxidized by *o*-quinones, and therefore indirectly by tyrosinase in the presence of phenols or *o*-diphenols.[24] Since the oxidation of these compounds to azo derivatives leads to their rapid cleavage and release of amino acids, we concluded that a hydrazine linker may be utilized in construction of tyrosinase-activated anti-melanoma prodrugs. To prove the feasibility of this approach we decided to test the reaction with a hydrazine containing anticancer drug used in medical practice—procarbazine (*N*-isopropyl-4-[(*N*′-methylhydrazino)methyl]benzamide). Procarbazine was developed as an antitumor agent in the 1960s and used extensively against lymphoid malignancies, particularly Hodgkin's lymphomas and certain brain tumors. However, serious side effects, including secondary leukemias and sterility later led to its withdrawal, although it has been recently reintroduced in combination therapies and yielded impressive results.[25] Activation pathway of this drug starts with its oxidation to azoprocarbazine attributed primarily to cytochrome P450 monooxygenases and peroxidases. Later reactions include tautomerization to methylhydrazone, which can hydrolyze to *p*-formyl-*N*-isopropylbenzamide and methylhydrazine, or undergo further oxidation to azoxy derivatives.[26–29] All these compounds may decompose generating free radicals, which are thought to be responsible for the cytotoxic effects.[30,31]

We first analyzed the reaction of procarbazine (Sigma-Tau, Gaithersburg, MD, USA) with 4-*tert*-butyl-*o*-benzoquinone generated by the oxidation of 4-*tert*-butylcatechol (Fluka) with sodium periodate (POCh, Gliwice, Poland) monitoring it spectrophotometrically. Reactions were carried out as described previously for the amino acid phenylhydrazides.[24] 4-*tert*-butylcatechol (final concentration 0.25 or 0.5 mM, always in excess over the oxidizing agent) was mixed with a solution of sodium periodate (final concentration 0.1, 0.2 or 0.25 mM), the volume was adjusted to 2.6 ml with the buffer (100 mM sodium phosphate, pH 6.8) and the UV/vis spectrum from 300 to 600 nm was recorded after 1 min. In a parallel reaction, the same components were mixed and after 1 min a solution of procarbazine (final concentration 0.1 or 0.25 mM) was added to a final volume of 2.6 ml. UV/vis spectra were recorded immediately after adding procarbazine and then in 2 min intervals until no changes were detected. This procedure was applied to avoid dilution of the first reaction with the solution of procarbazine. The addition of procarbazine led to instantaneous disappearance of 4-*tert*-butyl-*o*-quinone reflected by a loss of absorbance in the 400 nm region (Fig. 1). This contrasted markedly with the reaction of amino acid phenylhydrazides, which required 30–60 min for completion.[24]

We next carried out enzymatic oxidations of 4-*tert*-butylcatechol (0.1 or 0.25 mM final concentration) by 5 or 20 μg of mushroom tyrosinase (specific activity 3216 U/mg, Sigma–Aldrich) without or with procarbazine (0.1, 0.2 or 0.3 mM final concentration). UV/vis spectra recorded in the 300–600 nm range showed that the final concentration of 4-*tert*-butyl-*o*-benzoquinone (determined from the absorbance at 406 nm) was not significantly affected by the presence of procarbazine, but its formation was delayed (Fig. 2).

Reactions of procarbazine with tyrosinase were then monitored by oxygen consumption measurements.[24] Reaction mixtures contained procarbazine (0.025, 0.05, 0.1, 0.2 or 0.3 mM), 4-*tert*-butylcatechol or *N*-acetyl-L-

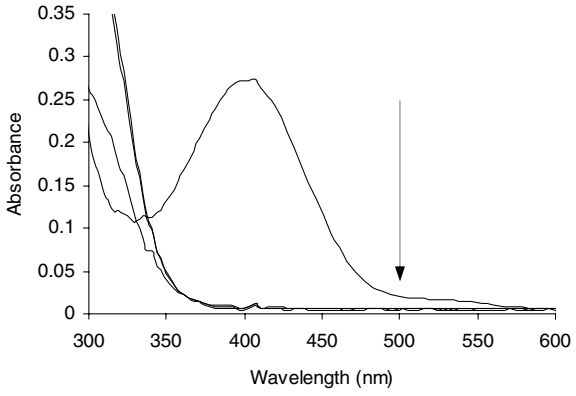


Figure 1. Spectral changes after the addition of 0.25 mM procarbazine to 4-*tert*-butyl-*o*-benzoquinone generated by oxidation of 0.5 mM 4-*tert*-butylcatechol with 0.25 mM $NaIO_4$. The spectra were recorded at 2 min intervals. The arrow indicates the direction of spectral changes (decrease of 4-*tert*-butyl-*o*-benzoquinone absorbance).

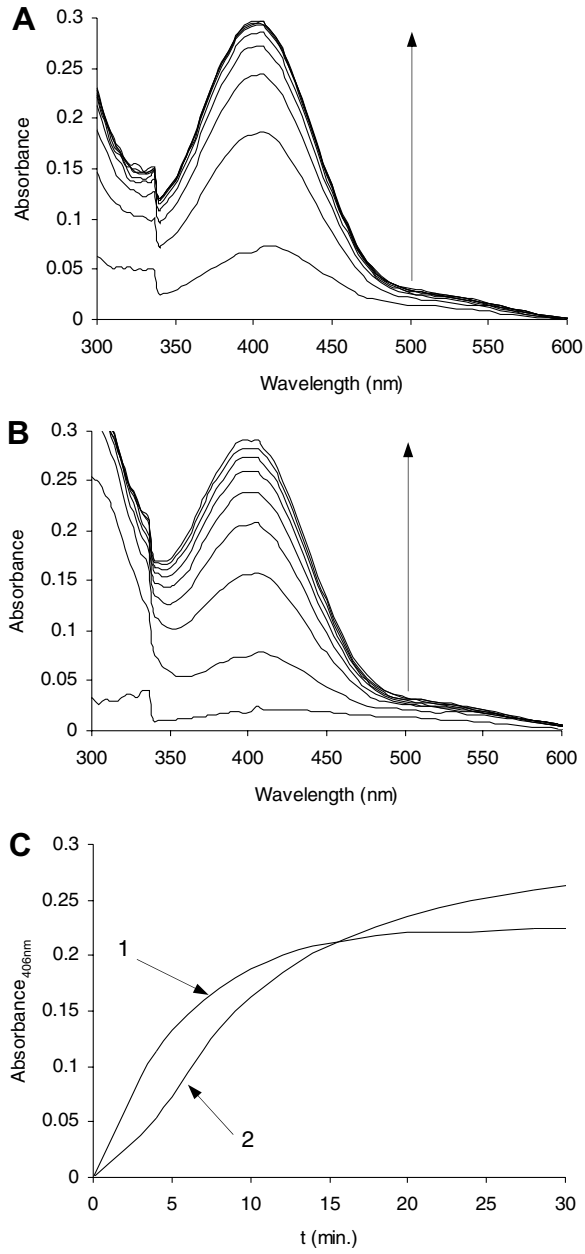


Figure 2. Spectral changes during the oxidation of 0.25 mM 4-*tert*-butylcatechol (A), and a mixture of 0.25 mM 4-*tert*-butylcatechol with 0.1 mM procarbazine (B) by 5 μg of tyrosinase. Spectra were recorded at 4 min intervals. Changes of absorbance at 406 nm during oxidation 0.25 mM 4-*tert*-butylcatechol (1), and a mixture of 0.25 mM 4-*tert*-butylcatechol with 0.1 mM procarbazine (2) by 5 μg of tyrosinase (C). The arrow in (A) and (B) indicates the direction of spectral changes (increase of 4-*tert*-butyl-*o*-benzoquinone absorbance).

tyrosine (0, 0.01, 0.025, 0.05 or 0.1 mM) and 42, 84, 126 or 168 μg of tyrosinase (to keep the enzyme concentration the same as in the spectrophotometric measurements). Two sets of experiments were performed: the concentration of procarbazine was kept constant and the concentration of the phenolic substrate was varied or vice versa. All the reactions were monitored for 60 min. Oxygen consumption in mixtures of procarbazine with the enzyme alone was very slow (3.17 nM/s). However, in the presence of 4-*tert*-butylcatechol the rate of oxygen consumption increased greatly and it was approximately proportional to the concentration of the phenolic substrate (Fig. 3)—44.1, 89.5, 140, and 260 nM/s for 0.01, 0.025, 0.05 and 0.1 mM of 4-*tert*-butylcatechol, respectively. Total oxygen consumption corresponded well to the concentration change required for the oxidation of both compounds (e.g., 0.18 mM for 0.1 mM of catechol, whereas the theoretical value equals 0.2 mM) and far exceed that required for the oxidation of 4-*tert*-butylcatechol alone (0.05 mM).

Similar experiments were performed with *N*-acetyl-L-tyrosine. Oxidation of this monophenolic, non-cyclizing substrate displays a characteristic lag phase. The presence of procarbazine in the reaction mixture reduced the lag period significantly, indicating that it acted as a reducing agent (Fig. 4). After the lag phase the reaction rates depended solely on the concentration of the phenolic substrate and not on the concentration of procarbazine. When procarbazine was the major substrate and the concentration of *N*-acetyl-L-tyrosine varied, the reaction rates increased after the lag period, proportionally to the concentration of the phenolic substrate (Fig. 5).

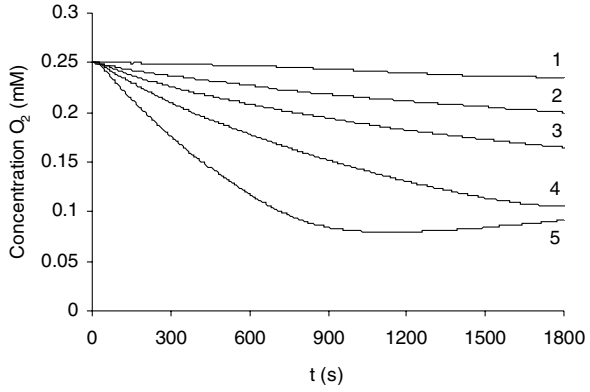


Figure 3. Oxygen consumption measurements during the oxidation of 0.3 mM procarbazine (1) and its mixtures with 0.01 mM (2), 0.025 mM (3), 0.05 mM (4) and 0.1 mM (5) 4-*tert*-butylcatechol by 42 μg of tyrosinase.

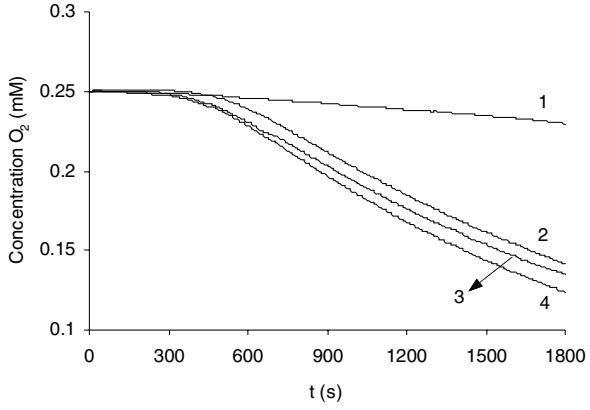


Figure 4. Oxygen consumption measurements during the oxidation of 0.2 mM *N*-acetyl-L-tyrosine (1) and its mixtures with 0.025 mM (2), 0.05 mM (3) and 0.1 mM (4) procarbazine by 84 μg of tyrosinase.

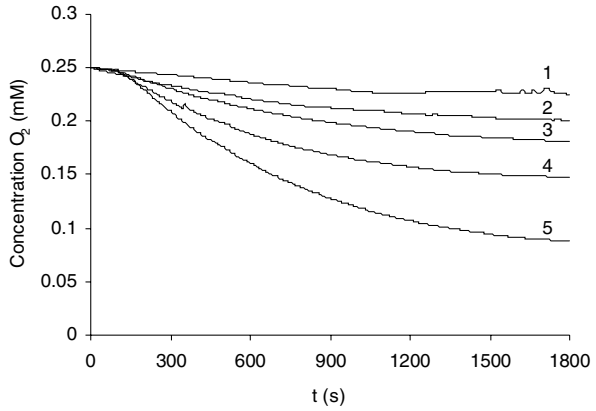


Figure 5. Oxygen consumption measurements during the oxidation of 0.3 mM procarbazine (1) and its mixtures with 0.01 mM (2), 0.025 mM (3) 0.05 mM (4) and 0.1 mM (5) *N*-acetyl-L-tyrosine by 168 μg of tyrosinase.

To identify the products formed in these reactions from procarbazine we performed HPLC analysis of a mixture of procarbazine and 4-*tert*-butylcatechol with tyrosinase. Reactions were carried in 1 ml of 100 mM sodium phosphate buffer, pH 6.8 with 5 μg of tyrosinase at various concentrations of the substrates (0.1–0.5 mM). Samples were analyzed immediately after the addition of the enzyme and then after 15, 30, and 60 min. Separations were performed on a ChromSep Inertsil 5 ODS-2 column (250 × 3 mm) connected to a Varian Polaris instrument with a dual wavelength UV/vis detector and a 20 μl sample loop with 10 mM sodium phosphate buffer, pH 6.8 and acetonitrile as a mobile phase (20–80% acetonitrile gradient), at a flow rate of 0.4 ml/min. Chromatograms were recorded at 230 and 280 nm. Again, in the presence of the phenolic substrate rapid disappearance of procarbazine was observed. It was replaced by one major (R_t = 16 min) and one minor product (R_t = 14.5 min) (Fig. 6B). The major product was isolated from a preparative reaction (0.4 mM procarbazine—12.8 mg, 0.1 mM 4-*tert*-butylchatechol and 1 mg of tyrosinase in 125 ml of 10 mM sodium phosphate buffer, pH 6.8). The reaction was monitored spectrophotometrically by taking the UV/vis spectra in the 300–600 nm region until no change in the absorption range of 4-*tert*-butyl-*o*-benzoquinone was observed. After completion, the reaction mixture was frozen, lyophilized, dissolved in 20 ml of water and separated on a 7 ml Bakerbond octadecyl column. After washing the column with water, products were eluted with 40% acetonitrile in water, 4 ml fractions were collected, analyzed by TLC and their UV/vis spectra were taken. The fraction containing the major product was lyophilized, dissolved in $CDCl_3$ and its ^1H NMR spectrum was taken (Bruker Avance 300 MHz). The product was unequivocally identified as azoprocarbazine. The signals of the methyl and methylene groups showed characteristic shifts from 2.81 to 3.80 and from 4.19 to 4.95 ppm, respectively.[29] The minor product could not be purified completely by standard gravitational chromatography. However, its chromatographic behavior (elution from a reverse-phase column slightly before azoprocarbazine[29]), and the UV/vis (maximum at 300 nm[26]) and

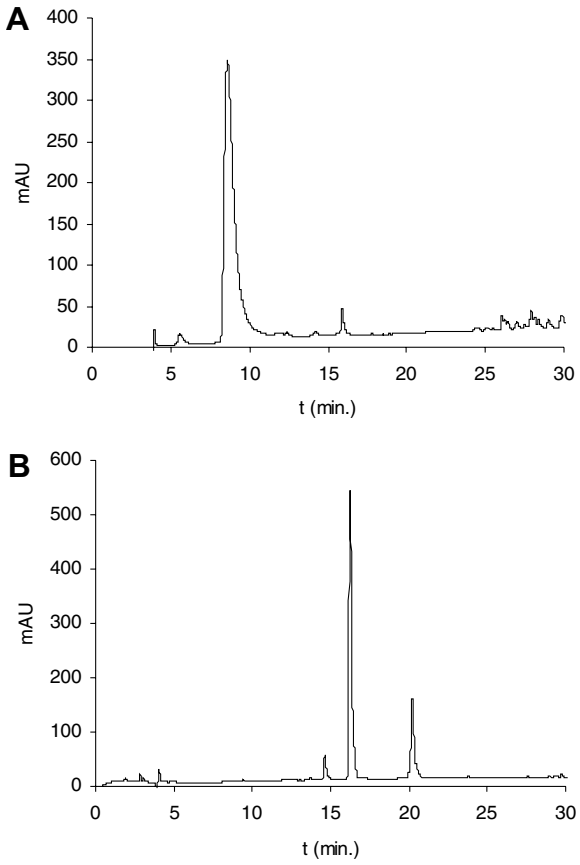
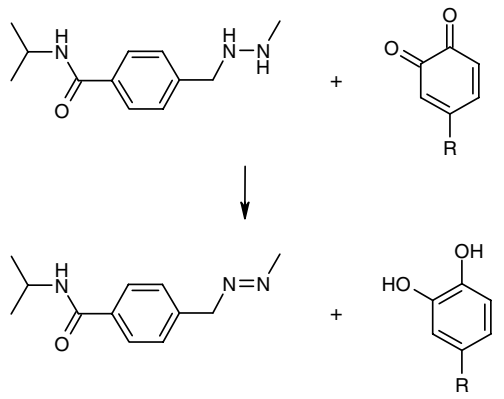


Figure 6. Chromatographic analysis of a mixture of 0.5 mM procarbazine with 5 μg of tyrosinase (A) and a mixture of 0.25 mM 4-*tert*-butylcatechol, 0.25 mM procarbazine and 5 μg of the enzyme (B) after 30 min incubation. Retention times determined by analysis of standards were 9 min for procarbazine, 20.5 min for 4-*tert*-butyl-*o*-benzoquinone, and 21.5 min for 4-*tert*-butylcatechol (not seen in the presented chromatogram). The major product with a retention time of 16 min has been identified as azoprocarbazine. The minor product with a retention time of 14.5 min has been tentatively identified as *p*-formyl-*N*-isopropylbenzamide methylhydrazone.

NMR (a singlet at 3.00 ppm) spectra of its mixture with the major product indicated that it was the hydrazone tautomer of azoprocarbazine (*p*-formyl-*N*-isopropylbenzamide methylhydrazone) decomposing slowly to the aldehyde (*p*-formyl-*N*-isopropylbenzamide—a small NMR signal at 10.08 ppm was also observed in these samples). As already indicated by oxygen consumption measurements, very little conversion of procarbazine was observed when the compound was incubated with tyrosinase alone (Fig. 6A)—the amount of azoprocarbazine was similar to that obtained as a result of autooxidation in the absence of the enzyme (data not shown). These results clearly demonstrate that in a mixture with monophenols or *o*-diphenols procarbazine is oxidized by tyrosinase indirectly through *o*-quinones (Scheme 1).

We believe that this reaction offers an alternative approach for the preparation of anti-melanoma prodrugs. Combination of a phenolic substrate with a hydrazine linker would create compounds from which the release of the active component after activation by tyrosinase would not occur as a result of a nucleophilic attack on



Scheme 1. Redox reaction occurring between procarbazine and *o*-quinones.

the *o*-quinone moiety but its redox exchange with the linker. Acyl hydrazine derivatives may be used for rapid release of a variety of cytotoxic agents, whereas alkyl hydrazines, for example, based on the structure of procarbazine, may serve as sources of alkyl radicals or carbocations. Preparation of such compounds is now under way.

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