European Journal of Medicinal Chemistry 71 (2014) 98–104

Contents lists available at ScienceDirect

European Journal of Medicinal Chemistry

journal homepage: http://www.elsevier.com/locate/ejmech

Original article

Synthesis and analysis of activity of a potential anti-melanoma prodrug with a hydrazine linker

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ARTICLE INFO

Article history:
Received 1 July 2013
Received in revised form
8 October 2013
Accepted 31 October 2013
Available online 9 November 2013

Keywords:
Anti-melanoma prodrug
Nitrogen mustard
Hydrazine
Tyrosinase

ABSTRACT

A potential anti-melanoma prodrug containing a phenolic activator, a hydrazine linker, and a nitrogen mustard effector – (*N*-{4-[bis-(2-chloroethyl)amino]benzoyl}-*N*′-(4-hydroxybenzyl)hydrazine) has been synthesized in seven steps. Spectrophotometric measurements of its oxidation by tyrosinase showed a rapid increase of absorbance at 337 nm. HPLC analysis demonstrated that two major products were formed. However, during the reaction one of the products was converted into the other. The stable product with a maximum of absorption at 337 nm was isolated and identified as 5,6-dihydroxy-1*H*-indazol-1-yl 4-[bis-(2-chloroethyl)amino]benzoate. It was formed by a cyclization of the enzymatically generated *o*-quinone. This reaction was unexpected, since the acylated hydrazine nitrogen atom should not be sufficiently nucleophilic to attack the *o*-quinone ring. This cyclization prevented the effector release from the enzyme-activated prodrug. As a result, the prodrug showed only limited specificity for B16–F10 murine melanoma cells compared to reference cell lines. When applied in solid tumors in mice it showed slightly higher activity than the parent mustard drug (4-[bis-(2-chloroethyl)amino]benzoic cid), but significantly lower activity than melphalan, a commercial mustard drug with a structure resembling tyrosine, occasionally used in the treatment of melanoma.

1. Introduction

Malignant melanoma is the most aggressive skin tumor resulting from neoplastic transformation of melanocytes, which shows a steadily increasing incidence [1]. For decades little progress in the therapy of this tumor has been made with dacarbazine (DTIC) remaining the major drug used in metastatic disease [2]. Recently, however, breakthroughs have been made with the discovery of selective BRAF inhibitors (vemurafenib, GSK2118436), which have been evaluated in clinical trials and demonstrated exceptional improvement in the treatment of this notoriously drug-resistant tumor in patients with mutations in this protein. Monoclonal antibodies against CTLA-4 (ipilimumab) also show significantly improved effectiveness compared to immunotherapy with interferon and interleukin 2 [3,4]. However, the respective drugs (Yervoy, Zelboraf) are effective in a limited subsets of patients. They are also extremely expensive and in many countries are not refunded by the national healthcare systems. Therefore, pathways for discovering new therapeutic agents for this tumor with better efficacy and lower side effects still remain open.

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 [5]. Tyrosinase can also convert other monophenols and *o*-diphenols to *o*-quinones, which are inherently cytotoxic. Tyrosinase activity in melanocytes seems to be correlated with their malignant

Abbreviations: DTIC, dacarbazine (5-(3,3-dimethyl-1-triazenyl)imidazole-4-carboxamide); MTT, (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PBS, phosphate buffered saline.

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http://dx.doi.org/10.1016/j.ejmech.2013.10.080

transformation [6,7]. Application of tyrosinase as a melanocyte-specific enzyme for activation of anti-melanoma prodrugs was therefore considered long time ago [8]. The concept of selectively releasing cytotoxic agents in melanocytes from tyrosinase-activated prodrugs was developed more than a decade ago and named Melanocyte-Directed Enzyme Prodrug Therapy (MDEPT) [9]. Initially, the cytotoxic agents were attached to the primary amino group of tyrosinase substrates (tyrosine, dopamine and related compounds) by carbamate or urea linkers [9–11]. However, later studies on such tyramine and dopamine derivatives showed that conversion of the amino group to amide, carbamate or urea derivatives made the nitrogen atom insufficiently nucleophilic for cyclization of the corresponding *o*-quinones to dihydroxydihydroindoles and therefore the release of the active group was unlikely [12]. In the meantime, new derivatives were developed, where the effector part was connected to the aromatic amino group of 4-aminophenol or 6-aminodopamine via a urea or thiourea linker [13]. Recently, tyramine and dopamine derivatives of triazenes (DTIC analogs) have been prepared as potential tyrosinase-activated anti-melanoma prodrugs [14]. Again, however, reduced nucleophilicity of the modified amine nitrogen hindered the release of the effector after enzymatic activation.

We have recently shown that the hydrazine group in amino acid phenylhydrazides [15] and in the antitumor drug procarbazine [16] can be oxidized by *o*-quinones and therefore indirectly by tyrosinase. Based on these results we have postulated that this redox exchange reaction can be utilized in activation of anti-melanoma prodrugs with a hydrazine linker (Scheme 1). Before designing target compounds we have first tested the concept with carbidopa, an approved drug containing both the catechol and hydrazine moieties. Detection of 6,7-dihydroxy-3-methylcinnoline as one of the major products of oxidation of his compound by tyrosinase demonstrated that the nucleophilic attack of the hydrazine group in the side-chain on the generated *o*-quinones, which led to cyclization, competed with the intramolecular redox exchange reaction between these two moieties [17]. This cyclization reaction is undesired from the point of view of designing anti-melanoma prodrugs, because it may reduce the yield of effector release. Therefore, after initial attempts, we have given up work on alkyl and dialkyl hydrazines and concentrated on acylated hydrazine derivatives. Here we report the synthesis of an aniline mustard prodrug with a hydrazine linker and a phenolic activator oxidizable by tyrosinase, analysis of its enzymatic activation and its effect on murine melanoma in vitro and in vivo.

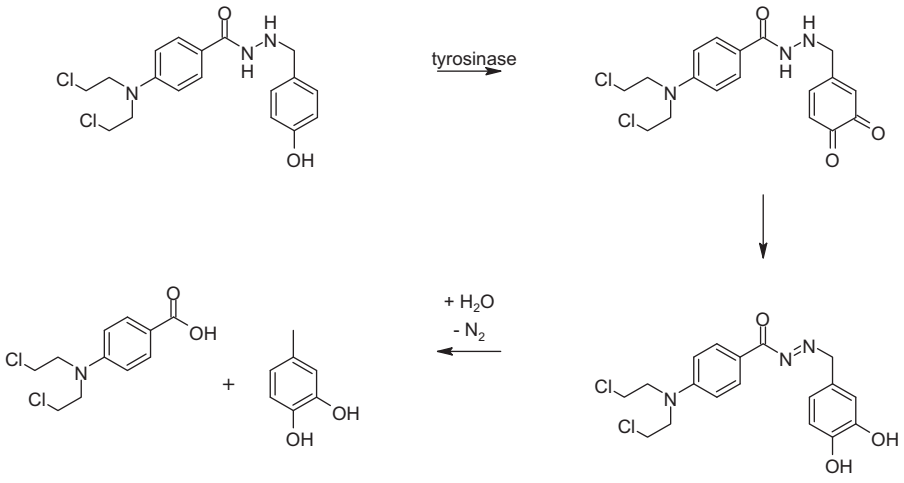
2. Results and discussion

2.1. Synthesis

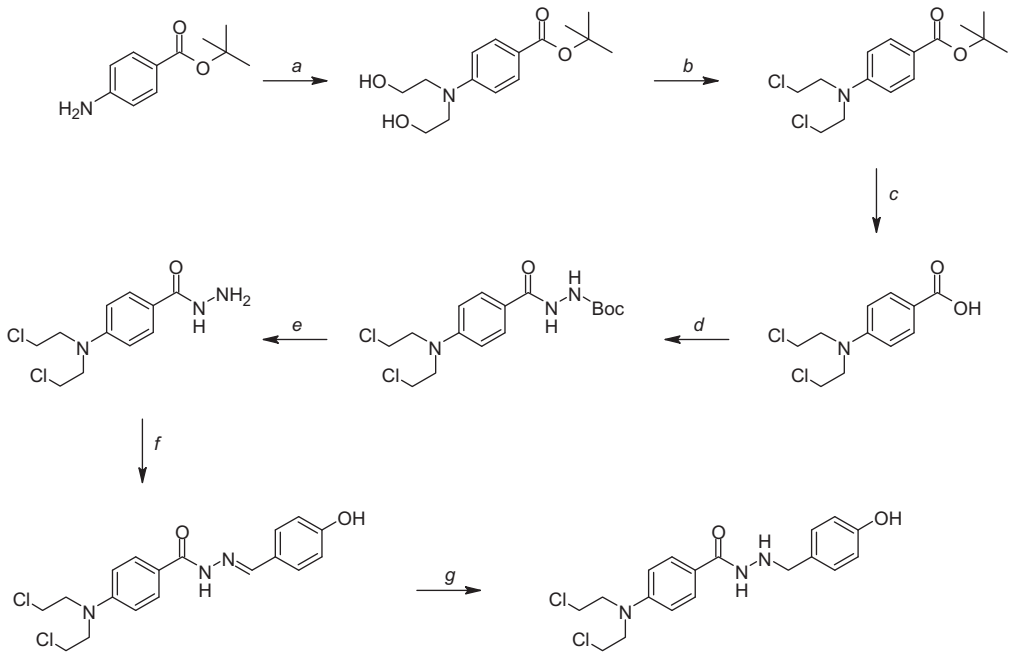
The synthesis of *N*-{4-[bis-(2-chloroethyl)amino]benzoyl}-*N*′-(4-hydroxybenzyl)hydrazine was accomplished in a 7-step procedure. First, *tert*-butyl 4-[bis-(2-hydroxyethyl)amino]benzoate was prepared from *tert*-butyl 4-aminobenzoate and ethylene oxide [18]. The hydroxyl groups were then replaced with chlorine atoms using methanesulfonyl chloride. The carboxylic group was deprotected and converted to a hydrazide in a reaction with *tert*-butoxycarbonylhydrazine. After removal of the protecting group, the hydrazide was coupled with 4-hydroxybenzaldehyde yielding a hydrazone, which was then reduced with hydrogen gas on a palladium catalyst (Scheme 2).

2.2. Analysis of the activation of the prodrug by tyrosinase

Spectrophotometric analysis of the oxidation of the synthesized compound by tyrosinase demonstrated that it served as a substrate of the enzyme – rapid changes in the UV–Vis spectrum occurred with a new maximum of absorption at 337 nm (Fig. 1). This spectrum did not correspond to *o*-quinones and suggested rather that an additional electron donating group was attached to the aromatic ring (i.e. intramolecular or intermolecular nucleophilic attack). HPLC analysis showed the presence of 2 major products (Fig. 2). Their proportions differed depending on the reaction conditions (time of the reaction, concentration of the substrate, proportion of the substrate to enzyme concentrations). Time-course analysis demonstrated that one of the products, eluting just behind the substrate, was eventually converted into the second product, whose UV–Vis spectrum closely resembled the spectrum of the reaction mixture, with a maximum of absorption at 337 nm. This compound was isolated by extraction and column chromatography and analyzed by NMR and high resolution mass spectrometry. Results of this analysis allowed its unequivocal identification as 5,6-dihydroxy-1*H*-indazol-1-yl 4-[bis-(2-chloroethyl)amino]benzoate – a cyclization product formed by a nucleophilic attack of the hydrazide nitrogen atom on the enzyme-generated *o*-quinone



Scheme 1. The concept of tyrosinase-activated anti-melanoma prodrugs with a hydrazine linker – postulated reactions occurring during oxidation of *N*-{4-[bis-(2-chloroethyl)amino]benzoyl}-*N*′-(4-hydroxybenzyl)hydrazine.



Scheme 2. Synthesis of *N*-{4-[bis-(2-chloroethyl)amino]benzoyl}-*N*′-(4-hydroxybenzyl)hydrazine: a) ethylene oxide, AcOH; b) MsCl, Py; c) 90% TFA in DCM; d) EDC, BtOH, Boc-$NHNH_2$; e) 1.25 M HCl in MeOH; f) 4-hydroxybenzaldehyde, EtOH; g) H_2, Pd/C, MeOH.

(Michael addition). This result was surprising, since we expected that the acylation of the nitrogen atom of the hydrazine linker would sufficiently lower its nucleophilicity to prevent cyclization. This reaction is, of course, undesired, since it prevents the effector release from the enzyme-oxidized prodrug. In fact, we were not able to detect the released mustard drug (4-[bis-(2-chloroethyl) amino]benzoic cid) in any reaction mixture by TLC, HPLC or MS analysis. The intermediate, eluting slightly behind the substrate in the HPLC analysis, was also isolated. Its mass and NMR spectra matched the spectra of the hydrazone – the last intermediate in the synthetic procedure, (*N*-{4-[bis-(2-chloroethyl)amino]benzoyl}-*N*′-(4-hydroxybenzylidene)hydrazine).

Based on these results we can propose the reactions occurring during the oxidation of *N*-{4-[bis-(2-chloroethyl)amino]benzoyl}-*N*′-(4-hydroxybenzyl)hydrazine by tyrosinase. The enzyme oxidizes the phenolic group of the activator to *o*-quinone. The hydrazide nitrogen can then attack the *o*-quinone generating a cyclic product, which is further oxidized undergoing aromatization – spontaneous or tyrosinase-catalyzed (Scheme 3). Alternatively the *o*-quinone can undergo a redox exchange reaction with a substrate molecule, generating the detected hydrazone. It is rather unlikely that this product is generated directly by the intermolecular redox exchange reaction. We therefore postulate that the diazene is formed first, which then undergoes tautomerization. A similar reaction sequence has been detected before during the metabolism of procarbazine [19]. We have also observed this azo-hydrazone tautomerization after oxidation of procarbazine by *o*-quinones (indirectly by tyrosinase), although in that case the azo compound was stable enough to be identified as the major product [16]. Benzylazoalkanes are generally considered less stable than their isomeric alkylhydrazones [19]. Studies of the degradation of the hydrazine-containing antidepressant isocarboxazid (*N*′-benzyl-5-methyl-1,2-oxazole-3-carbohydrazide) in vivo identified benzoic acid as one of the major metabolites, presumably formed via the diazene, hydrazone, and benzaldehyde intermediates [19]. In vitro oxidation of a model compound, *N*-(4-chlorobenzyl)-*N*′-benzoylhydrazine, by rat liver microsomes produced the corresponding hydrazone as the major product. In this case, however, the formation of this product was attributed mainly to *N*-hydroxylation catalyzed by cytochromes P450, followed by dehydration [20].

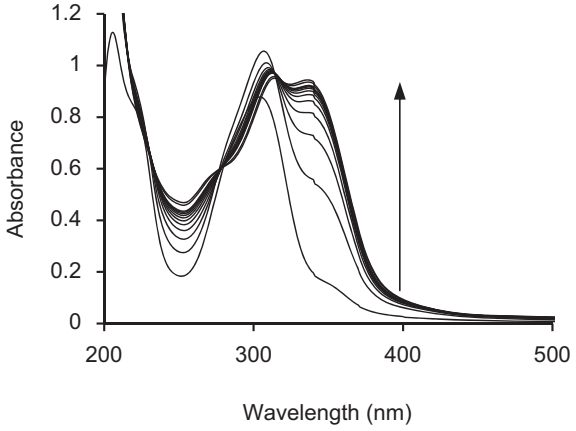


Fig. 1. UV–VIS spectra of a reaction mixture containing 0.05 mM *N*-{4-[bis-(2-chloroethyl)amino]benzoyl}-*N*′-(4-hydroxybenzyl)hydrazine and 2.5 μg of tyrosinase in 3 ml of 10 mM sodium phosphate buffer, pH 6.8. The spectra displayed were recorded for 20 min at 2 min intervals.

2.3. Effect of the synthesized compound on murine melanoma

The effect of the synthesized prodrug on cells in culture was tested with B16–F10 murine melanoma cells and HECa10 and NIH3T3 control cells. It was toxic to all three cell lines (lethal at 250 μM) and showed limited specificity for the melanoma cells (Fig. 3). It was then examined on solid tumors generated in mice. At 100 μg dose it showed moderate activity in reducing the tumor size, only slightly better than the parent mustard drug (4-[bis-(2-chloroethyl)amino]benzoic acid), but was significantly weaker than melphalan (Fig. 4). These results are consistent with the analysis of its enzymatic activation, which demonstrated that its oxidation by tyrosinase did not lead to effector release.

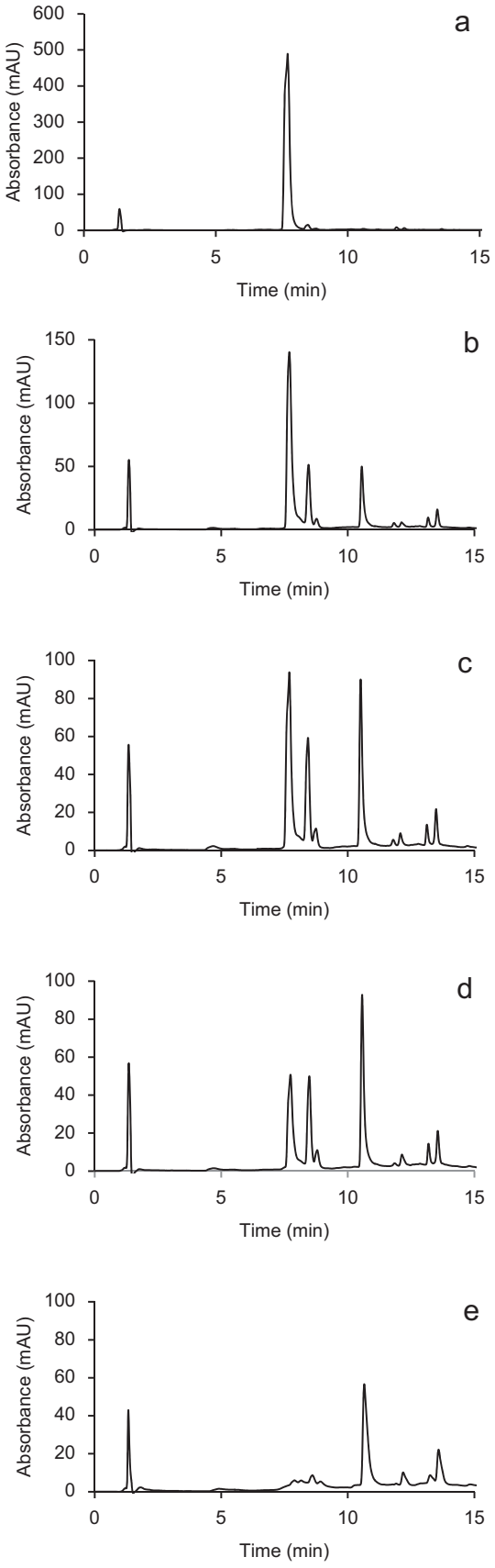


Fig. 2. HPLC separation of a reaction mixture obtained after oxidation of 0.1 mM *N*-{4-[bis-(2-chloroethyl)amino]benzoyl}-*N*′-(4-hydroxybenzyl)hydrazine by tyrosinase in 10 mM sodium phosphate buffer, pH 6.8. Analyses were performed immediately after mixing the reagents (a), and then after 30 min (b), 60 min (c), 120 min (d) with 250 μg of the enzyme in 50 ml of buffer, and after 120 min with 500 μg of the enzyme (e). The chromatograms displayed were recorded at 280 nm. Signals correspond to the following compounds identified by mass spectrometry, NMR analysis and/or comparison with synthetic standards: 7.7 min – the substrate, 8.5 min – *N*-{4-[bis-(2-chloroethyl)amino]benzoyl}-*N*′-(4-hydroxybenzylidene)hydrazine, 10.5 min – 5,6-dihydroxy-1*H*-indazol-1-yl 4-[bis-(2-chloroethyl)amino]benzoate.

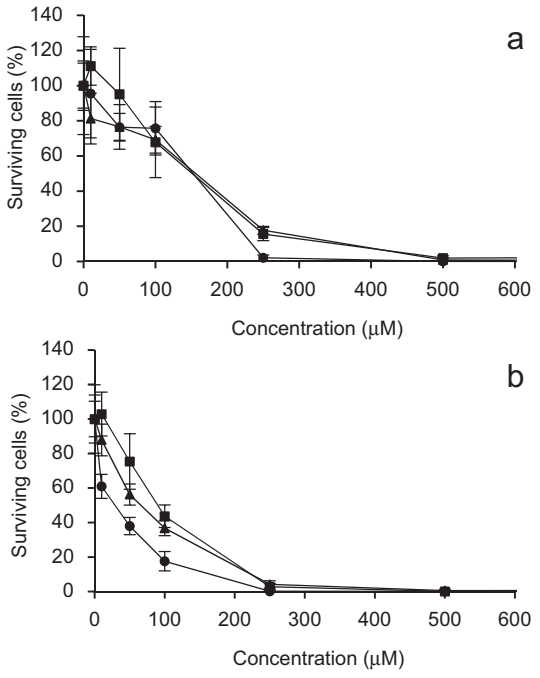


Fig. 3. Toxicity of *N*-{4-[bis-(2-chloroethyl)amino]benzoyl}-*N*′-(4-hydroxybenzyl)hydrazine against B16–F10 (●), NIH3T3 (▲), and HECa10 (■) cells in vitro after 24 h (a) and 48 h (b).

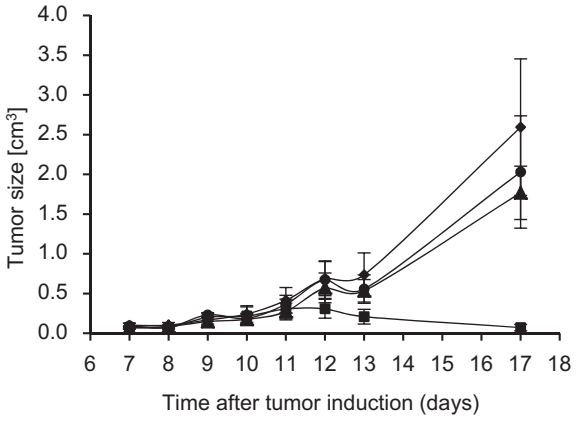
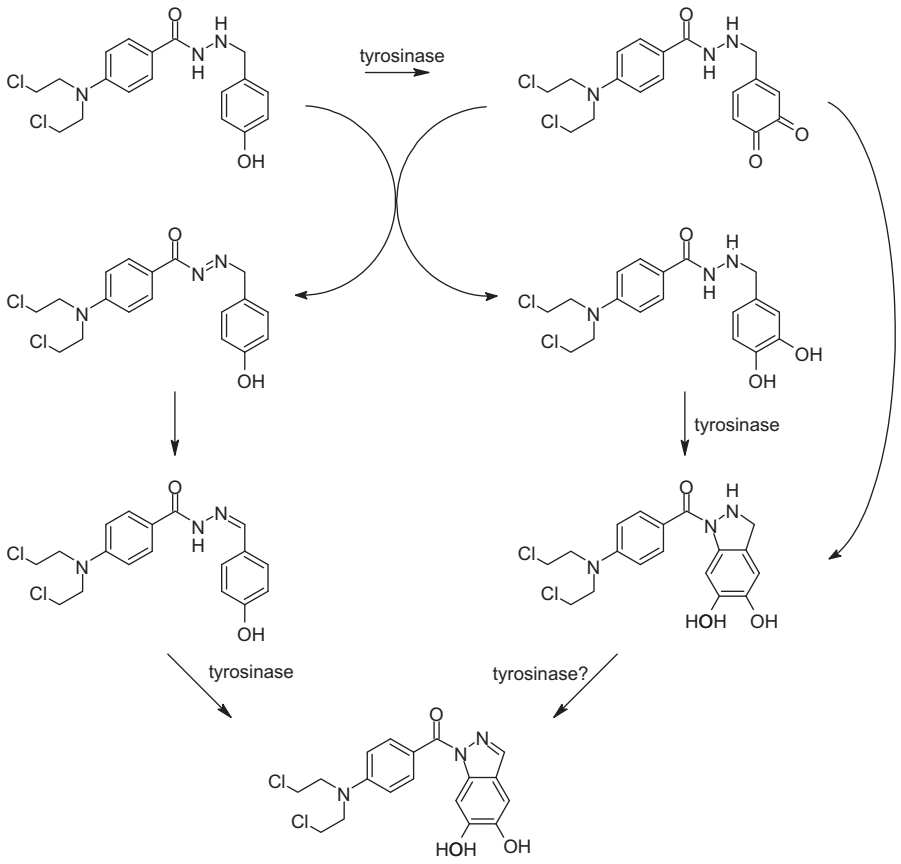


Fig. 4. Effect on the growth of melanoma tumors in mice: ◆ – control, ● – 4-[bis-(2-chloroethyl)amino]benzoic acid, ▲ – *N*-{4-[bis-(2-chloroethyl)amino]-benzoyl}-*N*′-(4-hydroxybenzyl)hydrazine, ■ – melphalan. Compounds were applied at 100 μg dose.

3. Conclusions

Our results have demonstrated that (*N*-{4-[bis-(2-chloroethyl) amino]benzoyl}-*N*′-(4-hydroxybenzyl)hydrazine) is effectively oxidized by tyrosinase. However, the *o*-quinone formed by its enzymatic oxidation does not undergo intramolecular redox exchange reaction with the hydrazine linker but a Michael addition of the acylated hydrazine nitrogen atom or an intermolecular redox exchange with the hydrazine group of a substrate molecule, which gives, either directly or indirectly, via a diazene intermediate, the



Scheme 3. Reactions occurring during oxidation of *N*-{4-[bis-(2-chloroethyl)amino]benzoyl}-*N*′-(4-hydroxybenzyl)hydrazine by tyrosinase based on structures of identified products.

corresponding hydrazone. Neither pathway, however, leads to release of the effector part from the prodrug. Application of carbazates or semicarbazides would probably not prevent the cyclization reaction. Phenylhydrazine derivatives are most likely not an option, either, because of their general toxicity and higher susceptibility to oxidation by a variety of enzymes. It seems therefore that anti-melanoma prodrugs with such a structure cannot be selectively activated by tyrosinase.

4. Experimental

4.1. Synthesis

4.1.1. General procedures for the synthesis of the prodrug

Chemicals were purchased from Sigma–Aldrich, Merck or POCH (Gliwice, Poland) and used without further purification. All chemicals and solvents were of reagent grade, except buffer components and HPLC solvents, which were of analytical grade. Analytical TLC was performed on precoated aluminum plates (Merck silica gel 60 F254) and visualized by UV fluorescence, potassium permanganate solution or ninhydrin solution. Preparative column chromatography was carried out on silica gel (60–120 mesh, Merck). Melting points (uncorrected) were determined on a Boetius apparatus. IR spectra were recorded on a Nicolet 6700 spectrometer. ^{1}H, ^{13}C, and correlation NMR spectra were obtained using a Bruker Avance 400 MHz spectrometer. Chemical shifts are given in ppm referenced to the TMS signal. Coupling constants, *J*, are expressed in Hz. High-resolution mass spectra (HRMS) were obtained on a Bruker Daltonik micrOTOF-Q II mass spectrometer equipped with an ESI source in the positive or negative ion mode. The mass spectrometer was calibrated with sodium formate clusters.

4.1.2. tert-Butyl 4-[bis-(2-hydroxyethyl)amino]benzoate

Yield: 83%; m.p.: 89–93 °C;

^{1}H NMR (DMSO) δ: 1.50 (s, 9H); 3.47 (m, 4H); 3.55 (m, 4H); 4.81 (t, 2H, $J = 5.21$ Hz); 6.70 (d, 2H, $J = 8.0$ Hz); 7.66 (d, 2H, $J = 8.0$ Hz);

^{13}C NMR (DMSO) δ: 29.61; 54.68; 59.53; 80.60; 111.92; 118.70; 132.34; 152.90; 166.85;

IR: 3308, 3007, 2976, 2943, 2880, 1705, 1604, 1160, 1111, 1068, 1055, 1040, 837, 771;

HRMS (+ESI): *m*/*z* calculated for $C_{15}H_{23}NNaO_4$ $(M + Na)^+$ 304.1519; *m*/*z* measured: 304.1546.

4.1.3. tert-Butyl 4-[bis-(2-chloroethyl)amino]benzoate

Yield: 78%; m.p: 68–70 °C;

^{1}H NMR (DMSO) δ: 1.51 (s, 9H); 3.78 (m, 8H); 6.80 (d, 2H, $J = 8.0$ Hz); 7.72 (d, 2H, $J = 8.0$ Hz);

^{13}C NMR (DMSO) δ: 27.85; 40.76; 51.62; 79.27; 110.89; 118.81; 130.86; 149.79; 164.91; IR: 3009, 2968, 2930, 2851, 1697, 1607, 1519, 1318, 1165, 770;

HRMS (+ESI): *m*/*z* calculated $C_{15}H_{22}Cl_2NO_2$ $(M + H)^+$ 318.1022; *m*/*z* measured: 318.1042.

4.1.4. 4-[Bis-(2-chloroethyl)amino]benzoic acid

Yield: 95%; m.p: 162–167 °C;

^{1}H NMR (DMSO) δ: 3.79 (m, 8H); 6.81 (d, 2H, $J = 8.0$ Hz); 7.77 (d, 2H, $J = 8.0$ Hz); 12.29 (bs, 1H);

^{13}C NMR (DMSO) δ: 40.75; 51.65; 110.87; 118.10; 131.19; 149.83; 167.18; IR: 3432, 2968, 1678, 1603, 1523, 1416, 1297, 1183, 767;

HRMS (-ESI): *m*/*z* calculated for $C_{11}H_{12}Cl_2NO_2$ $(M - H)^-$ 260.0240; *m*/*z* measured 260.0254.

4.1.5. N-{4-[Bis-(2-chloroethyl)amino]benzoyl}-N′-tert-butoxycarbonylhydrazine

Yield: 96%; m.p: 78–85 °C;

^{1}H NMR (DMSO) *δ*: 1.42 (s, 9H); 3.77 (m, 8H); 6.80 (d, 2H, *J* = 8.0 Hz); 7.75 (d, 2H, *J* = 8.0 Hz); 8.77 (bs, 1H); 9.89 (bs, 1H);

^{13}C NMR (DMSO) *δ*: 28.04; 40.90; 51.69; 78.87; 110.82; 120.04; 129.07; 148.97; 155.60; 165.52; IR: 3396, 3290, 2979, 2932, 1722, 1655, 1608, 1510, 1368, 1252, 1160, 762;

HRMS (+ESI): *m*/*z* calculated for $C_{16}H_{23}Cl_2N_3NaO_3$ $(M + Na)^+$ 398.1009; *m*/*z* measured: 398.0998.

4.1.6. 4-[Bis-(2-chloroethyl)amino]benzoic acid hydrazide

Yield: 90%; m.p: 123–128 °C;

^{1}H NMR (DMSO) *δ*: 3.75 (m, 8H); 4.50 (bs, 2H) 6.76 (d, 2H, *J* = 8.0 Hz); 7.71 (d, 2H, *J* = 8.0 Hz); 9.47 (bs, 1H);

^{13}C NMR (DMSO) *δ*: 40.51; 51.22; 110.65; 117.15; 129.25; 149.55; 165.19; IR: 3435, 2951, 2927, 2821, 2666, 1606, 1513, 1328, 831, 758;

HRMS (+ESI): *m*/*z* calculated for $C_{11}H_{16}Cl_2N_3O$ $(M + H)^+$ 276.0665; *m*/*z* measured: 276.0659.

4.1.7. N-{4-[Bis-(2-chloroethyl)amino]benzoyl}-N′-(4-hydroxybenzylidene)hydrazine

Yield: 72%; m.p: 119–122 °C;

^{1}H NMR (DMSO) *δ*: 3.80 (m, 8H); 6.83 (d, 2H, *J* = 8.0 Hz); 6.84 (d, 2H, *J* = 8.0 Hz) 7.54 (d, 2H, *J* = 8.0 Hz); 7.81 (d, 2H, *J* = 8.0 Hz); 8.33 (bs, 1H); 9.90 (bs, 1H); 11.41 (bs, 1H);

^{13}C NMR (DMSO) *δ*: 41.46; 52.22; 111.40; 116.11; 121.45; 126.03; 129.05; 129.79; 147.28; 149.48; 159.59; 162.88; IR: 3420, 3223, 1603, 1509, 1355, 1276, 1188, 1055, 920, 833, 756, 726;

HRMS (+ESI): *m*/*z* calculated for $C_{18}H_{20}Cl_2N_3O_2$ $(M + H)^+$ 380.0927; *m*/*z* measured: 380.0936.

4.1.8. N-{4-[Bis-(2-chloroethyl)amino]benzoil}-N′-(4-hydroxybenzyl)hydrazine

Yield: 74%; m.p: 116–122 °C;

^{1}H NMR (DMSO) *δ*: 3.76 (m, 10H); 5.11 (m, 1H); 6.71 (d, 2H, *J* = 8.4 Hz); 6.76 (d, 2H, *J* = 9.2 Hz); 7.14 (d, 2H, *J* = 8.4 Hz); 7.69 (d, 2H, *J* = 9.2 Hz); 9.30 (s, 1H); 9.77 (bs, 1H);

^{13}C NMR (DMSO) *δ*: 41.42; 52.20; 55.19; 111.30; 115.36; 121.26; 128.99; 129.19; 130.33; 149.13; 156.87; 165.76; IR: 3398, 3281, 2960, 2936, 1608, 1516, 1458, 1280, 830, 759, 730;

HRMS (+ESI): *m*/*z* calculated for $C_{18}H_{22}Cl_2N_3O_2$ $(M + H)^+$ 382.1084; *m*/*z* measured 382.1077.

4.2. Analysis of the enzymatic activation of the prodrug

4.2.1. Spectrophotometric analysis of the oxidation of the prodrug by tyrosinase

Mushroom tyrosinase was isolated as previously described [21]. A stock solution of *N*-{4-[bis-(2-chloroethyl)amino]benzoyl}-*N*′-(4-hydroxybenzyl)hydrazine was prepared in 40% methanol at 1 mM concentration. The substrate was then diluted to 0.05 mM in 10 mM sodium phosphate buffer, pH 6.8 giving 2% final methanol concentration, and 10 μg, 5 μg or 2.5 μg of tyrosinase from a 1 mg/ml stock in the same buffer was added. The final reaction volume was 3 ml. The spectra between 200 and 600 nm were recorded at 1 min intervals for 60 min in a Jasco V-650 UV–Vis spectrophotometer.

4.2.2. HPLC analysis

Products of the enzymatic reactions were analyzed on an Accucore C18 analytical column (100 mm length, 2.1 mm diameter, 2.6 μm particle size) from Thermo Scientific with a guard column connected to a Dionex UltiMate 3000 HPLC instrument with a UV–Vis detector with four channels. Separations were performed at 30 °C at a flow rate of 0.2 ml/min. Samples dissolved in methanol were injected into a 5 μL loop and then eluted with the following program: 30% acetonitrile in water for 2 min, 30–60% gradient of acetonitrile in water for 8 min, followed by 90% acetonitrile in water for 4 min. Chromatograms were recorded at 230, 280, 300, and 335 nm.

4.2.3. Isolation of the products of the enzymatic reaction

To identify the products of the oxidation of *N*-{4-[bis-(2-chloroethyl)amino]benzoyl}-*N*′-(4-hydroxybenzyl)hydrazine by tyrosinase 30.58 mg of the substrate (0.1 mM) was incubated in 800 ml of 10 mM sodium phosphate buffer, pH 6.8, with 4 mg of tyrosinase. The reaction mixture was stirred for up to 2 h (conditions were optimized for each of the products) and was then extracted with 2 × 400 ml of dichloromethane. Because the phases separated poorly, the mixture was transferred to 50 ml Falcon tubes and centrifuged at 7000×*g* for 5 min at room temperature in an Eppendorf 5810R centrifuge. The organic layer was recovered and dehydrated with anhydrous sodium sulfate. The solvent was evaporated, the residue was dissolved in 400 μl of acetonitrile and applied to a 10 g octadecyl column (Bakerbond® Octadecyl 40 μm Prep LC Packing). The sample was eluted with a stepwise gradient of acetonitrile in water (30 ml of each: 40%, 45%, 50%, 55%, 60%, 70%, 80%, 90% of acetonitrile in water and then with 100% acetonitrile). Fractions containing a pure product were pooled, the solvent was evaporated, the residue was dissolved in DMSO and analyzed by NMR (^{1}H, ^{13}C, HSQC, HMBC).

Separation on a reverse-phase column was not effective for the isolation of the second product, migrating in HPLC analysis close to the substrate. This compound was therefore isolated by column chromatography of the crude extract of the enzymatic reaction on silica gel (eluent: 1–3% methanol in dichloromethane).

For HPLC analysis reactions were carried out at 5 μmol scale (1.91 mg of the substrate in 50 ml of buffer) with 250 or 500 μg of tyrosinase for 15, 30, 60, and 120 min. After extraction with dichloromethane and evaporation of the solvent, samples were dissolved in 500 μL of methanol, a small portion was diluted 1:5 with methanol and analyzed as described above. The reaction mixture before extraction and both phases after extraction were analyzed by TLC with the substrate and the free mustard drug (4-[bis-(2-chloroethyl)amino]benzoic acid) as standards.

4.2.4. MS analysis of the products of the enzymatic reaction

For MS analysis the product purified by reverse phase chromatography was dissolved in acetonitrile–water (100 μl:50 μl) and 10 μl of 1 N sodium hydroxide was added. The second product isolated by normal phase chromatography was dissolve in acetonitrile-water (the same ratio). These samples were injected directly into the mass spectrometer.

4.2.4.1. 5,6-Dihydroxy-1H-indazol-1-yl 4-[bis-(2-chloroethyl)amino] benzoate. ^{1}H NMR (DMSO) *δ*: 3.80 (m, 8H); 6.86 (d, 2H, *J* = 8.0 Hz); 7.09 (s, 1H),7.80 (s, 1H), 8.00 (d, 2H, *J* = 8.0 Hz); 8.15 (s, 1H); 9.40 (bs, 2H);

^{13}C NMR (DMSO) *δ*: 41.39; 52.17; 101.01; 104.63; 111.03; 118.83; 120.59; 134.00; 135.14; 139.90; 144.98; 149.46; 150.14; 166.64;

HRMS (-ESI): *m*/*z* calculated for $C_{18}H_{16}Cl_2N_3O_3$ $(M - H)^-$ 392.0574; *m*/*z* measured: 392.0580;

4.3. Analysis of anti-melanoma activity

4.3.1. Cell culture

The following cell lines were used: B16–F10, NIH3T3 (all from ATCC); HECa10 (lymph node-derived and provided by Dr. D. Duś, Institute of Immunology and Experimental Therapy, Wrocław,

Poland). Cells were cultured using RMPI 1640 media supplemented with 10% FBS and using NUNCLON™ Surface (NUNC™) culture vessels. Cultures were kept in a humidified standard incubator (37 °C and 5% CO_2).

4.3.2. Drug cytotoxicity

Cells were grown in 96-well plates (NUNCLON™ Surface, 2×10^3 cells per well, 100 μL of RMPI 1640 supplemented with 10% FBS). After 24 h, the tested compound was added in quadruplicate to the culture medium using eleven different concentrations (10–2000 μM). The culture vessels were then placed in an incubator (5% CO_2, 37 °C) for further 24 h. Next, culture media were replaced with MTT/3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide solution (0.5 mg MTT/1 mL PBS^-) and cells were additionally incubated for 3 h at 37 °C. The formed formazan crystals were dissolved in acid isopropanol solution. Spectrophotometric measurements were performed using ELISA EL_x800 reader (Bio-Tek Instruments Inc.) at 570 nm. Percentage of live cells was estimated as: (absorbance at time *t*/initial absorbance) × 100%.

4.3.3. Effect on growth of melanoma tumors

All procedures involving animals were performed with the consent of the Local Ethics Committee, Medical Academy, Katowice, Poland. Therapy was performed using 6 to 8-week-old C57BL/6 mice from our own animal facility. Each animal was injected intradermally (dorsal side) with 2×10^5 B16–F10 cells/100 μL PBS^- (pH 7.2). When tumors reached ca. 3 × 3 mm (on the 6th day), mice received the tested compounds (100 μg or 200 μg) intratumorally for 3 days. Tumor size (caliper-measured), tumor growth rate as well as survival of animals were monitored.

Acknowledgments

This work was supported by a grant from the Polish Ministry of Science and Higher Education No. 2 P05F 00330.

Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version, at http://dx.doi.org/10.1016/j.ejmech.2013.10.080. These data include MOL files and InChiKeys of the most important compounds described in this article.

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