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Original article

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

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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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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.

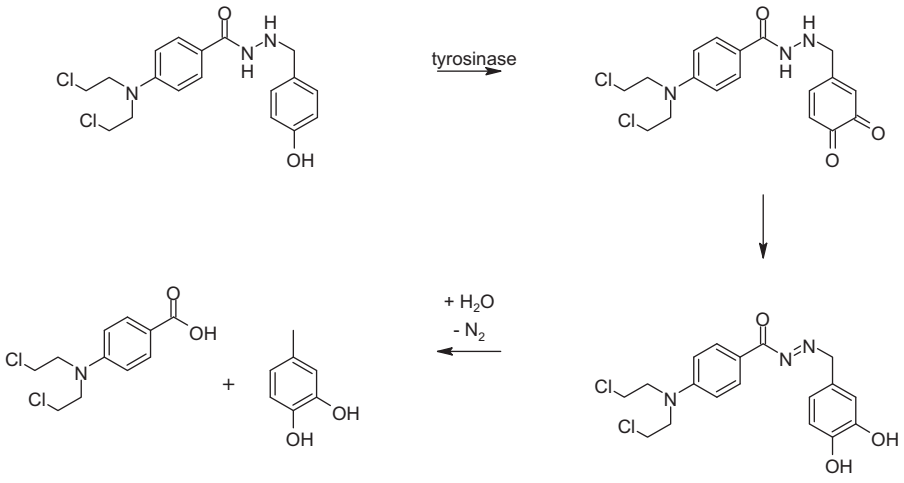
2. Results and discussion

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.