Neuroblastoma directed therapy by a rational prodrug design of etoposide as a substrate for tyrosine hydroxylase


Abstract

Tumor directed cytotoxic therapy is one of the major challenges for the success of chemotherapy. In order to accomplish this goal in neuroblastoma, we rationally designed a prodrug of etoposide as substrate for tyrosine hydroxylase, a well established neuroblastoma associated enzyme. Here, we report synthesis and characterization of a 3,4 dihydroxy-phenyl carbamate derivative of etoposide. In order to demonstrate activation by tyrosine hydroxylase, the coding sequence of murine tyrosine hydroxylase was generated by reverse transcriptase-polymerase chain reaction from NXS2 neuroblastoma cells and cloned into the pRSET-A bacterial expression vector. The enzyme was expressed in Escherichia coli, characterized by Western blot and enzymatic activity was demonstrated by conversion of tyrosine into DOPA in the presence of cofactors using reversed phase high-performance liquid chromatography. Under these enzymatic conditions, we demonstrate conversion of 3,4 dihydroxy-phenyl carbamate prodrug into free etoposide. This effect was clearly mediated by the enzyme since bacteria transformed with the empty vector were ineffective of prodrug activation. Furthermore, tyrosine hydroxylase positive cells exposed to the etoposide prodrug were effectively killed in contrast to tyrosine hydroxylase negative controls. These findings demonstrate that etoposide can be designed as a prodrug substrate for tyrosine hydroxylase and thereby establish proof of concept for neuroblastoma directed enzyme prodrug therapy.

Keywords: Tyrosine hydroxylase; Etoposide; Prodrug; Neuroblastoma; Chemotherapy

1. Introduction

Improved strategies for chemotherapy remain one of the major challenges for the effective treatment of cancer. This is particularly true for neuroblastoma, a disease in which chemotherapy has not yet lived up to its promise. Several strategies to improve tumor selectivity are currently under preclinical and clinical investigation, involving the use of monoclonal antibodies in order to target a cytotoxic agent into the tumor microenvironment [1]. An alternative approach to tumor selective chemotherapy is anti-
body-directed enzyme prodrug therapy (ADEPT), a promising approach towards improving the efficacy of chemotherapy while at the same time reducing its dose-limiting peripheral toxicity [2,3]. In this approach, an enzyme is targeted to the tumor site by conjugation to an antibody that binds a tumor-associated antigen or an angiogenic marker protein. Following systemic delivery of such a protein, its localization at the tumor site, and clearance of unbound conjugate, a designed prodrug is delivered systemically. Ideally, since the prodrug is not activated in the periphery and is non-toxic in the prodrug form, toxicity is localized to the tumor site thereby minimizing dose-limiting side effects. This is a three component system consisting of a drug activating enzyme, a prodrug(s), and a targeting antibody. With the availability of humanized or human antibodies against a range of tumor-associated antigens or angiogenic markers, the targeting component of this strategy has yet to be limiting. The enzyme and prodrug components of the strategy, however, present serious challenges to the practical application of this approach. The prodrug should be designed so that it may be catalytically converted to the active drug by the enzyme-antibody conjugate localized at the tumor site and not by endogenous enzymes. Further, since a wide range of drugs are used clinically in cancer therapy, the modification chemistry applied to inactivate the drug should be versatile and the enzyme used to activate the prodrug should ideally be capable of processing a wide variety of drugs. In order to meet the specific requirement that the prodrug not be activated by enzymes endogenous to humans, bacterial enzymes have typically been employed. Unfortunately, the use of non-human proteins in human therapy raises the issue of the immunogenicity of the drug-activating conjugate. Administration of a foreign protein typically elicits a vigorous antibody response that limits repeated administration of the protein. Indeed, immunogenicity has been problematic in clinical studies of ADEPT [4]. In order to suppress antibody responses against the enzyme-antibody conjugate, the immunosuppressive agent cyclosporin has been used in early clinical studies of the ADEPT approach [5]. Immunosuppression in cancer, however, is not desirable and offers only limited mitigation of an immune response against the conjugate. Thus, the immunogenicity of the enzyme component of ADEPT remains a major problem seriously limiting the use of this approach.

One direct strategy proposed to overcome the immunogenicity issue of the drug-activating enzyme is to use endogenous enzymes specifically expressed by the tumor. We selected tyrosine hydroxylase as endogenous tumor catalyst since it is highly expressed in neuroblastoma. The expression of tyrosine hydroxylase is selective for neuroblastoma since its natural expression occurs in sympathetic nervous tissue only, which is, except for the adrenal medulla, largely protected by the blood–brain barrier. Here we report the first prodrug of etoposide [6] activated by tyrosine hydroxylase to yield free etoposide thereby providing proof of concept for neuroblastoma directed enzyme prodrug therapy (NDEPT).

2. Materials and methods

2.1. Synthesis of ProVP16-VI

3-methoxybenzylalcohol was reacted with 4-nitrophenylchloroformate to give carbonate 1, which was further reacted with amine linker 2 to afford carbamate 3 (Fig. 1A). The latter was deprotected with TFA and immediately reacted with etoposide carbonate 4 to give the desired prodrug.

2.2. Bacterial expression of mouse tyrosine hydroxylase

Cloning of mouse tyrosine hydroxylase from NXS2 neuroblastoma cells was previously described [7]. EcoRI and Bg/II restriction enzymes were used for cloning of mTH from pCMV 3FUb mTH [7] into the bacterial expression vector pRSET A (Invitrogen, La Jolla, CA) designated mTH-pRSET A (mTH insert size: 1.5 Kb, pRSET A: 2.9 Kb, total: 3.4 Kb). The plasmid leads to expression of a 63 KD fusion protein containing a 6xHis epitope for downstream purification (Fig. 2A). Tyrosine hydroxylase protein expression was accomplished in BL21 Star(DE3) Escherichia coli stab. Briefly, LB medium (50 ml) containing 0.1 mM ferrous sulfate and 50 μg/ml Ampicillin was inoculated with 0.5 ml of a fresh overnight culture of BL21 Star(DE3) bacteria transformed with mTH-pRSET A. Protein expression was
induced by adding isopropyl-β-D-thiogalactopyranoside (IPTG) (1 mM final concentration) during the log phase of bacterial growth (OD$_{600}$ = 0.3–0.4). Samples for protein expression analysis by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) were taken 3 h after IPTG induction and compared to control samples taken before IPTG induction.

2.3. Purification of 6xHis-mTH recombinant protein

For purification of 6xHis-mTH, the ProBond Purification System (Invitrogen, La Jolla, CA) was used. For this purpose, bacteria were harvested from 50 ml culture by centrifugation. Lysis was accomplished after resuspension of the pellet in 10 ml of ‘Native Buffer’ supplemented with egg white lysozyme and four subsequent sonication-freeze (methanol/dry-ice) – thaw (37°C) – cycles. RNase and DNase was added to a final concentration of 5 μg/ml on ice for 15 min. Insoluble debris was removed by centrifugation (3000 × g, 15 min) and by passage through a 0.8 μm filter. Binding of the mTH protein to the column was accomplished by application of 5 ml
lysate- aliquots to the pre-equilibrated column. Each application was followed by three wash steps with ‘Native Binding Buffer’ (pH 7.8) and ‘Wash Buffer’ pH 6.0 and 5.5, respectively. The mTH protein was eluted with 5 ml of ‘Native pH Elution Buffer’. Fractions of 1 ml volume were collected and protein content determined by OD" reading.

Protein samples were reduced (50 mM DTT, 85°C, 2 min) and analyzed by standard SDS-PAGE and Western blot using rabbit anti-mouse tyrosine hydroxylase antibody (top) and anti-6xHis antibody (bottom) (U: Uninduced, I: Induced, P: Purified).

2.4. Determination of mTH activity by high-performance liquid chromatography (HPLC)

Enzyme reaction condition used to determine conversion of tyrosine and ProVP16 VI included 0.1 μl dihydropertedrine (3.8 U/ml), 1 μl catalase(1400 U/μl), 1 μl glycocol (0.2 M), 5 μl 6MpH4 (10 mM), 5 μl NADPH4 (10 mM), 10 μl mTH, 68 μl 0.2 M acetate buffer (pH 6.8). Reaction was started at 37°C by the addition of 10 μl Tyr (100 μM) or 10 μl ProVP16 VI (100 μM), respectively, and terminated by adding 50 μl of 0.4 M perchloric acid [8]. After centrifugation (10 000 g, 5 min) a 20 μl aliquot of the supernatant was subjected to HPLC analysis. The hydroxylation of tyrosine to DOPA was measured by HPLC as previously described [9]. For this purpose, a Waters dual pump HPLC unit (Model 715 Ultra Wisp) with a multiwavelength detector was used. Separation of tyrosine, DOPA, VP-16 and ProVP16 IV was accomplished on a reversed phase C18 analytical column (TSK-GEL HPLC C18 column, 4.6 × 250 mm, TosoHaas GmbH, Stuttgart, Germany) at a flow rate of 0.8 ml/min in 2% (v/v) ACN-H₂O (pH 4.5). Specific peaks for each compound were obtained and retention times were 7.5 min (DOPA), 12.8 min (tyrosine) and 5.4 min (VP16) and 16.3 min (ProVP16 IV).

2.5. Cytotoxicity assay

Cytotoxicity was determined by the XTT tetrazolium/formazan assay as previously described [10].
Briefly, cells were seeded in 96 flat bottom well plates at a density of $10^4$/well in 100 μl media 24 h before exposure to drug concentrations ranging from $10^{-4}$ to $10^{-1}$ M. After 72 h, cell viability was assessed by adding 50 μl XTT-reagent (1 mg/ml in serum free RPMI) activated with 0.2% v/v PMS (1.53 mg/ml in phosphate buffered saline) incubated at 37°C for 4 h. Plates were analyzed in a Thermomax (Molecular Devices) micro plate reader at 450 nm. OD values were plotted as a function of drug concentration and the curves were integrated using the softmax software to obtain the IC$_{50}$ concentration values.

3. Results and discussion

3.1. Expression and purification of mouse tyrosine hydroxylase

Following subcloning of mouse tyrosine hydroxylase cDNA into bacterial expression vector pRSET A, the integrity of the plasmid DNA was determined by EcoRI and BglII restriction enzyme digest (Figs. 2A, B). The expected fragment sizes were 1.5 kb (mTH) and 2.9 kb (pRSET), respectively, and both fragments were confirmed by agarose gel electrophoresis (Fig. 2B, lanes 1–3). Bacterial expression was determined following transformation of E. coli BL21 star (DE3) and induction of protein expression with IPTG. Samples were subjected to Western blot analysis before and after purification. The highest protein content was obtained in fraction 4 with a concentration of 0.46 mg/ml (Fig. 2C). Western blot-analysis with anti-tyrosine hydroxylase antibody (Fig. 2D top) and anti-6xHis antibody (Fig. 2D bottom) revealed bands at around 63KD characteristic for mTH.

3.2. Activation of ProVP16-IV by tyrosine hydroxylase

ProVP16-IV (Fig. 1, compound I) was designed to be specifically activated by tyrosine hydroxylase catalytic mechanism. The phenol functionality was masked through an $N,N$-dimethylethylendiamine spacer, linked to 3-methoxybenzylalcohol. We reasoned that hydroxylation of the aromatic ring in the ortho position to the methoxy group (Fig. 1, compound II) will trigger a self-immolative reaction-sequence that consequently will lead to the release of free etoposide. Thus, insertion of a hydroxyl group in the ring initiates rearrangement leading to the release of quinone methide species, spontaneous decarboxylation and cyclization reaction to form a dimethyl urea derivative and the free drug. The activity of 6xHis-tyrosine hydroxylase expressed by E. coli BL21 star (DE3) was demonstrated by determination of the enzymatic conversion of Tyrosine to Dopa. The Km of this reaction was at 69 μM similar to results previously reported [8]. The activation of ProVP16-IV was subsequently analyzed using the same enzyme reaction conditions. The generation of free VP-16 was clearly demonstrated over time with a Km of 58 μM (data not shown). Bacteria transformed with empty vector were used as negative controls with no detectable prodrug activation. These results show that tyrosine hydroxylase converts ProVP16 IV to free VP-16 and demonstrate that ProVP16 IV is a prodrug. This is the first time that a hydroxylation reaction by an enzyme is being applied for the concept of selective prodrug activation.

3.3. Cytotoxicity of ProVP16-IV against neuroblastoma cells

Based on the finding of tyrosine hydroxylase mediated activation of ProVP16-IV, we tested the cytotoxic activity of the drug against tyrosine hydroxylase positive neuroblastoma cell lines NXS2 and SK-N-AS. Results were compared to a control
cell line Molt-3 negative for tyrosine hydroxylase expression (Fig. 3). We demonstrate that the neuroblastoma cells were killed, but no effect was observed in tyrosine hydroxylase negative Molt-3 cells. However, the calculated IC₅₀ for ProVP16-IV in NXS2 and SK-N-AS cells of 5 × 10⁻⁵ and 3 × 10⁻⁵ M, respectively, are 2 logs greater than the IC₅₀ of free VP-16 against the same cell lines (see Lange et al. in this issue). Determination of anti-tumor effects and the therapeutic window for ProVP16-IV in vivo is subject to ongoing investigations. This involves the determination of the maximum tolerated dose and anti-tumor response in relevant preclinical models.

4. Conclusion

In summary, we report the first prodrug of etoposide activated by tyrosine hydroxylase, an enzyme highly expressed by the majority of neuroblastomas. Cytotoxic effects in the presence of intracellular tyrosine hydroxylase expression provide the first example for the concept of NDEPT.

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References