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ORIGINAL ARTICLE

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Thymidine phosphorylase as a target for imaging and therapy with thymine analogs

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Abstract *Purpose*: Thymidine phosphorylase (TPase; platelet-derived endothelial cell growth factor) is an attractive target for imaging and therapy because of the strong relationship between its expression in tumor biopsies and clinical outcome in many tumor types. Although the mechanism has yet to be explained, expression of TPase is highly associated with angiogenesis. *Methods*: Tumor cells were phenotyped for TPase activity, and incubated with thymine or its analogs (5-X-Ura). After intracellular conversion to thymidine analogs via the reverse reaction for TPase, these molecules were phosphorylated and incorporated into DNA. *Results*: Preferential localization was found in cells with high TPase, e.g. U937. Incorporation was enhanced in cells with high TPase by coincubation with modulators such as deoxyuridine. *Conclusions*: 5-X-Ura molecules can be readily labeled with positron emitters, and this finding provides support for further evaluation in vivo of their potential as probes for noninvasive external imaging of TPase, both at the time of diagnosis and during maneuvers intended to manipulate TPase. If the 5-X-Ura molecules were labeled with a therapeutic isotope, e.g. ^{125}I or ^{211}At, selective cytotoxicity would be expected in cells with high TPase expression. However, direct evaluation of the safety in vivo of the therapeutic approach is required. The 5-X-Ura compounds constitute a novel approach to both imaging and therapy directed towards TPase. Further, there are distinct advantages to using the imaging mode to identify tumors likely to benefit from therapy with the same set of molecules.

Keywords Thymine analogs · Thymidine phosphorylase · Angiogenesis · PET imaging

Abbreviations *BrUra:* bromouracil · *dIno:* deoxyinosine · *dR-P:* deoxyribose-1-phosphate · *dThd:* thymidine · *dUrd:* deoxyuridine · *PET:* positron emission tomography · *Thy:* thymine · *TPase:* thymidine phosphorylase · *UPase:* uridine phosphorylase · *Ura:* uracil · *5-X-Ura:* thymine analogs

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Introduction

The expression of thymidine phosphorylase (TPase; EC 2.4.2.4), a well-known enzyme for pyrimidine catabolism, is strongly associated with clinical outcome for many tumor types. High expression of TPase is associated with an extremely poor overall survival compared with low TPase, as illustrated by representative reports on four different cancer types: colorectal [22], head/neck [11], bladder [1], and cervical [5]. TPase has also emerged in an unanticipated new role as a growth factor associated with angiogenesis [9]. A large series of growth factors (e.g. VEGF, EGF, FGF, TNF, and PDECGF) and their receptors have been implicated in angiogenesis, and the elucidation of their roles/interrelationships is an ongoing story. PDECGF (platelet-derived endothelial cell growth factor) has been identified, surprisingly, as TPase [20, 22].

The ability to image factors such as TPase could have enormous potential as a guide to therapeutic development, as well as in the treatment of individual patients. Non-invasive, external imaging methods avoid the need for biopsies, and also have the capability of scanning large areas of the body, indeed, the entire body if necessary. Multiple time-points can be obtained during a single imaging session, and longitudinal evaluations (before and after therapy) are feasible.

At one level, monitoring of TPase could serve as a broad, nonspecific biomarker for evaluation of treatment. If demonstrated in prospective studies, a reduction in TPase activity could be interpreted as successful therapy, and encourage its continuation. Conversely, failure to reduce TPase could lead to a recommendation to change the therapeutic approach. Perhaps more importantly, imaging could identify particular tumors that might benefit from therapy directed specifically at TPase as a target. For example, Fukushima et al. [6] have reported that an inhibitor of TPase ["TPI", 5-chloro-6-(2-iminopyrrolidin-1-yl)methyl-2,4(1H,3H)-pyrimidinedione] shrinks tumors in mice.

In the study reported here, we explored the underlying rationale for using increased expression of TPase within tumors as a mechanism for intracellular conversion of Thy-based prodrugs into dThd analogs that might be noninvasively imaged and/or be cytotoxic. As a first step, we provide data from in vitro studies that support these concepts and lay the groundwork for further evaluation in vivo.

We present the following rationale for the use of Thy analogs to image and treat tumors with high TPase, the enzyme that splits dThd into Thy and dR-P:

$$\mathrm{dThd} + \mathrm{P_i} \rightarrow \mathrm{Thy} + \mathrm{dR-P}$$

This reaction is not helpful for trapping dThd-based probes within the tumor, because Thy readily leaves the cell. However, TPase also catalyzes the reverse reaction (converts Thy to dThd). Because nucleosides are efficiently phosphorylated within the cell, Thy molecules that are converted to dThd by TPase should be trapped within the cell, either as nucleotides or via subsequent processing and incorporation into DNA. If the products are trapped intracellularly, the accumulation within cells will track the rate of conversion by TPase.

$$\mathrm{Thy} + \mathrm{dR-P} \rightarrow \mathrm{dThd}$$

$$\mathrm{dThd} \rightarrow \mathrm{dTMP} \rightarrow\rightarrow \mathrm{DNA}$$

Thus, we hypothesized that tumor cells with high levels of TPase expression would trap Thy more readily than cells with low levels of TPase expression.

A variety of Thy analogs (5-X-Ura; Fig. 1) are also substrates for TPase, which increases the set of potential probes. In general, a suitable Thy analog could include any substituent at the 5-position of the Ura base. In addition to Thy itself, familiar Thy analogs include 5-Br-Ura, 5-I-Ura, and 5-CF_3-Ura (trifluorothymine). Ura and 5-FUra are also substrates for TPase, but are less desirable for the current application because they are also substrates for UPase. Thus, it would not be feasible to separate the contributions of TPase and UPase as determinants for intracellular accumulation of Ura and 5-FUra.

Preferential trapping of 5-X-Ura within cells provides opportunities for both imaging and therapy. Thus, in one aspect, by radiolabeling a Thy analog with a positron emitter, we now have a probe to noninvasively monitor the localization of TPase within the tumor and/or normal host tissue by use of imaging technologies such as a PET scanner. In another aspect, a Thy analog labeled with a therapeutic isotope would be useful for therapy because it is preferentially localized in tumors with high expression of TPase. The tumors that are found via imaging to have high expression of TPase are also the tumors likely to benefit from therapy with the 5-X-Ura compounds.

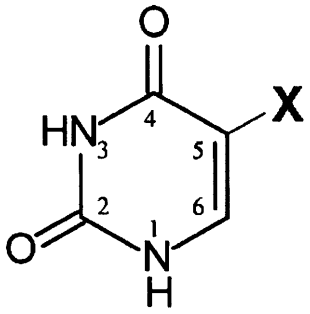

Fig. 1 Structure of 5-X-Ura (Thy and its analogs). X = CH_3, Br, I, CF_3, etc

The reverse reaction of TPase is much slower in cells than the forward reaction. It appears that the major limitation is the intracellular supply of the co-substrate for the reverse reaction, dR-P. dR-P does not cross cell membranes, so its intracellular supply must be increased via a precursor. Deoxyribonucleosides are converted by the forward reaction of TPase or purine nucleoside phosphorylase into dR-P and its corresponding base. Thus, compounds such as dIno, dUrd, and dThd are all effective in cell culture at increasing the rate of the reverse reaction for TPase.

Materials and methods

Materials

Human tumor-derived cell lines were obtained from the American Type Culture Collection (Fairfax, Va.). [Me-^{3}H]-dThd (65 Ci/mmol), [Me-^{3}H]-Thy (65 Ci/mmol), [6-^{3}H]-BrUra (20 Ci/mmol), [2-^{14}C]-CF_3Ura (56 mCi/mmol), and [2-^{14}C]-iodouracil (56 mCi/mmol) were obtained from Moravek Biochemicals (Brea, Calif.). The Bio-Rad protein assay kit was from Bio-Rad Laboratories (Hercules, Calif.). Nucleosides and all other reagents were obtained from Sigma Chemical Co. (St. Louis, Mo.).

Phenotyping cell lines for TPase

Five human tumor-derived cell lines were assayed for TPase activity by the method of Gan et al. [7]. Cells were suspended in Tris-HCl (10 m*M*) and extracts were prepared by five freeze-thaw cycles in dry ice/propanol and a 37°C water-bath. The protein content of the supernatant was determined with the Bio-Rad kit. An equal volume of extract was mixed with 20 m*M* sodium phosphate at pH 7.4 and incubated with tritiated dThd (0.25 m*M*) for 1 h at 37°C. The rate of formation of tritiated Thy was measured by HPLC with an on-line radioactivity detector. Thy and dThd were separated on a 4.6×250 mm, 5 μm, reverse-phase column. An isocratic mobile phase of 6% acetonitrile in 10 m*M* sodium acetate, pH 5, was used at a flow rate of 1 ml/min.

Conversion of Thy analogs into dThd analogs, and incorporation into DNA

Thy or BrUra at a concentration of 20 μ*M* and labeled with tritium was incubated with each of the cell lines for 24 h. The initial density was 300,000 cells/ml. Following the incubation period, our standard methods [10] were used for isolation and digestion of DNA, and HPLC analysis of its nucleosides.

Modulation of incorporation into DNA by dR-P prodrugs

The effects of dR-P prodrugs were examined in U937 cells incubated for 24 h with 20 μ*M* BrUra and 20 μ*M* of either dR-P or one of five different deoxyribonucleosides.

Results

Phenotyping cell lines for TPase

As shown in Table 1, three levels of activity were found. TPase activity in U937 cell extracts was the highest for all cell lines tested and tenfold higher than for Raji cells. In turn, TPase activity in Raji cells was at least sevenfold higher than in CEM, MOLT4 and K562 cells.

Conversion of Thy analogs into dThd analogs, with subsequent incorporation into DNA

Thy and BrUra were converted into dThd and bromodeoxyuridine, and subsequently incorporated into DNA. Figure 2 presents a comparison of incorporation into DNA of Thy (20 μ*M*) and BrUra (20 μ*M*) for the five human-derived tumor cell lines. The trends for Thy and BrUra were identical. U937 cells had the highest TPase activity and the greatest incorporation into DNA. Raji cells had intermediate TPase activity and intermediate DNA incorporation.

The three cell lines with lowest TPase activity (CEM, MOLT4, and K562) also had the lowest levels of incorporation into DNA. The five cell lines used in this work had identical proliferation rates (doubling times of about 24 h), but the incorporation of Thy or BrUra varied more than tenfold, and was directly associated with the amount of TPase in each cell line. If Thy and BrUra were proliferation markers, identical incorporation would have been observed in all cell lines.

Table 1 Phenotyping of cells for TPase. Cell extracts were incubated with [^{3}H]-dThd (0.25 m*M*) for 1 h. Enzyme activity was determined by measuring the rate of formation of [^{3}H]-Thy, with separation by HPLC. Values are means ± SD from triplicate experiments

Cell type	TPase activity (nmol/h/mg protein)
CEM	< 0.3
MOLT4	< 0.3
K562	< 0.3
Raji	2.0 ± 0.3
U937	24.9 ± 2.8

In addition to these studies with Thy and BrUra, pilot studies for iodouracil and trifluorothymine in U937 cells yielded similar results (data not shown).

Modulation of incorporation into DNA by dR-P prodrugs

The effects of dR-P prodrugs were examined in U937 cells incubated for 24 h with 20 μ*M* BrUra and 20 μ*M* of dR-P or one of five different deoxyribonucleosides. As illustrated in Fig. 3, the maximum enhancement of BrUra trapping in DNA was 24-fold for dUrd. An increase of sixfold was found for dIno.

When 20 μ*M* dUrd was added to 20 μ*M* BrUra in all cell lines, the dramatic increase in incorporation into DNA for U937 cells was more attenuated in Raji cells, and nonexistent in the three cell lines with low TPase (Fig. 4).

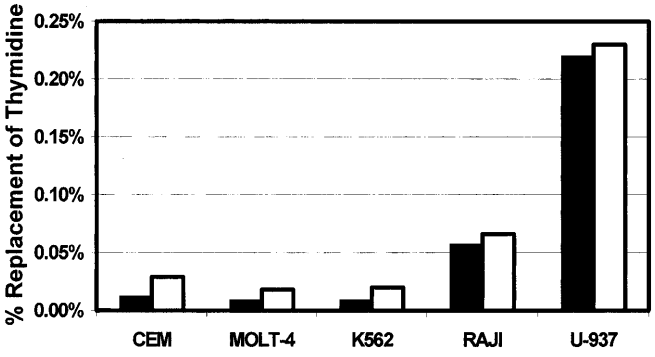

Fig. 2 Incorporation of 20 μ*M* BrUra (*black bars*) or Thy (*white bars*) into DNA after incubation with cell lines for 24 h. Experiments were conducted in duplicate; replicates differed by 5% or less

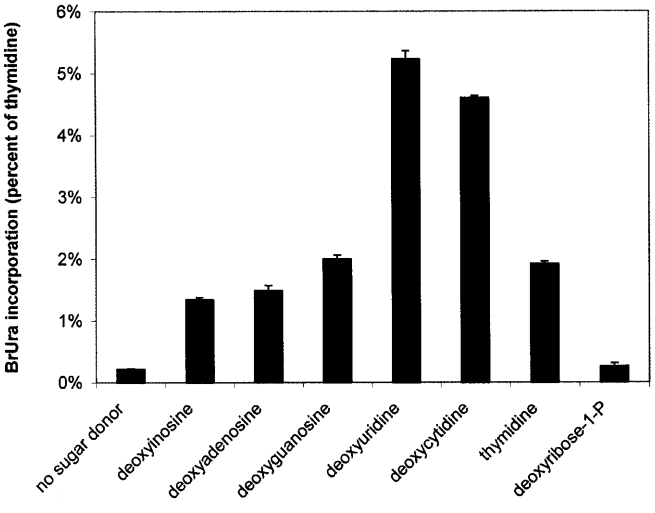

Fig. 3 Impact of deoxyribose-P prodrugs on incorporation of BrUra (20 μ*M*) into DNA. dR-P or one of the nucleosides were coincubated at 20 μ*M* for 24 h

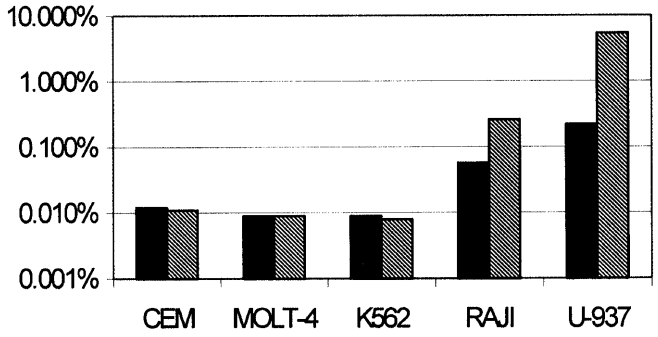

Fig. 4. Impact of dUrd (20 μ*M*) on incorporation of BrUra (20 μ*M*) into DNA (*solid bars* absence of dUrd, *hatched bars* dUrd present). Experiments conducted in duplicate; replicates differed by 10% or less. *Note:* logarithmic scale

Discussion

TPase has emerged as a surprisingly novel target for imaging and therapy of tumors. This report has introduced the underlying rationale for using Thy-based prodrugs (5-X-Ura) that might be noninvasively imaged and/or be cytotoxic. Data from studies in vitro have laid the groundwork for further evaluation in vivo. The data in Fig. 2 demonstrate that Thy and its analogs are not simply probes for cellular proliferation. 5-X-Ura localization inside cells was related to TPase activity, under circumstances in which cellular proliferation rates were identical.

Noninvasive imaging with 5-X-Ura

If confirmed by studies in vivo, PET imaging with 5-X-Ura could permit evaluation of the three-dimensional distribution of TPase activity throughout the body. Comparison of TPase activity before and after administration of a TPase inhibitor could yield an assessment of the effectiveness of TPase inhibition at any location in the body, and could be evaluated over a series of time-points. Several inhibitors of TPase, including acyclothymine [8] and 6-amino-thymine [14], have demonstrated effectiveness in isolated enzyme preparations. "TPI" has produced decreases in average or whole body TPase in mice and monkeys, as demonstrated by an increase in plasma concentrations when a dThd analog (trifluridine, 5-CF_3-dUrd) was administered [6].

The same approach could be applied to imaging of induction or upregulation of TPase activity. A diverse set of interventions produces increased TPase activity, e.g. interferon [12], taxanes [16], cyclophosphamide [4], radiation therapy [17]. These interventions have been demonstrated in cell culture or in animals, but not in humans. PET imaging for TPase at its intended target, the tumor, would permit comparison of baseline TPase activity and activity over a series of time-points after administration of a TPase modulator.

Although TPase is widely known as an inactivator of dThd analogs, capecitabine is an example of a prodrug that was designed to be activated by TPase in the tumor [18, 19]. TPase catalyzes the final step for the conversion of capecitabine to fluorouracil. Thus, the ability to monitor expression of TPase activity in the tumor can be a tool for predicting whether a preferential response to capecitabine is likely. Further, the optimal timing of when to administer capecitabine following an inducer/upregulator can be determined from the time of peak expression of TPase activity.

Treatment of tumors with 5-X-Ura

Once an imaging study has identified individual tumors that have high rates of incorporation of Thy analogs into DNA, these tumors become natural targets for therapeutic approaches that exploit this incorporation. The 5-X substituent could include a cytotoxic isotope, e.g. [^{211}At], [^{125}I], [^{131}I]. These radioactive forms of 5-X-Ura are very potent, and provide several options for biophysical modes of cell killing. For a weak beta emitter such as [^{125}I], only cells that have this isotope incorporated into DNA would be successfully treated. In contrast, when some alpha particles are generated, as for [^{211}At] [21], the region of tissue toxicity produced by the radioisotope extends beyond the borders of the cell in which it is localized. This "bystander effect" is critical for killing tumor cells that are quiescent at the time of exposure. Of course, there is a preferred range of cytotoxic effect; otherwise, adjacent normal tissue will be impacted adversely. Direct evaluation of the safety of the therapeutic approach in vivo is essential.

Combining 5-X-Ura with nucleosides to increase the intracellular supply of dR-P

Our studies with dUrd and dIno confirm literature reports (e.g. reference 3) that the reverse reaction rate of TPase can be increased by expanding the intracellular supply of the co-substrate, dR-P, with deoxyribonucleosides. This increase in reaction rate yields greater intracellular trapping of Thy analogs. For diagnostic uses, this effect translates into improved imaging sensitivity. For therapeutic uses, the effect is increased cytotoxicity.

Although any deoxyribonucleoside seems to be effective in cell culture, dIno is the only one that has been shown to be effective in vivo. In a study conducted in rats bearing tumors, Ciccolini et al. [3] have demonstrated that co-administration of dIno with 5-FUra increases the intracellular concentrations of fluorodeoxyuridine-derived anabolites, indicating that dIno shifts intracellular metabolism towards preferential utilization of the TPase pathway, rather than the UPase pathways that dominate 5-FUra anabolism.

Linkages of TPase imaging to clinical outcomes

Because of the strong association between angiogenesis and TPase activity, the ability to evaluate TPase activity provides an assessment of angiogenic activity and a potential tool for monitoring the impact of antiangiogenic treatments. Imaging of TPase would serve as a biomarker for angiogenesis. A biomarker would be valuable for determining which tumors have active angiogenesis, i.e. are candidates for treatment with antiangiogenic agents. In addition, the biomarker could help to determine whether antiangiogenic treatment is producing an effect.

Although a number of antiangiogenesis drugs are now being tested in patients with cancer, it has been difficult to evaluate their success. With classic clinical endpoints (e.g. survival), these trials will be very long, cumbersome, and expensive. Our most frequently used intermediate measure for cytotoxic drugs, tumor shrinkage, is not necessarily anticipated to be as relevant for antiangiogenic agents. Imaging of changes in TPase activity can provide an alternative window into therapeutic effectiveness.

By radiolabeling a Thy analog with a positron emitter, we have a probe that has the potential to noninvasively monitor the localization of TPase within or near the tumor by use of imaging. Immunohistochemical techniques have shown localization of TPase inside tumor cells and supporting stroma. For these sites, the results from our evaluation in vitro are reasonable initial models. On the other hand, if TPase is extracellular, localization might be reduced or eliminated as the circulation washes away products formed by TPase. Localization may be preserved, to some extent, because any locally generated nucleosides would be rapidly taken up into tumor cells, and then trapped as if they were formed intracellularly.

Because PET imaging can provide three-dimensional information with high sensitivity, positron emitters are particularly desirable for imaging probes. Positron-emitting isotopes with a variety of half-lives can be incorporated into Thy analogs, including: [^{124}I], 4 days; [^{76}Br], 16 h; [^{18}F], about 2 h; and [^{11}C], 20 min. This range of physical half-lives provides the optimal opportunity to match the isotope to the underlying biochemical process. For phenomena that occur on a time scale of an hour or less, the short half-life of [^{11}C] (20 min), provides rapid elimination from the body, thereby permitting serial "snapshots" of changes in tumor activity. For phenomena that are more readily tracked over a period of a day, [5-^{76}Br]-BrUra would provide the ideal match because its physical half-life is 16 h.

One of the 5-X-Ura probes, [2-^{11}C]-Thy, has already been directly administered to humans as part of a study to model the kinetic behavior of [2-^{11}C]-dThd [13]. The subjects were healthy volunteers, so no assessment of tumor-specific parameters was possible. In addition, many patients have been exposed to [2-^{11}C]-Thy as a metabolite when [2-^{11}C]-dThd was administered. Based upon prior human experience, the safety aspects of this class of probes are not expected to be problematic, but dosimetry must be evaluated separately for each probe. One of the major issues for modeling and interpreting imaging data from [2-^{11}C]-Thy is the formation and retention of multiple radiolabeled species, including [^{11}C]-bicarbonate and [^{11}C]-CO_2. Although Mankoff et al. [13] have demonstrated that these problems can be overcome, the effort is enormous. Other groups have addressed this issue by blocking the catabolism (see below).

The mechanism for the association of TPase and angiogenesis is undetermined. Of course, even if the association of TPase with angiogenesis was an epiphenomenon, TPase would still be a potentially useful target for identifying areas of active angiogenesis and the impact of treatments. Perhaps more importantly, independently of its relationship to angiogenesis, TPase is an attractive target for imaging, based upon its strong relationship to clinical outcome in many tumor types. The 5-X-Ura compounds constitute a novel approach to both imaging and therapy directed towards TPase. Further, there are distinct advantages to being able to use the imaging mode to identify tumors likely to benefit from therapy with the same set of molecules.

Although the concept is promising and the data in vitro are supportive, there are many obstacles to successful translation to the clinic. For all probes, these include considerations of drug delivery factors (tissue perfusion, cellular influx and efflux) and competing processes such as metabolism and excretion from the body. For the 5-X-Ura class of molecules, we are especially concerned about catabolites, which might accumulate and interfere with imaging by raising the nonspecific background radioactivity in tissues.

Dihydropyrimidine dehydrogenase is a highly active enzyme for catabolism of 5-X-Ura. As mentioned above, radiolabeled catabolites are a substantial obstacle to imaging. Saleem et al. [15] have reported the use of eniluracil to inhibit this enzyme to improve the imaging of 5-[^{18}F]-Ura in patients. Bading et al. [2] have explored the same concept for studies of 5-[^{18}F]-Ura in rats. Building upon the experience of these groups, the next step for our research is to extend these studies of 5-X-Ura from cell culture to an animal model in vivo, with and without an enzyme inhibitor.

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