Published in final edited form as:
ACS Med Chem Lett. ; 3(4): 268–272. doi:10.1021/ml2002554.

Ribonuclease-Activated Cancer Prodrug

Gregory A. Ellis[†,‡], **Nicholas A. McGrath**[§,‡], **Michael J. Palte**[‖], and **Ronald T. Raines**[*,†,§]

[†]Department of Biochemistry, University of Wisconsin–Madison, Madison, Wisconsin 53706, United States

[§]Department of Chemistry, University of Wisconsin–Madison, Madison, Wisconsin 53706, United States

[‖]Medical Scientist Training Program, and Molecular & Cellular Pharmacology Graduate Training Program, University of Wisconsin–Madison, Madison, Wisconsin 53706, United States

Abstract

Cancer chemotherapeutic agents often have a narrow therapeutic index that challenges the maintenance of a safe and effective dose. Consistent plasma concentrations of a drug can be obtained by using a timed-release prodrug strategy. We reasoned that a ribonucleoside 3′-phosphate could serve as a pro-moiety that also increases the hydrophilicity of a cancer chemotherapeutic agent. Herein, we report an efficient route for the synthesis of the prodrug uridine 3′-(4-hydroxytamoxifen phosphate) (UpHT). UpHT demonstrates timed-released activation kinetics with a half-life of approximately 4 h at the approximate plasma concentration of human pancreatic ribonuclease (RNase 1). MCF-7 breast cancer cells treated with UpHT showed decreased proliferation upon co-incubation with RNase 1, consistent with the release of the active drug—4-hydroxytamoxifen. These data demonstrate the utility of a human plasma enzyme as a useful activator of a prodrug.

Keywords

human pancreatic ribonuclease; 4-hydroxytamoxifen; pharmacokinetics; plasma; tamoxifen; timed-release

Many drug candidates have demonstrable therapeutic potential *in vitro* but fail *in vivo* because of poor pharmacokinetic behavior. [1,2] The dosing of chemotherapeutic agents for cancer, in particular, is made difficult by narrow therapeutic indices.[3,4] Following parenteral administration of a drug, there is a spike in drug plasma concentration, followed by a slow decline in concentration as the drug is eliminated or metabolized, complicating maintenance of the drug at a beneficial concentration. [3,5] Timed-release prodrug technology provides one potential means to overcome this problem. A pro-moiety renders the drug inactive until liberation by an enzyme-catalyzed or nonenzymatic process. Ideally, such timed-release modulates near-toxic peaks or near-ineffective troughs in the concentration of active drug in plasma.[1,3,5–7] Although many promoieties exist,[1–7] few provide timed-release in plasma.

[*]**Corresponding Author**, Tel: 608-262-8588. rtraines@wisc.edu.
[‡]These authors contributed equally to this work.

ASSOCIATED CONTENT

Supporting Information

Analytical data and experimental protocols. This material is available free of charge via the Internet at http://pubs.acs.org.

We sought a pro-moiety that would not only inactivate the parent drug, but also be released during catalysis by an endogenous plasma enzyme. Fulfilling these criteria is difficult, as few enzymes have adequate plasma concentrations and many that do have high specificity for a native substrate. Human pancreatic ribonuclease (RNase 1[8]; EC 3.1.27.5) is an exception. Contrary to its name, RNase 1 is expressed in tissues other than pancreas,[9] and circulates in human plasma at a concentration of ~0.4 mg/L.[10] Moreover, like its renowned homolog bovine pancreatic ribonuclease (RNase A[11,12]), RNase 1 catalyzes the cleavage of RNA by a transphosphorylation reaction[13–15] and has little specificity for its leaving group.[16–20] This promiscuity is the basis for the tumor-targeted activation of a phenolic nitrogen mustard from a ribonucleoside 3′-phosphate prodrug using an antibody–RNase 1 variant in an antibody-directed enzyme prodrug therapy (ADEPT) strategy.[20]

Due to the promiscuous activity of ribonucleases, we reasoned that a chemotherapeutic drug condensed with a ribonucleoside 3′-phosphate pro-moiety would be released upon catalysis by RNase 1. We were aware that the use of a ribonucleoside 3′-phosphate as a pro-moiety would be facilitated by extant, highly optimized phosphoramidite chemistry,[21,22] making the prodrug readily accessible on a laboratory or industrial scale. A pendant ribonucleoside 3′-phosphate could render inactive a small-molecule drug by hindering the interaction with its target. The hydrophilicity of a ribonucleoside 3′-phosphate could impart improved pharmacokinetics to hydrophobic drugs.[23] Additionally, small molecules with anionic groups are endowed with reduced rates of cytosolic uptake and glomerular filtration.[24–31]

For our proof-of-concept studies, we chose the model parent drug 4-hydroxytamoxifen (HT). HT is the activated form of tamoxifen (oxidized by cytochrome P450 enzymes[32]) and is significantly more potent than tamoxifen as an anti-proliferative agent against breast cancer cells.[33] Tamoxifen acts as an anti-estrogen and is one of the most commonly used hormonal drugs for the prevention and treatment of breast cancer.[34,35] Unfortunately, tamoxifen can have off-target effects and is linked to an increased risk (2–3%) of endometrial carcinoma and pulmonary embolism.[36] Presumably, these side effects could be attenuated by delivering tamoxifen at a consistent, low dose.[37–41] Tamoxifen-encapsulated liposomes have been developed for this purpose,[41] but liposomal delivery has, in general, demonstrated only modest efficacy in the clinic.[42] Hence, we elected to attach HT to uridine 3′-phosphate and analyze the activation of this model prodrug by RNase 1 (Figure 1).

Uridine 3′-(4-hydroxytamoxifen phosphate) (UpHT) was synthesized in five steps from commercially available HT (≥70% *Z* isomer, which is the more active form[43,44]) and uridine phosphoramidite (Scheme 1). Briefly, HT was coupled to uridine phosphoramidite by using *N*-methylbenzimidazolium triflate as a catalyst.[45] The coupled product was oxidized with iodine and deprotected stepwise. The final product was purified by reverse-phase HPLC on C18 resin to provide UpHT in an overall yield of 58%.

We expected the uridine 3′-phosphate moiety of UpHT to endow the prodrug with greater hydrophilicity than the parent drug, which could improve pharmacokinetic behavior. To investigate this issue, we calculated the partition (logP) and distribution (logD) coefficients of UpHT and HT.[46] The calculated logP and logD values of UpHT were indeed significantly lower than those of the parent drug HT (Table 1), indicative of increased hydrophilicity.

To be the basis for an effective timed-release prodrug strategy, the pro-moiety needs to be released by the activating enzyme over time. Hence, we assessed the RNase 1-catalyzed rate of HT-release from UpHT. To do so, RNase 1 (final concentration: ~0.15 μg/mL) was added to 0.10 M MES–NaOH buffer, pH 6.0, containing NaCl (0.10 M) and UpHT (0.090 mM).[47–49] The reaction mixture was incubated at 37 °C, and aliquots were withdrawn at known times and assayed for HT by HPLC. Under these conditions, which are typical for

assays of ribonucleolytic activity,[11,47] HT was released with a half-life of ~4 h (Figure 2). Importantly, UpHT was stable in the absence of RNase 1; after 11 h at 37 °C, <6% of UpHT had degraded to HT.

To assess the unmasking of UpHT under more physiological conditions, HT-release from UpHT was monitored in cell-culture medium (Figure 3A). In medium without added ribonucleases, HT was released with a half-life of ~9 h. To validate that UpHT is inherently unstable at pH 7.4 (as opposed to the medium containing contaminating ribonucleases), the stability of UpHT was assessed in ribonuclease-free 0.10 M sodium phosphate buffer, pH 7.4, containing NaCl (0.10 M). Again, the half-life was ~9 h. The instability of UpHT at pH 7.4 is consistent with HT being a good leaving group, as its hydroxyl group has pK_a ~9.3.[46] By comparison, the P–O$^{5'}$ bond in RNA has a half-life of 4 y.[51]

To demonstrate the efficacy of UpHT *in cellulo*, we monitored its effect on the proliferation of MCF-7 breast cancer cells, which are known to be vulnerable to HT.[33] UpHT was made more anti-proliferative by the presence of added RNase 1 (Figure 3B), indicating that UpHT is a ribonuclease-activatable prodrug. Thus, we have demonstrated proof-of-concept for a prodrug strategy that employs a human plasma enzyme to release a cancer chemotherapeutic agent in a timed-release manner (Figure 1).

In addition to the attributes evident in UpHT, the RNase 1/ribonucleoside 3′-phosphate prodrug system has versatile modularity. For example, the leaving group need not be an aryloxy group. Pancreatic-type ribonucleases catalyze the cleavage of P–O bonds to alkoxy groups, which could include a self-immolative linker to an amino group.[4] RNase 1 is known to cleave RNA faster after pyrimidine than purine nucleobases.[52] Hence, cytidine- and uridine-masked drugs are likely to be activated more rapidly than adenosine- and guanosine-masked drugs. In addition, synergistic drugs could be conjugated to different ribonucleoside 3′-phosphates to achieve simultaneous release of drugs at desired concentrations. These same effects could be used to optimize simultaneous plasma concentrations of chemoprotective drugs and chemotherapeutic drugs. The pharmacokinetics of the drug could be tuned further by modification of the ribose 5′-hydroxyl group. For instance, this hydroxyl group could be PEGylated to enhance serum half-life, extended with additional nucleoside 3′-phosphates to increase hydrophilicity, or alkylated with the intent of increasing hydrophobicity.[53]

Finally, we note that nucleoside 3′-phosphate pro-moities could impart selective activation of chemotherapeutic agents near tumor sites. Although RNase 1 was employed herein due to its abundance in plasma,[8,9,54,55] RNase 1 homologues might also activate prodrugs like UpHT *in situ*.[8] One such homologue is eosinophil-derived neurotoxin (RNase 2), which is carried and released by eosinophils.[8] These cells are known to accumulate and degranulate at tumor sites.[56–58] We anticipate that, akin to prodrug monotherapy (PMT) in which prodrugs are activated by endogenous enzymes found in abundance near tumors,[59] a prodrug strategy reliant on RNase 2 could be used to generate active drugs at adventitious sites. Studies to probe the versatility of the RNase 1/ribonucleoside 3′-phosphate prodrug system are underway in our laboratory.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors acknowledge M. L. Hornung for early contributions to this work.

Funding

This work was supported by grant R01 CA073808 (NIH). N.A.M. was supported by the postdoctoral fellowship F32 GM096712 (NIH). M.J.P. was supported by Molecular and Cellular Pharmacology Training Grant T32 GM008688 (NIH) and predoctoral fellowship 09PRE2260125 (American Heart Association). This study made use of the National Magnetic Resonance Facility at Madison, which is supported by NIH grants P41RR02301 (BRTP/NCRR) and P41GM66326 (NIGMS). Additional equipment was purchased with funds from the University of Wisconsin, the NIH (RR02781, RR08438), the NSF (DMB-8415048, OIA-9977486, BIR-9214394), and the USDA.

ABBREVIATIONS

HT	hydroxytamoxifen
MES	2-(*N*-morpholino)ethanesulfonic acid
RNase 1	human pancreatic ribonuclease
U>p	uridine 2′,3′-cyclic phosphate
UpHT	uridine 3′-(4-hydroxytamoxifen phosphate)

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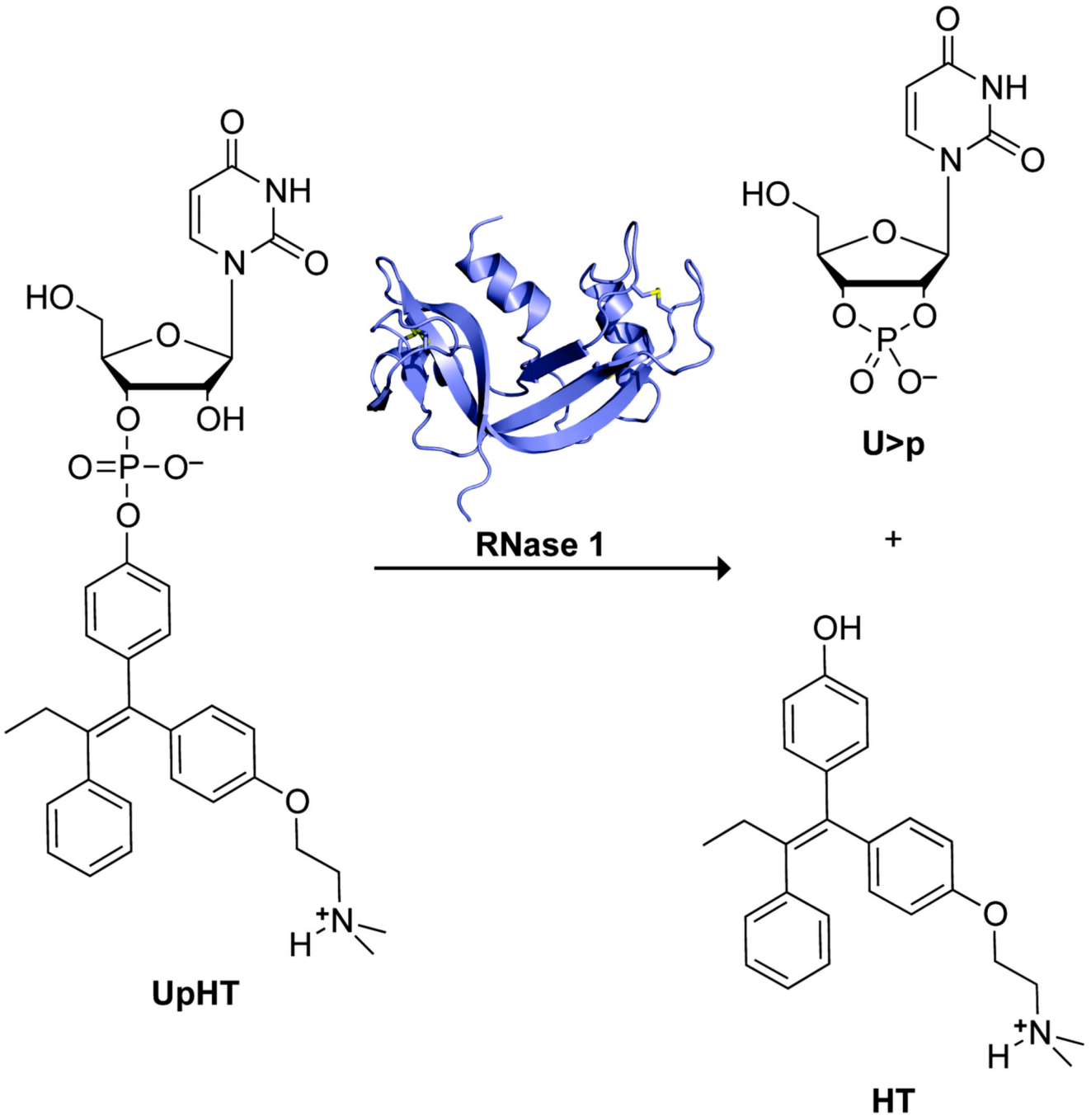


Figure 1.
Scheme showing the cleavage of prodrug UpHT by RNase 1 to yield uridine 2′,3′-cyclic phosphate (U>p) and HT.

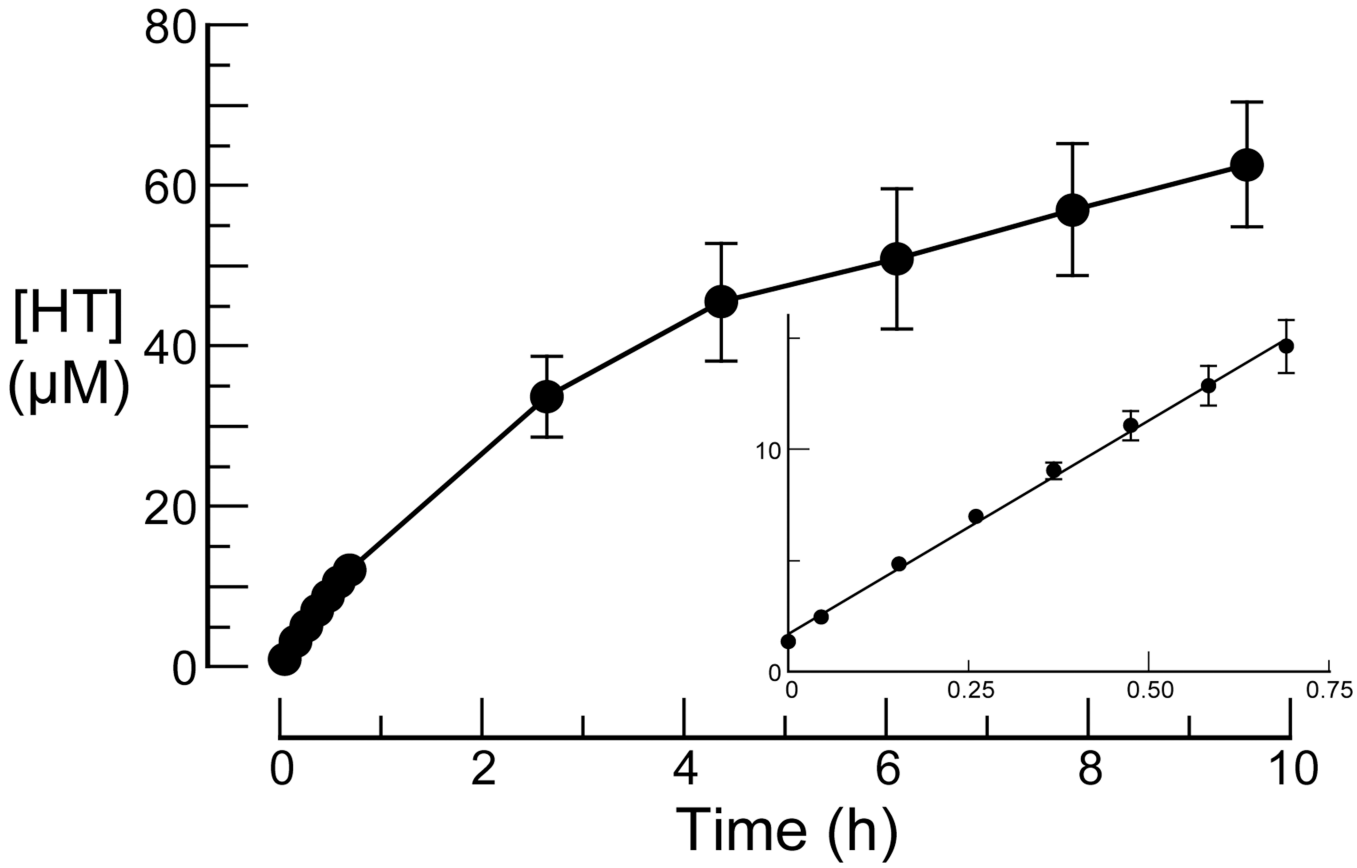


Figure 2.
Progress curve for the release of HT from UpHT (0.090 mM) by RNase 1 (~0.15 μg/mL) in 0.10 M MES–NaOH buffer, pH 6.0, containing NaCl (0.10 M) at 37 °C. Inset: $t < 1$ h.

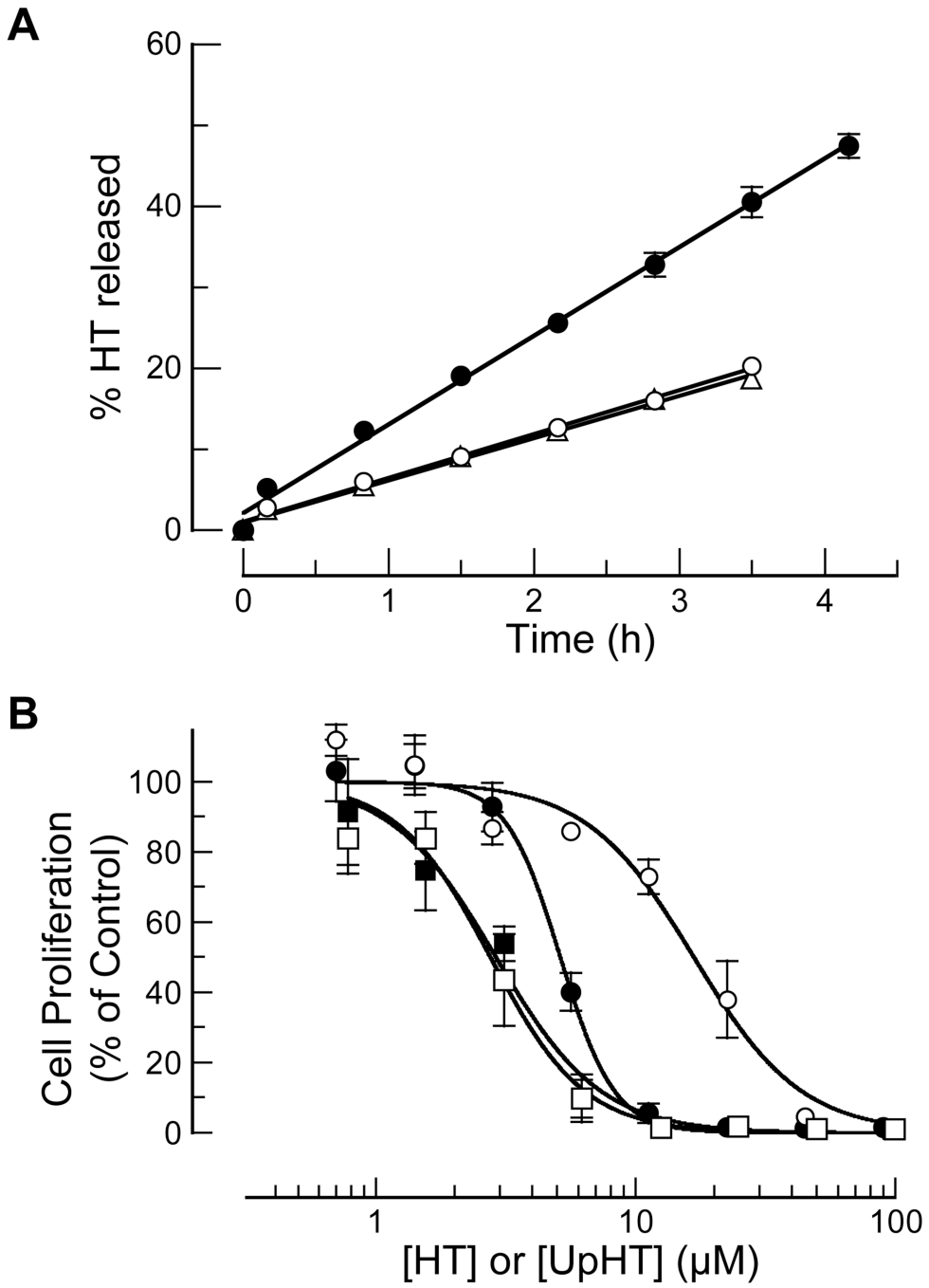
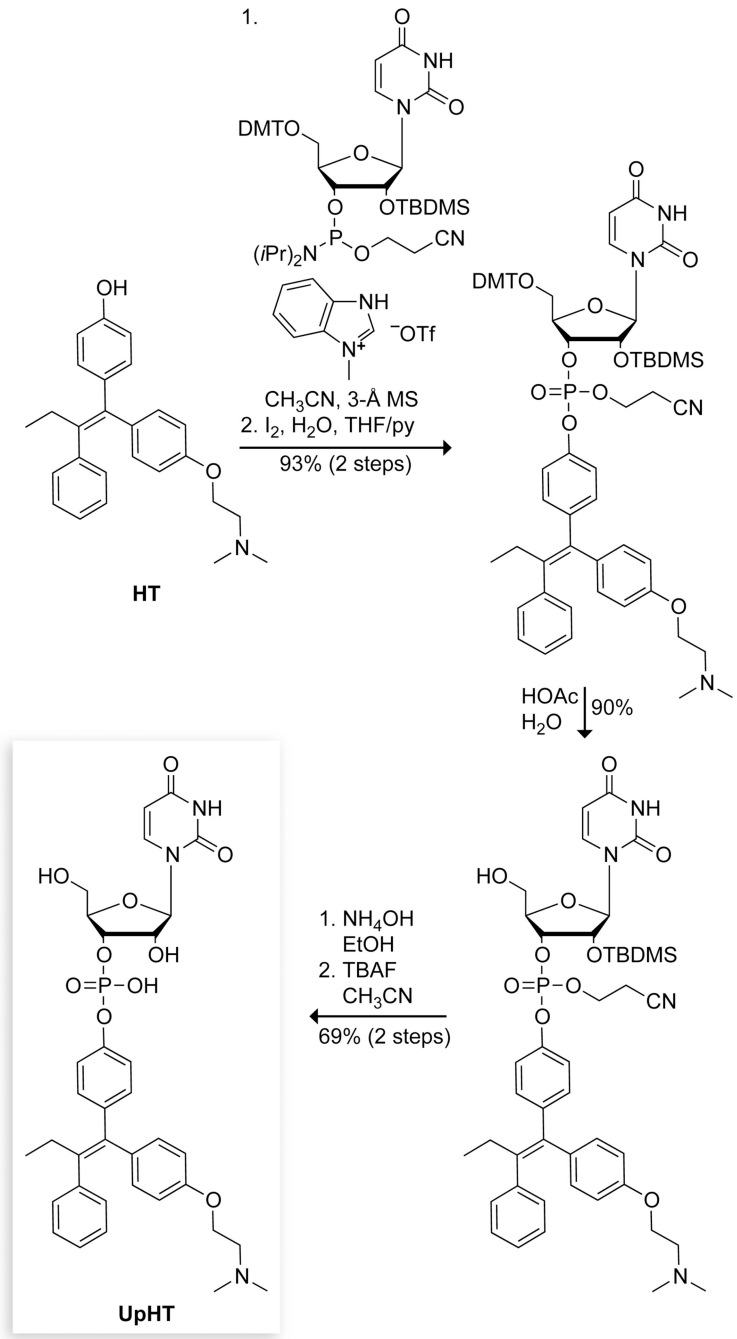


Figure 3.

Stability of UpHT and effect of UpHT on the proliferation of MCF-7 cells. All data points are the mean (±SE) of separate experiments carried out in triplicate. (A) Progress curves for the release of HT from UpHT (40 μM) at 37 °C in ribonuclease-free 0.10 M sodium phosphate buffer, pH 7.4, containing NaCl (0.10 M) (Δ, $t_{½}$ = 9.4 h), and serum-free[50] medium in the absence (○; $t_{½}$ = 9.0 h) and presence (●; $t_{½}$ = 4.4 h) of RNase 1 (0.4 μg/mL). (B) Proliferation of MCF-7 cells in serum-free[50] medium, monitored by the incorporation of [*methyl*-^{3}H]thymidine into cellular DNA. UpHT in the absence (○; IC_{50} = 16.7 ± 0.8 μM) and presence (●; IC_{50} = 5.2 ± 0.2 μM) of RNase 1 (6.2 μg/mL). HT in the absence (□; IC_{50} = 2.7 ± 0.1 μM) and presence (■; IC_{50} = 2.7 ± 0.4 μM) of RNase 1.



Scheme 1.
Synthesis of UpHT

Table 1

Calculated partition and distribution coefficients of HT and UpHT[46]

Coefficient	HT	UpHT
log*P* (non-ionized)	6.05	3.88
log*P* (ionized)	2.55	−2.00
log*D* (pH = 7.4)	4.66	0.12
log*D* (pH = p*I*[a])	5.69	−1.79

[a]HT, p*I* 9.01; UpHT, p*I* 5.00.