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(54) Title: ENZYME CATALYZED THERAPEUTIC ACTIVATION

(57) Abstract: This invention provides novel substrate compounds that selectively inhibit the proliferation of pathological cells, for example, pathological cells that endogenously overexpress a target enzyme that confers resistance to biologic and chemotherapeutic agents. The enzyme acts on a substrate compound to 1) convert it to a cellular toxin and/or 2) release a toxic byproduct. In one embodiment, the activity of the target enzyme has been greatly enhanced in a target cell as a result of loss of tumor suppressor function and/or selection resulting from previous exposure to chemotherapy. In another embodiment, the pathological cell contains a target enzyme that is an expression product of an infectious agent in the cell. Further provided by this invention is a method for treating a subject by delivering to the subject a prodrug as described herein. The prodrugs of this invention may be used alone or in combination with other chemotherapeutics or alternative anti-cancer therapies such as radiation.

# ENZYME CATALYZED THERAPEUTIC ACTIVATION

## CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims priority under 35 U.S.C. § 119(e) to the following U.S. provisional applications, U.S. Serial Nos.: 60/145,356; 60/145,437; and 60/191,315, filed July 22, 1999; July 23, 1999 and March 21, 2000, respectively, the contents of which are hereby incorporated by reference into the present disclosure.

## TECHNICAL FIELD

The present invention relates to the field of drug discovery and specifically, the design of prodrugs that are substrates for endogenous intracellular enzymes that are overexpressed in pathological cells.

## BACKGROUND OF THE INVENTION

Throughout and within this disclosure, various publications are referenced by first author and date, patent number or publication number. The full bibliographic citation for each reference can be found within the specification or at the end of this application, immediately preceding the claims. The disclosures of these publications are hereby incorporated by reference into this disclosure to more fully describe the state of the art to which this invention pertains.

Cancer is one of the most commonly fatal human diseases worldwide. Treatment with anticancer drugs is an option of steadily increasing importance, especially for systemic malignancies or for metastatic cancers that have passed the state of surgical curability. Unfortunately, the subset of human cancer types that are amenable to curative treatment today is still rather small (Haskell, C.M. (1995)) resulting in about 600,000 deaths per year. See, Cancer Facts & Figures, 1999 American Cancer Society. Progress in the development of drugs that can cure human cancer is slow, with success limited to a few hematological malignancies and fewer solid tumor types (Dorr and Van Hoff (1994)). Progress in discovering therapies that

are based upon disease mechanism offers opportunities for future success. (Cobleigh, M.A. et al. (1999); and Roth, J.A. et al. (1999)).

The heterogeneity of malignant tumors with respect to their genetics, biology and biochemistry as well as primary or treatment-induced resistance to therapy mitigate against curative treatment. Moreover, many anticancer drugs display only a low degree of selectivity, causing often severe or even life threatening toxic side effects, thus preventing the application of doses high enough to kill all cancer cells. Searching for anti-neoplastic agents with improved selectivity to treatment-resistant pathological, malignant cells remains therefore a central task for drug development.

Cancer cells are characterized by uncontrolled growth, de-differentiation and genetic instability. The instability expresses itself as aberrant chromosome number, chromosome deletions, rearrangements, loss or duplication beyond the normal diploid number. (Wilson, J.D. et al. (1991)). This genomic instability may be caused by several factors. One of the best characterized is the enhanced genomic plasticity which occurs upon loss of tumor suppressor gene function (e.g., Almasan, A. et al. (1995a) and Almasan, A. et al. (1995b)). The genomic plasticity lends itself to adaptability of tumor cells to their changing environment, and may allow for the more frequent mutation, amplification of genes, and the formation of extrachromosomal elements (Smith, K.A. et al. (1995) and Wilson, J.D. et al. (1991)). These characteristics provide for mechanisms resulting in more aggressive malignancy because it allows the tumors to rapidly develop resistance to natural host defense mechanisms, biologic therapies (see, Wilson, J.D. et al. (1991) and Shepard, H.M. et al. (1988)), as well as to chemotherapeutics (see, Almasan, A. et al. (1995a); and Almasan, A. et al. (1995b)).

In addition, the clinical usefulness of a chemotherapeutic agent may be severely limited by the emergence of malignant cells resistant to that drug. A number of cellular mechanisms are probably involved in drug resistance, e.g., altered metabolism of the drugs, impermeability of the cell to the active compound or accelerated drug elimination from the cell, altered specificity of an inhibited enzyme, increased production of a target molecule, increased repair of cytotoxic lesions, or the bypassing of an inhibited reaction by alternative biochemical pathways. In some cases, resistance to one drug may confer resistance to other, biochemically distinct drugs. An alternative mechanism of resistance to cancer chemotherapeutics occurs *via* the functional loss of tumor suppressor genes. The best characterized of these are p53, RB and p16. (Funk,

J.O. 1999; and Teh, B.T. (1999)). Loss of function of these gene products leads to depressed expression of enzymes commonly targeted by anti-cancer drugs (e.g., 5-fluorouridyl ("5FU")/thymidylate synthase and methotrexate/dihydrofolate reductase). (Lee, V. et al. (1997); Lenz, H.J.et al. (1998); and Fan, J. and Bertino, J. (1987)). Amplification of certain genes is involved in resistance to biologic and chemotherapy. Amplification of the gene encoding dihydrofolate reductase is related to resistance to methotrexate, while overexpression/amplification of the gene encoding thymidylate synthase is related to resistance to treatment with 5-fluoropyrimidines. (Smith, K.A. et al. (1995)). Table 1 summarizes some prominent enzymes in resistance to biologic and chemotherapy.

**Table 1**

**Enzymes Overexpressed in Resistance to Cancer Chemotherapy**

| Enzyme | Biologic or Chemotherapy | Referenced (Examples) |
|---|---|---|
| Thymidylate synthase | Uracil-based<br>Folate-based<br>Quinazoline-based | Lönn, U. et al. (1996)<br>Kobayashi, H. et al. (1995)<br>Jackman, A.L. et al. (1995a) |
| Dihydrofolate reductase | Folate-based | Banerjee, D. et al. (1995)<br>Bertino, J.R. et al. (1996) |
| Tyrosine kinases | TNF-alpha<br>Multidrug resistance | Hudziak, R.M. et al. (1988)<br>Stühlinger, M. et al. (1994) |
| MDR-associated proteins (ABC P-gp proteins) | Multidrug resistance | Simon, S.M. and Schindler, M. (1994)<br>Gottesman, M.M. et al. (1995) |
| CAD* | PALLA** | Smith, K.A. et. Al. (1995)<br>Dorr, R.T. and Von Hoff, D.D., eds. (1994) |
| Topoisomerase I (Colon & Prostate Cancers) | Camptothecin | Husain et al. (1994) |
| Ribonucleotide reductase | Hydroxyurea | Wettergren, Y. et al. (1994)<br>Yen, Y. et al. (1994) |

* CAD = carbamyl-P synthase, aspartate transcarbamylase, dihydroorotase
** PALA = N-(phosphonacetyl)-L-aspartate

The poor selectivity of anticancer agents has been recognized for a long time and attempts to improve selectivity and allow greater doses to be administered have been numerous. One approach has been the development of prodrugs. Prodrugs are compounds that are toxicologically benign but which may be converted *in vivo* to therapeutically active products. In some cases, the activation occurs through the action of a non-endogenous enzyme delivered to the target cell by antibody ("ADEPT" or antibody-dependent enzyme prodrug therapy (U.S. Patent No.4,975,278)) or gene

targeting ("GDEPT" or gene dependent enzyme-prodrug therapy (Melton, R.G. and Sherwood, R.E. (1996)). These technologies have severe limitations with respect to their ability to exit the blood and penetrate tumors. See, Connors, T.A. and Knox, R.J. (1995).

A number of nucleotide and nucleoside analogs have been developed and tested as both anti-tumor and anti-viral agents. For example 5-flurouracil (5FU) and 5-fluoro-deoxyuridine (5FUdR) have been widely used as chemotherapeutic agents based on their ability to be converted intracellularly into inhibitors of the thymidylate synthase (TS) enzyme. As with many other enzyme inhibitory chemotherapeutic agents, cancer therapy using 5FU frequently leads to selection for aggressive drug resistant tumor cells which are refractory to further treatment with this drug. (Aschele, C. et al. (1994); Mader, R.M. et al. (1998); Lönn, U. et al. (1996); Paradiso, A. et al. (2000); and Edler, D. et al. (2000)).

Particular attention has also been paid to halogenated nucleoside analogs such as (E)-5-(2-Bromovinyl)-2'-deoxyuridine (BVdU). These types of compounds were originally developed as anti-viral agents based on the observation that they required phosphorylation to the monophosphate nucleotide form in order to elicit cytotoxic responses and this phosphorylation was preferentially accomplished by herpes virus thymidine kinase (TK) enzymes. BVdU and related compounds have consistently shown limited toxicity to normal mammalian cells, while they have been effective at killing virally infected cells. (DeClerq, E. et al. (1997)).

Throughout the extensive testing of nucleoside analogs such as BVDU as anti-viral and anti-cancer agents, they have consistently been reported to have minimal toxicity to both normal and cancer cells. These observations have been supported by results demonstrating the preferential phosphorylation of such compounds by viral thymidine kinase enzymes, which explains the low toxicity of these compounds for mammalian cells. Thus, BVdU and related nucleoside analogs have not been developed as anti-cancer therapeutic agents. (De Clerq, E. et al. (1997)).

## DISCLOSURE OF THE INVENTION

This invention provides novel compounds that selectively inhibit the proliferation of pathological cells, for example, pathological cells that endogenously overexpress a target enzyme that confers resistance to biologic and chemotherapeutic agents. The

enzyme acts on a prodrug compound of this invention to 1) convert it to a cellular toxin and/or 2) release a toxic byproduct. In another aspect of this invention, the product of an initial reaction with a resistance enzyme, is subsequently fully activated by a common cellular enzyme such as an acylase, phosphatase or other "housekeeping" enzyme (Voet, et al. (1995)) or common cellular constituent (e.g., water) to release the toxic byproduct from the prodrug.

In one embodiment, the activity of the target enzyme has been greatly enhanced in a target cell as a result of loss of tumor suppressor function and/or selection resulting from previous exposure to chemotherapy. In another embodiment, the pathological cell contains a target enzyme that is an expression product of an infectious agent in the cell. The expression product can confer antibiotic resistance.

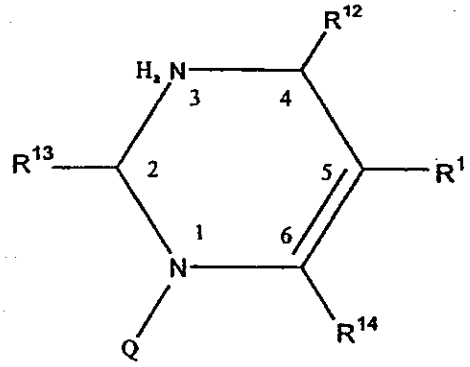
Another aspect of this invention includes assays for screening for new prodrugs that can be activated by target enzymes. Kits to perform such assays containing the reagents and instructions necessary to complete the assay and analyze the results are also provided by this invention.

Further provided by this invention is a method for treating a subject by delivering to the subject a prodrug as described herein. The prodrugs of this invention may be used alone or in combination with other chemotherapeutics or alternative anti-cancer therapies such as radiation.

A further aspect of this invention is the preparation of a medicament for use in treating a subject suffering from a pathology characterized by cells expressing a target enzyme.

A still further aspect of this invention is a method for identifying the optimal therapeutic for a subject, by isolating cells expressing a target enzyme and contacting the cells with at least one of the prodrugs of this invention, and then identifying which of the one or more prodrugs inhibits the proliferation or kills the cells, thereby identifying the optimal therapeutic for the subject.

The prodrugs of this invention have the following structure:




or tautomers thereof;

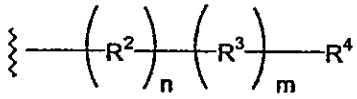
wherein $R^{12}$ or $R^{13}$ may be the same or different and are selected from the group consisting of oxo, OH or $NHNH_2$;

wherein if a is 0 or 1, providing that if a is 0 and $R^{13}$ is oxo, then a double bond exits between position 3 and 4 and $R^{12}$ is $NHNH_2$ or

if a is 0 and $R^{12}$ is oxo, then a double bond exists between position 2 and 3 and $R^{13}$ is $NHNH_2$; or

if a is 1, then $R^{12}$ and $R^{13}$ are both oxo;

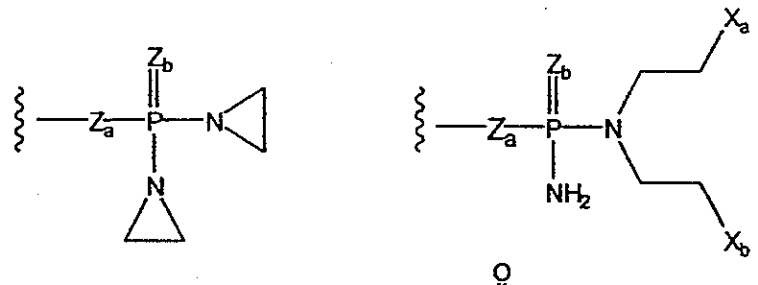
wherein $R^1$ is a substituent having the formula:

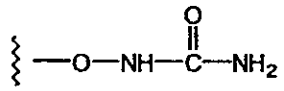


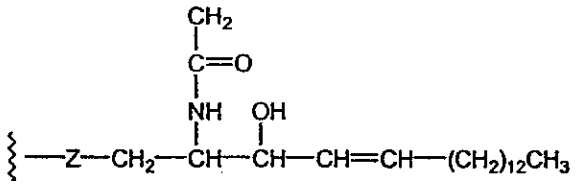
wherein $R^2$ and $R^3$ are independently an unsaturated or a saturated hydrocarbyl group;

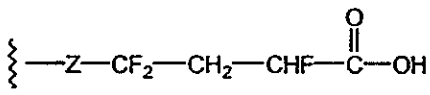
wherein n is 0 or an integer from 1 to 10 and m is 0 or 1; and

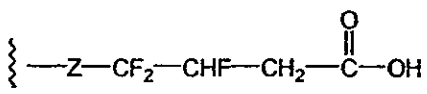
wherein $R^4$ is selected from the group consisting of F, Cl, Br, I, CN, $SO_3H$, $CO_2H$, $CO_2CH_2CH_3$, $CO_2CH_3$, $SI(CH_3)_3$, CHO, $NO_2$, $CF_3$, $CCl_3$, $CH{=}C(R^{15})_2$ or a substituent having the structure:

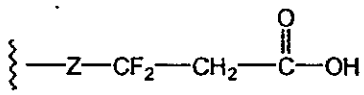


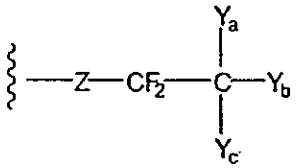


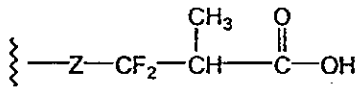


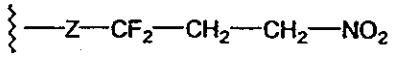


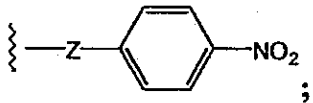









and


;

wherein $X_a$ and $X_b$ are independently the same or different and are selected from the group consisting of Cl, Br, I, and a potent leaving group;

wherein $Y_a$, $Y_b$ or $Y_c$ are independently the same or different and are H or F;

wherein Z, $Z_a$ and $Z_b$ are independently the same or different and are O or S; and

wherein $R^{14}$ is H or F, providing if $R^{14}$ is F, then a is 1 and $R^{12}$ and $R^{13}$ are both oxo; and

wherein Q is selected from the group consisting of a sugar, a carbocyclic, and an acyclic compound, or a masked phosphate derivative or phosphoramidate derivative thereof.

## BRIEF DESCRIPTION OF THE FIGURES

The concepts described herein are displayed graphically in Figure 1A and 1B using the example of the thymidylate synthase enzyme and the compound NB1011™. Figure 1A shows how higher levels of TS in tumor cells can lead to preferential generation of toxin. Figure 1B shows the conversion of NB1011™ to BVdUMP, and subsequent interaction with TS to generate nucleotide toxin.

Figures 2A and 2B are flow charts illustrating a high throughput screen of this invention.

Figure 3A shows relative TS levels in various human tissues. Figure 3B shows a comparison of average TS mRNA levels between normal and human colon tumor tissues.

Figure 4 shows the synthetic scheme for the furo[2,3-d]pyrimidinone nucleosides of this invention. The synthetic protocol is described in detail, *infra*.

## MODES FOR CARRYING OUT THE INVENTION

The practice of the present invention will employ, unless otherwise indicated, conventional techniques of molecular biology, microbiology, cell biology, organic chemistry, medicinal chemistry and recombinant DNA, which are within the skill of the art. See, *e.g.*, Sambrook et al., MOLECULAR CLONING: A LABORATORY MANUAL, 2nd edition (1989); CURRENT PROTOCOLS IN MOLECULAR BIOLOGY, F.M. Ausubel et al. eds., (1987); the series METHODS IN ENZYMOLOGY, Academic Press, Inc.; PCR 2: A PRACTICAL APPROACH, M.J. MacPherson et al., eds. (1995); Spector, D.L. et al. (1998) CELLS: A LABORATORY MANUAL, Vols I to III, Cold Spring Harbor Press; and ANIMAL CELL CULTURE, R.I. Freshney, ed. (1987).

As used in the specification and claims, the singular form "a," "an" and "the" include plural references unless the context clearly dictates otherwise. For example, the term "a cell" includes a plurality of cells, including mixtures thereof.

As used herein, the term "comprising" is intended to mean that the compositions and methods include the recited elements, but not excluding others. "Consisting essentially of' when used to define compositions and methods, shall mean excluding other elements of any essential significance to the combination. Thus, a composition consisting essentially of the elements as defined herein would not exclude trace contaminants from the isolation and purification method and pharmaceutically acceptable carriers, such as phosphate buffered saline, preservatives, and the like. "Consisting of" shall mean excluding more than trace elements of other ingredients and substantial method steps for administering the compositions of this invention. Embodiments defined by each of these transition terms are within the scope of this invention.

The term "overexpression" shall mean at least 2 fold, preferably 3 fold, and more preferably 4 fold and most preferably 5 fold or more expression over normal levels or levels measured from normal or non-pathological cells.

A "composition" is intended to mean a combination of active agent and another compound or composition, inert (for example, a detectable agent or label) or active, such as an adjuvant.

A "pharmaceutical composition" is intended to include the combination of an active agent with a carrier, inert or active, making the composition suitable for diagnostic or therapeutic use *in vitro*, *in vivo* or *ex vivo*.

As used herein, the term "pharmaceutically acceptable carrier" encompasses any of the standard pharmaceutical carriers, such as a phosphate buffered saline solution, water, and emulsions, such as an oil/water or water/oil emulsion, and various types of wetting agents. The compositions also can include stabilizers and preservatives. For examples of carriers, stabilizers and adjuvants, see Martin REMINGTON'S PHARM. SCI., 15th Ed. (Mack Publ. Co., Easton (1975)).

An "effective amount" is an amount sufficient to effect beneficial or desired results. An effective amount can be administered in one or more administrations, applications or dosages.

"Target" or "pathological" cells include hyperproliferative cells that are de-differentiated, immortalized, neoplastic, malignant, metastatic or transformed. Examples include, but are not limited to cancer cells such as sarcoma cells, leukemia cells, carcinoma cells, or adenocarcinoma cells. Specified cancers include, but are not limited to breast cancer cells, hepatoma cells, liver cancer cells, pancreatic carcinoma cells, oesophageal carcinoma cells, bladder cancer cells, gastrointestinal cancer cells, ovarian cancer cells, skin cancer cells, prostate cancer cells, and gastric cancer cells.

Target or pathological cells overexpress an intracellular enzyme that is related to any of a loss of tumor suppressor gene product function, drug resistance or genetic instability. Alternatively, resistance to one drug may confer resistance to other, biochemically distinct drugs. Unlike prior art therapies directed to creating more potent inhibitors of endogenous, intracellular enzymes, this invention exploits the higher enzyme activity associated with therapy-resistant diseased cells and tissues versus normal cells and tissues and does not rely on inhibiting the enzyme. The term "target enzyme" is used herein to define enzymes having one or more of the above noted characteristics.

Gene products activated or overexpressed and related to drug resistance include, but are not limited to thymidylate synthase (TS) (Lönn, U. et al. (1996); Kobayashi, H. et al. (1995); and Jackman, A. L. et al. (1995b)), dihydrofolate reductase (Banerjee, D. et al. (1995) and Bertino, J. R. et al. (1996)), tyrosine kinases (TNF-α, Hudziak, R. M. et al. (1988)) and multidrug resistance (Stühlinger, M. et al. (1994)); Akdas, A. et al.

(1996); and (Tannock, I.F. (1996)); and ATP-dependent multidrug resistance associated proteins (Simon, S.M. and Schindler, M. (1994)) and, in some diseases including colon and prostate cancer, topoisomerase I (Husain et al. (1994)).

Amplification of dihydrofolate reductase (DHFR) is related to resistance to methotrexate while amplification of the gene encoding thymidylate synthase is related to resistance to tumor treatment with 5-fluoropyrimidine. Amplification of genes associated with drug resistance can be detected and monitored by a modified polymerase chain reaction (PCR) as described in Kashini-Sabet, et al. (1988), U.S.Patent No. 5,085,983, or the method described herein. Acquired drug resistance can be monitored by the detection of cytogenetic abnormalities, such as homogeneous chromosome staining regions and double minute chromosomes both of which are associated with gene amplification. Alternative assays include direct or indirect enzyme activity assays and each of which are associated with gene amplification (e.g., Carreras and Santi (1995)); other methodologies (e.g. polymerase chain reaction, Houze, T. A. et al. (1997) or immunohistochemistry (Johnson, P. G. et al. (1997)).

The enzyme glutathione-S-transferase has been shown to be occasionally elevated in some human tumors (Morgan, A. S. et al. (1998)), but nevertheless is excluded from "target enzyme" as used herein because it is a member of a gene family encoding enzymes with overlapping specificities.

In sum, the prodrugs of the subject invention are distinguishable from conventional therapeutics on the basis that the target enzymes of this invention are commonly overexpressed, over-accumulated or activated in pathological cells versus normal cells. The most important principles that distinguishes the current invention from other approaches are:

(1) This invention describes the synthesis of substrates for enzymes like thymidylate synthase. The overexpressed enzyme will convert prodrugs to toxin, preferentially in diseased cells. Previous approaches have mostly relied on inhibitors of these enzymes. The inhibitors lead to amplified expression of the enzyme, and subsequent resistance to treatment (see, e.g., Lönn, U. et al. (1996); Paradiso, A. et al. (2000); and Edler, D. (2000)).

(2) The current approach is also distinguishable from other "substrate-prodrug" approaches, e.g., the glutathione-S-transferase enzymes (see, e.g., Morgan, A.S. et al. (1998)). The enzymes of the GST family are expressed at increased levels in

response to toxic insult to the cell. The GST family of enzymes has overlapping substrate specificities, which makes it difficult to design a substrate reactive with only a single species of enzyme with elevated expression in a cancer cell (Morgan, A.S. et al. (1998)). Because the target enzymes of the current invention (e.g., thymidylate synthase, dihydrofolate reductase and thymidine kinase) are unique with respect to its structure and substrate specificity, it is facile to design unique substrates. Several examples of substrates for thymidylate synthase are provided infra.

(3) In some cases the gene encoding the target enzyme (e.g., thymidylate synthase) may have undergone mutation to give resistance to inhibitors, (Barbour, K.W. et al. (1992) and Dicken, A.P. et al. (1993)) but will still be capable of carrying out reaction with non-inhibitor substrate prodrugs.

(4) An advantage of this approach is that loss of tumor suppressor function is critical to development of malignancy. The majority of tumor cells have lost one of the p53, RB or p16 tumor suppressor functions. (Funk, J.O. (1999); Banerjee, D. (1998); and Teh, B.T. et al. (1999)). Such a loss results in increased expression of resistance enzymes (*e.g.*, thymidylate synthase), independent of previous exposure to chemotherapy. The prodrugs described herein will be useful in treating early stages of malignancy, as well as disease previously treated with chemotherapy. Substrates for enzymes like GST include many compounds unrelated to chemotherapy. (Whalen and Boyer (1998)).

The concepts described above are displayed graphically in Figure 1A and 1B using the example of the thymidylate synthase enzyme and the compound NB1011™.

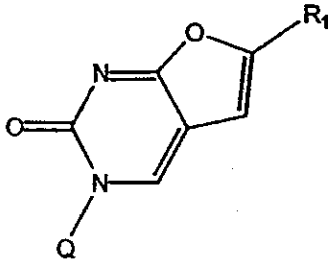
In an alternative embodiment, the target cell is defined by its resistance to a drug or compound unrelated to cancer. In this case, the cell is infected with a microorganism that confers resistance to an antibiotic, e.g. beta-lactamase. Organisms include bacteria, yeast and parasites, such as trypanosomes. Table 2 provides a list of enzymes targeted by this approach in infectious disease.

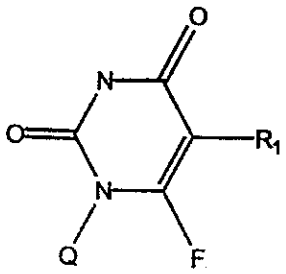
| Table 2<br>Enzymes Overexpressed in Infectious Disease, and which Contribute to Drug Resistance | |
|---|---|
| Enzyme | Provides increased Resistance to: |
| Beta-lactamases | Penicillin and other beta-lactam containing antibiotics |
| Aminoglycosidase, or aminoglycoside midifying enzymes | Aminoglycoside antibiotics (e.g., streptomycin, gentamycin) |
| Chloramphenicol transacetylase | Chloramphenicol |
| Dihydrofolate reductase | Trimethoprim |
| Reference: Mechanisms of Microbial Disease, 2nd Ed., M. Schaechter, G. Medloff, B.I. Eisenstein, Editor TS Satterfield. Publ. Williams and Wilkins, p. 973 (1993). | |

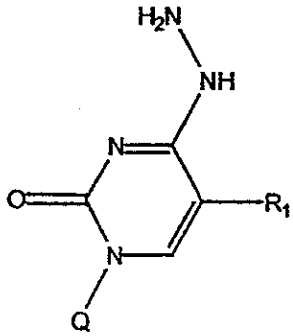
The prodrugs of this invention are selected from the group consisting of the L and D isomers of compounds having the structures:

5

I or II. or III.

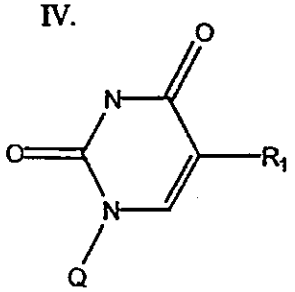


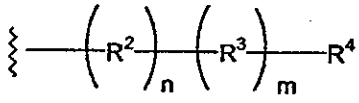

or

IV.



wherein:

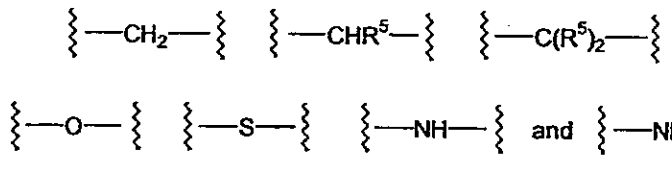
$R^1$ is a moiety of the formula:



with the proviso that in compound I, n can be 0.

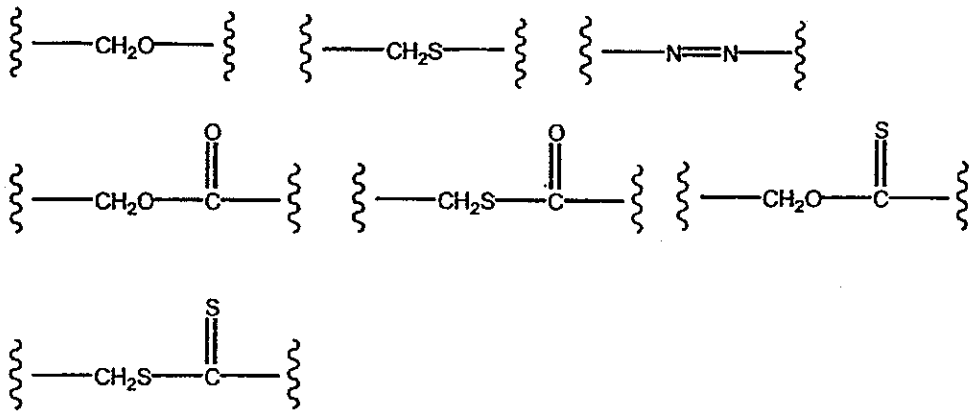
$R^2$ is a divalent electron conduit moiety selected from the group consisting of an unsaturated hydrocarbyl group; an aromatic hydrocarbyl group comprising one or more unsaturated hydrocarbyl groups; and, a heteroaromatic group comprising one or more unsaturated hydrocarbyl groups;

$R^3$ is selected from the group consisting of:

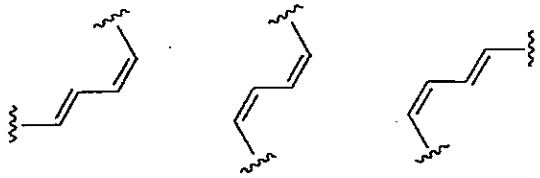


$R^5$ may be the same or different and is independently a linear or branched alkyl group having from 1 to 10 carbon atoms, or a cycloalkyl group having from 3 to 10 carbon atoms, or a halogen (e.g., F, Cl, Br, I);

or the group:

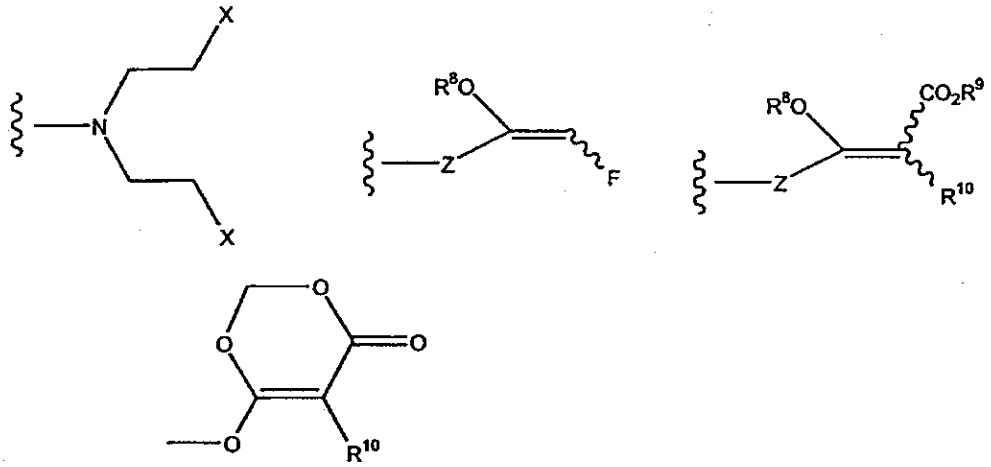


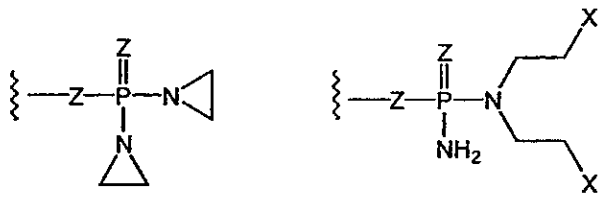
or the group:

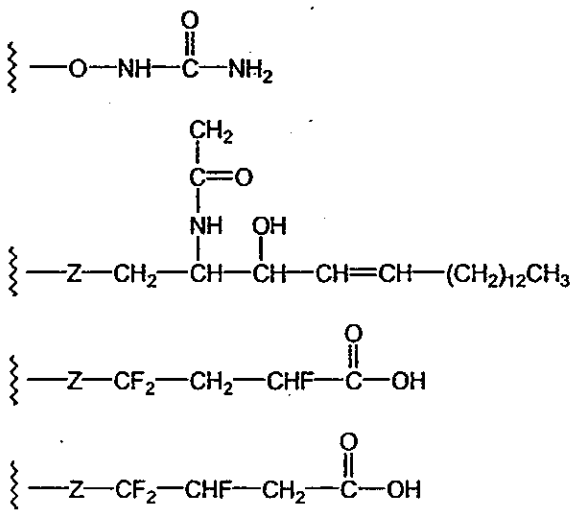


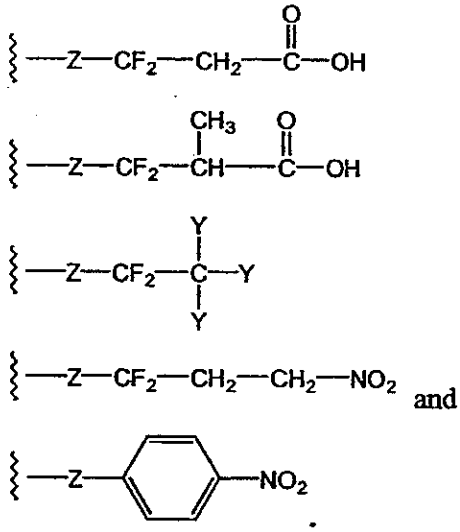
wherein n is 0 or an integer from 1 to 10 and m is 0 or 1;

wherein $R^4$ can be a substituent selected from the group consisting of





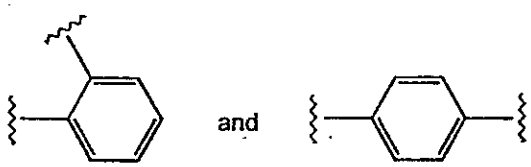




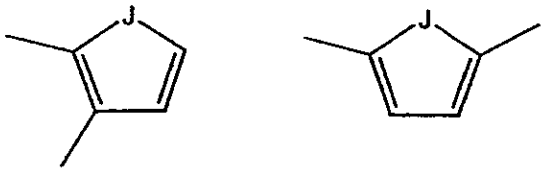
wherein $R^8$ and $R^9$ are lower alkyls and $R^{10}$ is H or $CH_3$ and each X substituent is independently the same or different from the other and is -Cl, -Br, -I, or other potent leaving group, with the proviso that when $R^7$ is -H, and m is zero, then $R^4$ is not a halogen or when m is zero and n is zero, then $R^4$ is not a halogen;

wherein each Y substituent is independently the same or different from the other and is independently -H or –F and each Z substituent is independently the same or different from the other and is -O- or -S-.

In one aspect, m is 0 and $R^2$ is an aromatic hydrocarbyl group selected from the group consisting of:



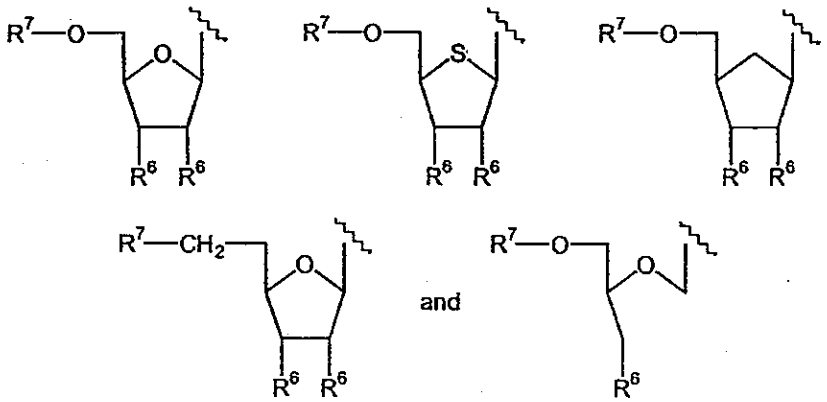
In an alternative aspect, m is 0 and $R^2$ is a heteroaromatic group selected from the group consisting of:



wherein J is selected from the group consisting of -O-, -S-, -Se-, -NH-, and -$NR^{ALK}$- and wherein $R^{ALK}$ is a linear or branched alkyl having 1 to 10 carbon atoms or a cycloalkyl group having 3 to 10 carbon atoms.

In one specific aspect, $R^4$ is selected from the group consisting of:

Q is a selected from the group consisting of a sugar, a carbocylic, an acyclic compound and masked phosphate or phosphoramidate derivatives thereof. The masked phosphate or phosphoramidate derivation is attached to Q at the 5' position of the compound, shown in more detail below. For example, Q is a moiety selected from the group consisting of:

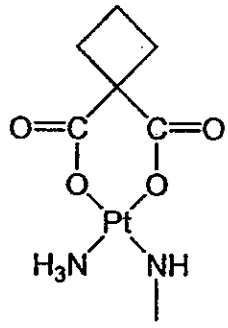


wherein each $R^6$ is independently the same or different from the other and is selected from the group consisting of -H, -OH, $-OC(=O)CH_3$, F, and other protected hydroxyl group; and,

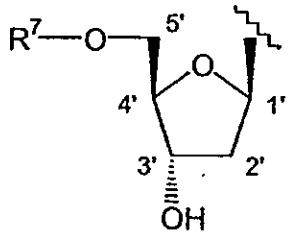
$R^7$ is hydrogen, a phosphate group, a phosphodiester group, or a phosphoramidate group attached to Q at the 5' position.

It should be understood, although not explicitely stated that any of the prodrugs of this invention may be in any enantiomeric, diasteriomeric, or stereoisomeric form, including, D-form, L-form, α-anomeric form, and β-anomeric form.

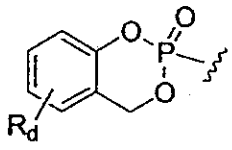
In one embodiment, $R^4$ is or contains a chemical entity selected from the group consisting of: -Br, -I, -O-alkyl, -O-aryl, O-heteroaryl, -S-alkyl, -S-aryl, -S-heteroaryl, -CN, -OCN, -SCN, $-NH_2$, -NH-alkyl, $-N(alkyl)_2$, -NHCHO, -NHOH, -NHO-alkyl, $NH_2CONHO$-, $NHNH_2$, $-N_3$, and a derivative of cis-platin, such as:



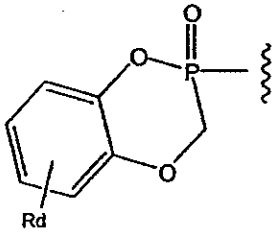
In one embodiment, Q is:



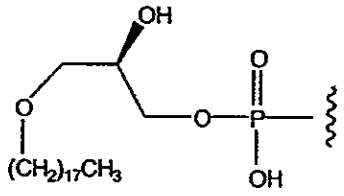
wherein $R^7$ is as described above, or more specifically, a structure such as:



or a compound having the structure:

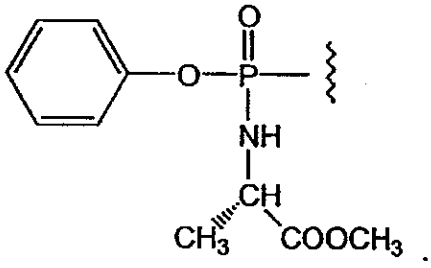



and



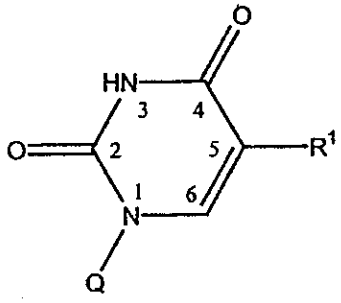

wherein $R_d$ is an aromatic substituent.

In an alternative embodiment, $R^7$ is a phosphoramidate group derived from an amino acid, including, for example, the twenty naturally occurring amino acids. In one embodiment, $R^7$ is a phosphoramidate group derived from alanine. In one embodiment, $R^7$ is or contains a group having the structure:



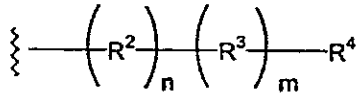

The above group, and methods for its preparation, are described in McGuigan, et al. (1993), and McGuigan, et al. (1996).

This invention also provides an L-or D- isomer of the compound having the structure:



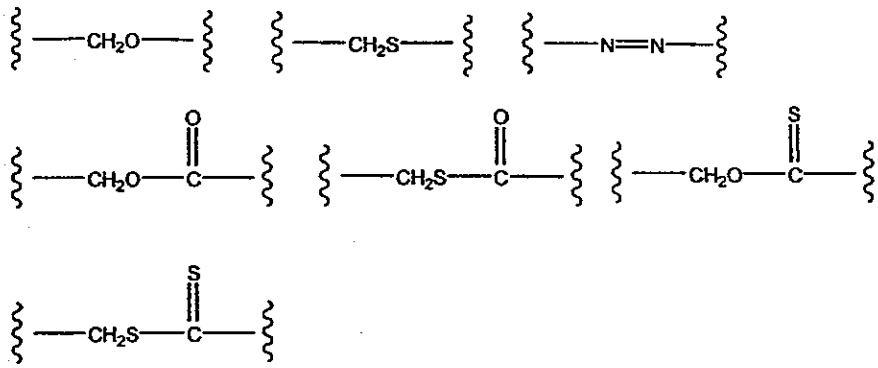

or tautomers thereof,

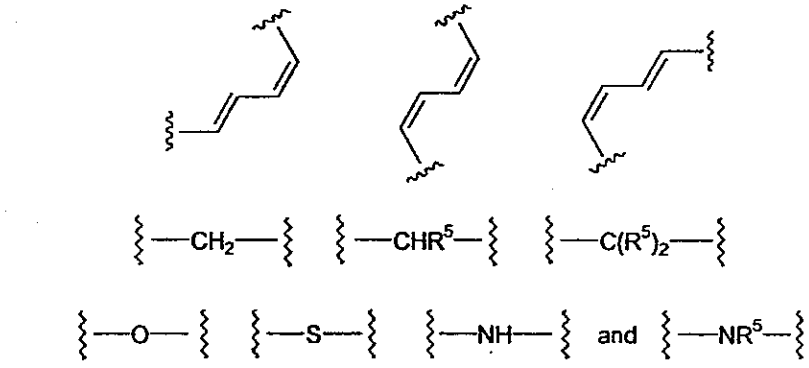
wherein $R^1$ is a substitutent having the formula:



wherein $R^2$ and $R^3$ are the same or different and are independently an unsaturated or a saturated hydrocarbyl group; wherein $R^4$ is selected from the group consisting of F, Cl, Br, I, CN, $SO_3H$, $CO_2H$, $CO_2CH_2CH_3$, $SI(CH_3)_3$, CHO, $NO_2$, $CF_3$, $CCl_3$, $C(R^5)_2$; wherein $R^5$ is selected from the group consisting of F, Cl, Br, and I; wherein n is 0 or an integer from 1 to 10 and m is 0 or 1; and Q is as defined above.

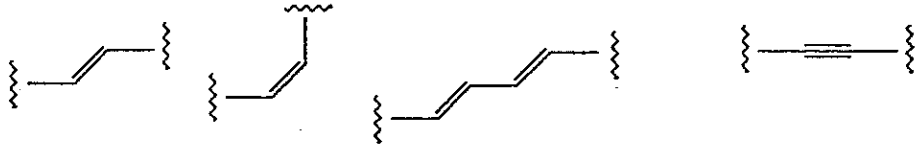
In one aspect, m is 0 and $R^2$ is selected from the group consisting of:

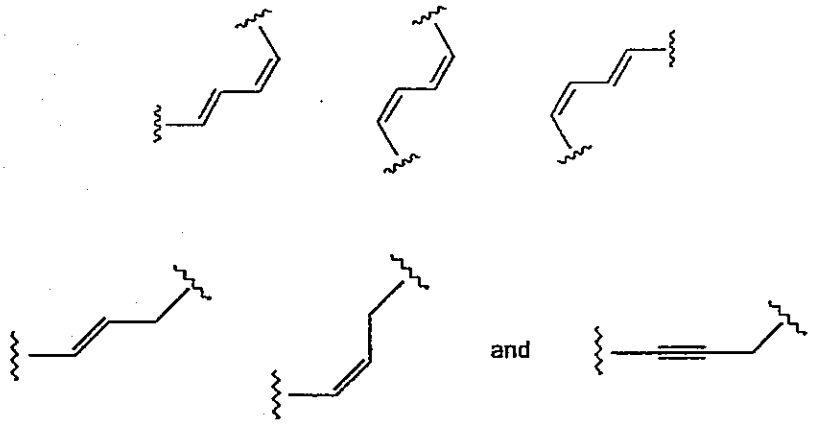




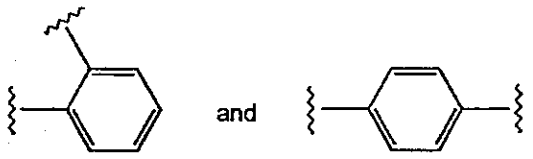
wherein $R^5$ is independently the same or different and is selected from the group consisting of a linear or branched alkyl group having from 1 to 10 carbon atoms, a cycloalkyl group having from 3 to 10 carbon atoms, CN and a halogen.

In another aspect, $R^2$ and $R^3$ taken together is an alkenyl or alkynyl and is selected from the group consisting of:

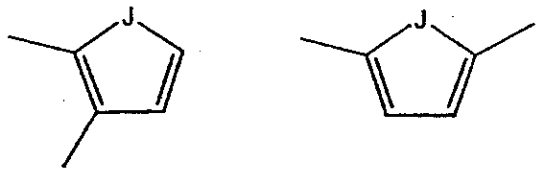





In another aspect, m is 0 and $R^2$ is an aromatic hydrocarbyl group selected from the group consisting of:



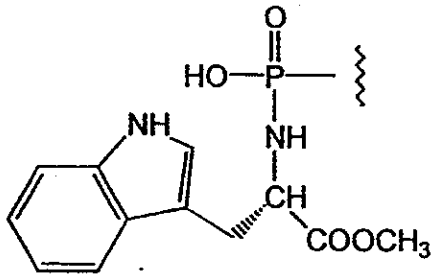

In a further aspect, m is 0 and $R^2$ is a heteroaromatic group selected from the group consisting of:




wherein J is selected from the group consisting of -O-, -S-, -Se-, -NH-, and -$NR^{ALK}$- and wherein $R^{ALK}$ is a linear or branched alkyl having 1 to 10 carbon atoms or a cycloalkyl group having 3 to 10 carbon atoms.

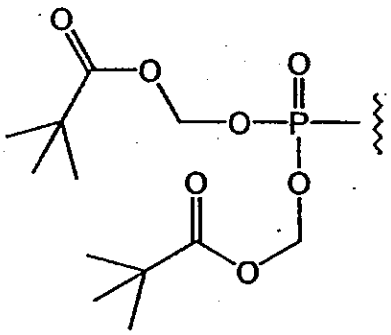
For these embodiment, $R^7$ is as described above.

In a specific embodiment, $R^7$ is:

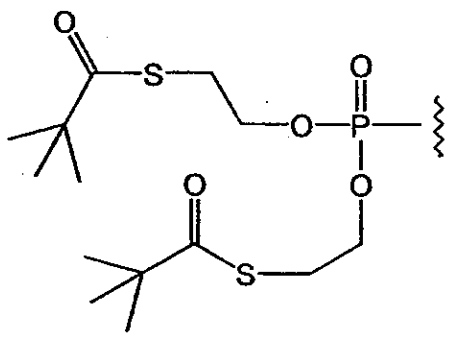


The above group, and methods for its preparation, are described in Abraham, et al. (1996).

In one embodiment, $R^7$ is a phosphate group or is or contains a group having a structure selected from the group consisting of:

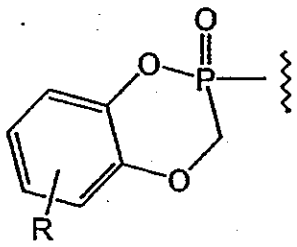


and

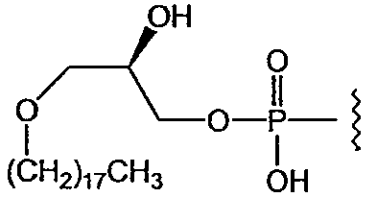


The first of the two above groups, and methods for its preparation, are described in Freed, et al. (1989); Sastry, et al., (1992); Farquhar, et al. (1994), and Farquhar, et al. (1995). The second of the two above groups, and methods for its preparation, are described in Valette, et al. (1996); and Benzaria, et al. (1996).

In one embodiment, $R^7$ is or contains a group having a structure selected from the group consisting of:



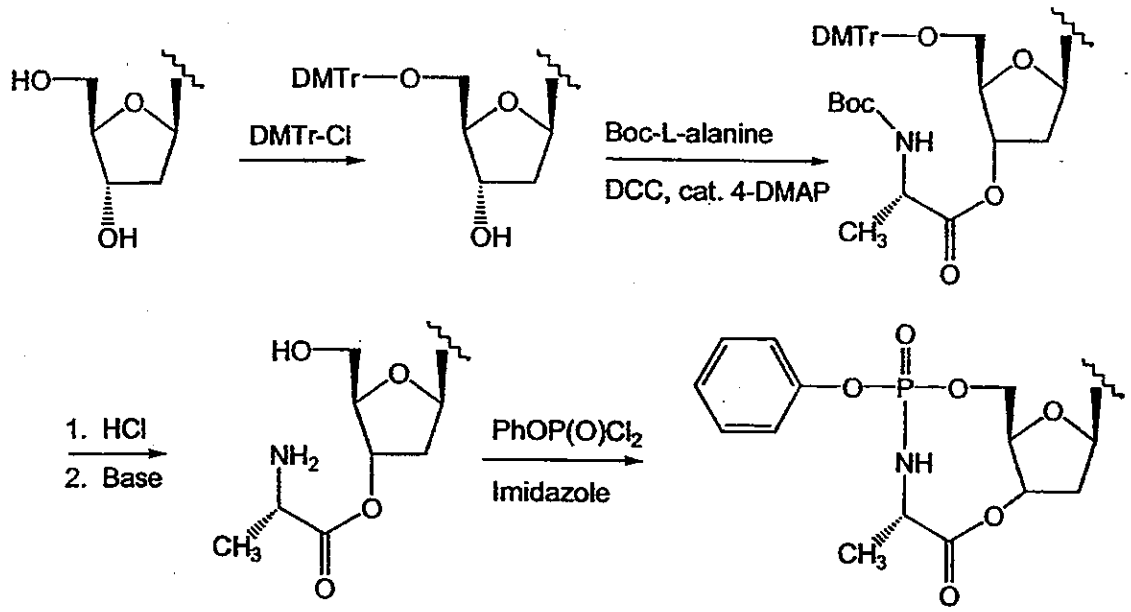
and



where R is an aromatic substituent the first of the two above groups, and methods for its preparation, are described in Meier, et al. (1997). The second of the two above

groups, and methods for its preparation, are described in Hostetler, et al. (1997); and Hostetler, et al., published International Patent Application No. WO 96/40088 (1996).

In one embodiment, the $R^7$ forms a cyclic group within Q. One such embodiment, and a method for its preparation, is shown below (where DMTr is 4,4'-dimethoxytrityl, Boc is t-butyloxycarbonyl, DCC is 1,3-dicyclohexylcarbodiimide, and 4-DMAP is 4-dimethylaminopyridine):




In one embodiment, the compound may be in a salt form, or in a protected or prodrug form, or a combination thereof, for example, as a salt, an ether, or an ester.

In a separate embodiment, the above structures are further modified to possess thiophosphodiaziridine instead of phosphodiaziridine groups, using the methods described below.

Synthesis of the above noted 5-substituted pyrimidine derivatives can be accomplished by methods that are well-known in the art. For example, treatment of 5-chloromercuri-2'-deoxyuridine with haloalkyl compounds, haloacetates or haloalkenes in the presence of $Li_2PdCl_4$ results in the formation, through an organopalladium intermediate, of the 5-alkyl, 5-acetyl or 5-alkene derivative, respectively. Wataya, et al. (1979) and Bergstrom, et al. (1981). Another example of C5-modification of pyrimidine nucleosides and nucleotides is the formation of C5-*trans*-styryl derivatives by treatment of unprotected nucleotide with mercuric acetate followed by addition of styrene or ring-substituted styrenes in the presence of $Li_2PdCl_4$. Bigge, et al. (1980).

Pyrimidine deoxyribonucleoside triphosphates were derivatized with mercury at the 5 position of the pyrimidine ring by treatment with mercuric acetate in acetate buffer at 50° for 3 hours. Dale, et al. (1973). Such treatment also would be expected to be effective for modification of monophosphates; alternatively, a modified triphosphate could be converted enzymatically to a modified monophosphate, for example, by controlled treatment with alkaline phosphatase followed by purification of monophosphate. Other moieties, organic or nonorganic, with molecular properties similar to mercury but with preferred pharmacological properties could be substituted. For general methods for synthesis of substituted pyrimidines, see for example, U.S. Patent Nos. 4,247,544; 4,267,171; and 4,948,882; and Bergstrom, et al. (1981). The above methods would also be applicable to the synthesis of derivatives of 5-substituted pyrimidine nucleosides and nucleotides containing sugars other than ribose or 2'-deoxyribose, for example 2'-3'-dideoxyribose, arabinose, furanose, lyxose, pentose, hexose, heptose, and pyranose. An example of a 5-position substituent is the halovinyl group, e.g. E-5-(2-bromovinyl)-2'-deoxyuridylate. Barr, P.J. et al. (1983).

Alternatively, 5-bromodeoxyuridine, 5-iododeoxyuridine, and their monophosphate derivatives are available commercially from Glen Research, Sterling, VA (USA), Sigma-Aldrich Corporation, St. Louis, MO (USA), Moravek Biochemicals, Inc., Brea, CA (USA), ICN, Costa Mesa, CA (USA) and New England Nuclear, Boston, MA (USA). Commercially-available 5-bromodeoxyuridine and 5-iododeoxyuridine can be converted to their monophosphates either chemically or enzymatically, though the action of a kinase enzyme using commercial available reagents from Glen Research, Sterling, VA (USA) and ICN, Costa Mesa, CA (USA). These halogen derivatives could be combined with other substituents to create novel and more potent antimetabolites.

The structures at the 5-position of uracil are referred to as the tethers because they connect the proposed leaving group (toxophore) to the heterocycle. Upon activation of the heterocycle by reaction with the Cys residue in the active site of a human enz, TS, for example, a negative charge is conducted from the 6-position of uracil into the tether. This mechanism has been described for the 5'-monophosphorylated versions of (*E*)-5-(bromovinyl)-2'-deoxyuridine (BVdU) by Barr, P. J. et al. (1983) and of (*E*)-5-(3,3,3-trifluoro-1-propenyl)-2'-deoxyuridine (TFPe-dUrd) by Wataya, et al. (1979), Santi (1980); and Bergstrom, et al. (1984).

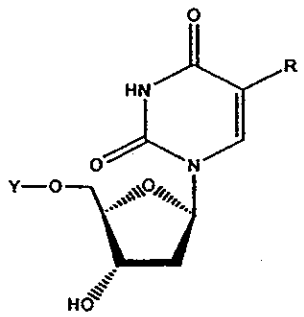
The tether "spacer" between the toxin and dNMP must be unsaturated so that it can conduct the toxin-labilizing negative charge supplied by the TS-Cys-sulfhydryl attack. Of the many unsaturated organic functionalities available for this purpose, the vinyl, allyl, and propargyl units are simple, small, and readily accessible synthetically. The vinyl and allyl units have the advantage that they can be prepared in either of two non-interconvertible geometric isomeric forms. Thus, they can be used as "probes" of prodrug accommodation by the enzyme active site. On the other hand, the propargyl unit has the advantage of being cylindrically symmetrical, so that enz-catalyzed toxin release from this type of tether does not depend upon its orientation with respect to dUMP's uracil ring, as is the case with the vinyl and allyl molecules.

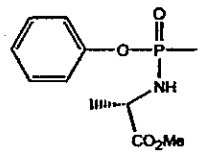
Two distinct approaches have been taken to design of some of the nucleotide-based prodrugs of this invention. One is based on the structure of BVdU monophosphate and features a leaving group/toxin directly attached to the terminus of a (poly)vinyl substituent at C5 of dUMP. This is the vinyl tether approach. The other is based on the structure of TFPe-dUMP and is similar to the first but has a methylene unit separating the leaving group/toxin and the unsaturated unit and thus contains an allyl or propargyl unit. This is the allyl tether approach.

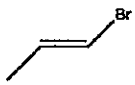
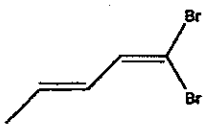
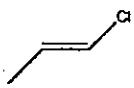
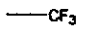
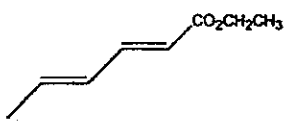
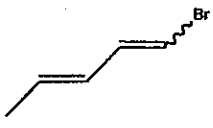
The mechanism of activation of a propargyl version of the allyl tether approach has a precedent in the interaction of both 5-ethynyl-2'-deoxyuridine 5'-monophosphate (EdUMP) and 5-(3-hydroxy-l-propynyl)-2'deoxyuridine 5'-monophosphate (HOPdUMP) with TS (Barr, et al. (1981); Barr and Robins 1983). EdUMP is a potent inhibitor of TS (Ki = 0.1 TM), and likely forms an allene-based species at the active site. HOPdUMP (Ki = 3.0 TM) shows unusual inhibition kinetics, which might be due to formation of a cumulene-based species at the active site.

5-Alkylidenated 5,6-dihydrouracils similar in structure to the intermediate common to both the vinyl and allyl tether approach mechanisms have been synthesized recently (Anglada et al. 1996). These were shown to be highly electrophilic. Their ready reaction with ethanol to generate 5-(ethoxymethyl)uracil is a precedent for the water addition that regenerates catalytically competent TS. Even more recently, the existence of the long-elusive C5 methylene intermediate produced by TS was demonstrated by trapping studies (Barrett, et al. (1998)).

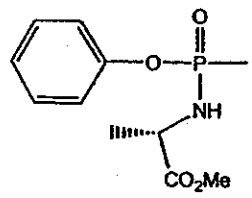
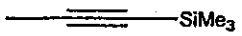
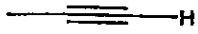
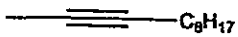
Particular embodiments include the compounds having the L or D structures shown below. Compounds are identified by structure and a numerical designation.





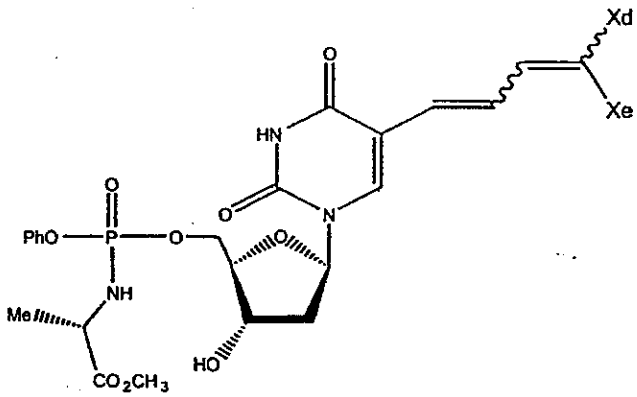
| R | Y = | Y = H |
|---|---|---|
|  | NB1011 | NB1015 (BVdU) |
|  | NB1012 | – |
|  | NB1013 | NB1020 |
|  | NB1014 | NB1027 |
|  | NB1016 | NB1021 |
|  | NB1017 | NB1024 |

(Continued from Previous Page)

| R | Y = |  Y = H |
|---|---|---|
|  | NB1018 | NB1022 |
|  | NB1019 | NB1023 |
|  | – | NB102 |
| -H | NB1025 | NB1026 |
| | – | NB102 |

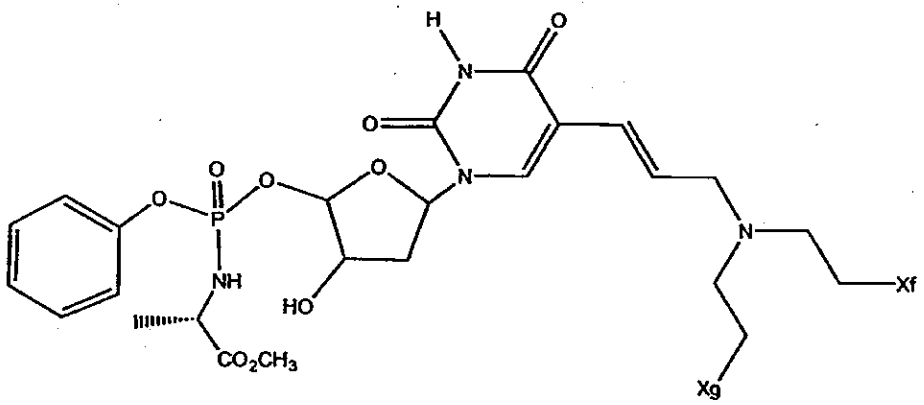
Preferred embodiments are shown below:

A compound having the structure:



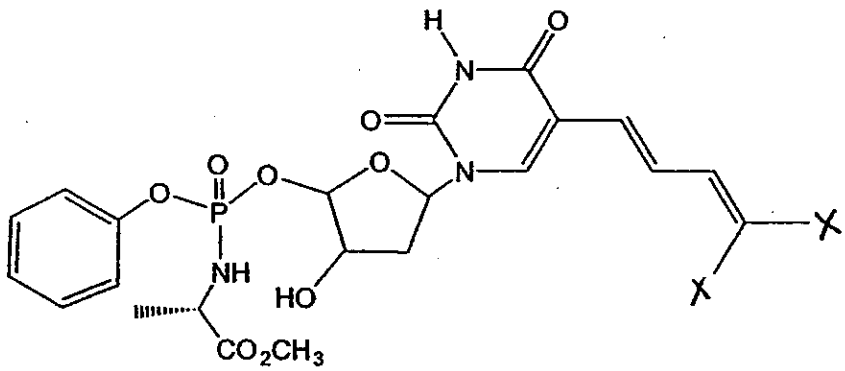
wherein $X_d$ and $X_e$ are independently the same or different and are selected from the group consisting of Cl, Br, I, and CN or the nucleoside analogs thereof. In a more preferred aspect, $X_d$ is Cl or Br and $X_e$ is H.

A compound having the structure:




wherein $X_f$ and $X_g$ are independently the same or different and are selected from the group consisting of Cl, Br, I, and CN or the nucleoside analogs thereof. In a preferred embodiment, $X_f$ and $X_g$ are the same and are each is Cl or Br.

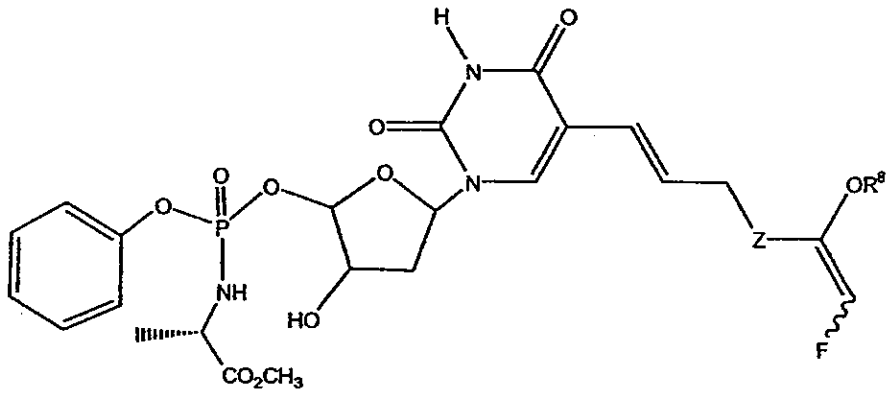
A compound having the structure of the formula:




wherein each X is selected from the group consisting of Cl, Br, I, and CN;

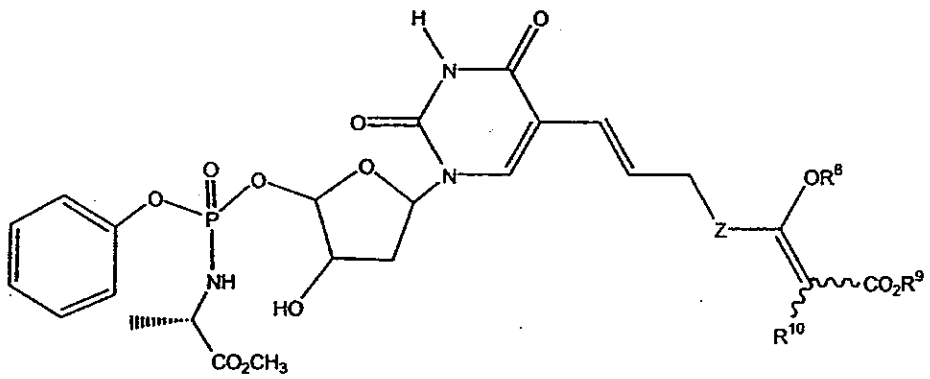
or the nucleoside analogs thereof. In a preferred embodiment, X is Cl or Br and in a more preferred embodiment, X is Br.

A compound having the structure:



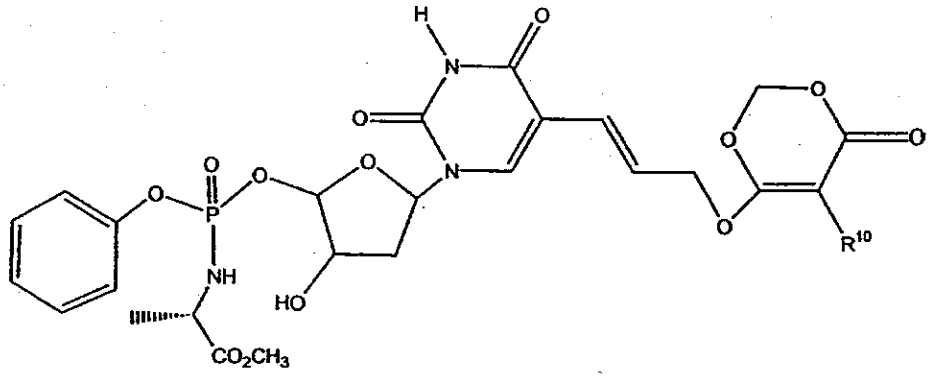
wherein $R^8$ is a lower straight or brainched chain alkyl, or the nucleoside analogs thereof.

A compound having the structure:



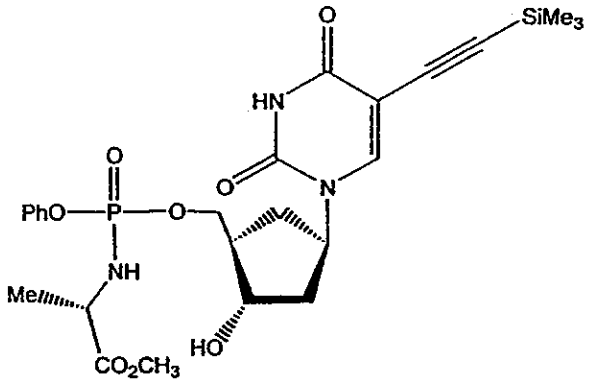
wherein $R^8$ and $R^9$ are lower straight or branched chain alkyls and $R^{10}$ is H or $CH_3$ or the nucleoside analogs thereof.

A compound having the structure:

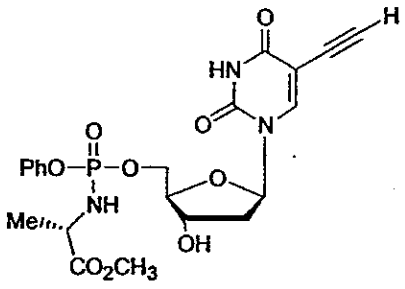


wherein $R^{10}$ is H or $CH_3$;or the nucleoside analog thereof.

A compound having the structure:

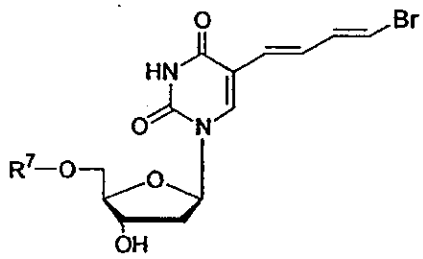


A compound having the structure:

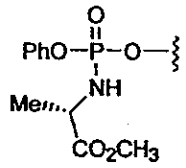


or the nucleoside analog thereof.

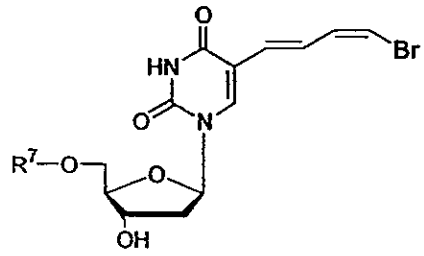
A compound as described herein, wherein the compound has the structure:



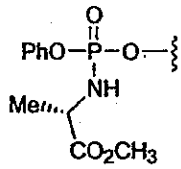
In one aspect, $R^7 = H$. In another aspect, $R^7$ is a substituent having the structure:



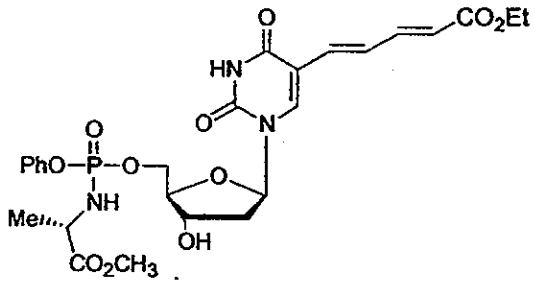
A compound as described herein wherein the compound has the structure:



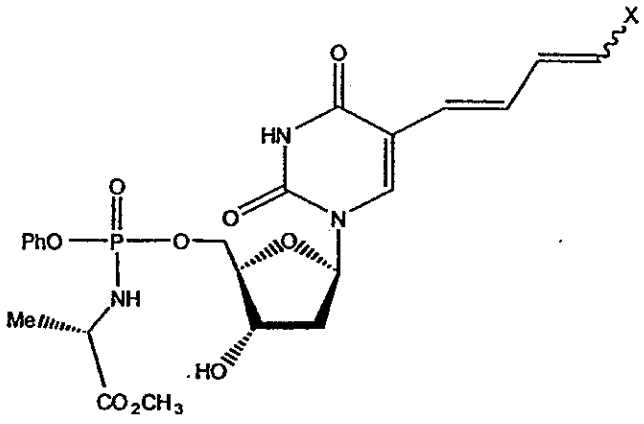
wherein $R^7 = H$ or $R^7$ is a substituent having the structure



A compound having the structure:

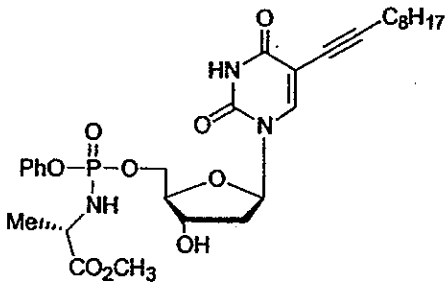


A compound having the structure:



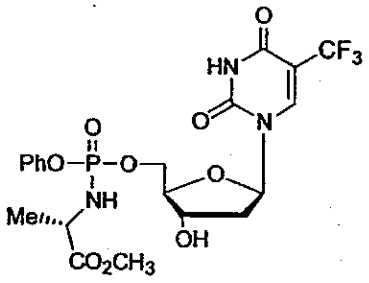
wherein each X is selected from the group consisting of $CO_2Et$, Cl, and Br; or the nucleoside analog thereof.

A compound having the structure:



or the nucleoside analog thereof.

A compound having the structure:



or the nucleoside analog thereof.

The prodrugs can be combined with a carrier, such as a pharmaceutically acceptable carrier, for use *in vitro* and *in vivo*.

This invention also provides a quick and simple screening assay that will enable initial identification of novel compounds with at least some of the desired characteristics (see Figure 2A and 2B). The assay requires at least two cell types, the first being a control cell in which the target enzyme is not expressed or is expressed at a low level, e.g., a normal cell. The second cell type is the test cell in which the target enzyme is expressed at a detectable level, e.g., a high level. This cell can be a tumor cell line that is selected for enhanced levels of target enzymes. Alternatively, a cell genetically modified to differentially express the target enzyme or enzymes (containing the appropriate species of target enzyme) can be used. Transfection of host cells with polynucleotides encoding the target enzyme is either transient or permanent using procedures well known in the art and described in Chen, L. et al. (1996); Hudziak, R.M. et al. (1988); or Carter, P. et al. (1992), and in the experimental section below. The cells can be procaryotic (bacterial such as *E. coli*) or eucaryotic. The cells can be mammalian or non-mammalian cells, e.g., mouse cells, rat cells, human cells, fungi (e.g., yeast) or parasites (e.g., *Pneumocystis* or *Leishmania*) which cause disease.

Suitable vectors for insertion of the cDNA are commercially available from Stratagene, La Jolla, CA and other vendors. The amount of expression can be regulated by the number of copies of the expression cassette introduced into the cell or by varying promoter usage. The level of expression of enzyme in each transfected cell line can be monitored by immunoblot and enzyme assay in cell lysates, using monoclonal or polyclonal antibody previously raised against the enzyme for immuno-detection.

(Chen, L. et al. (1996)). Enzymatic assays to detect the amount of expressed enzyme also can be performed as reviewed by Carreras, C.W. and Santi, D.V. (1995), or the method described in the experimental section below.

In a further aspect, more than one species of enzyme can be used to separately transduce separate host cells, so that the effect of the candidate drug on a target enzyme can be simultaneously compared to its effect on another enzyme or a corresponding enzyme from another species.

In another embodiment, a third target cell is used as a control because it receives an effective amount of a prodrug compound of this invention. This embodiment is particularly useful to screen for new agents that are activated by thymidylate synthase. In yet a further aspect, at least one additional test cell system is set up to test the synergistic potential of the test therapeutic in combination with a known therapy or agent.

For the purposes of this invention, the successful candidate drug will block the growth or kill the test cell type, but leave the control cell type unharmed. Growth assays can be performed by standard methods as described by Miller (1992); Sugarman, B. J. et al. (1985) and Spector, D. L. et al. (1998), or using the methods described in the experimental section below.

It will be understood by those skilled in the art that the screen can be applied broadly for the discovery of antibiotics. For example, thymidylate synthase from yeast could be substituted for that of human or *E. coli* forms of the enzyme. This would allow the discovery of specific antifungal antibiotics targeting yeast related pathogens. In addition, other enzymes can be subjected to this treatment. For example, prodrugs that target specifically the dihydrofolate reductase activity of infectious agents, like *Pneumocystis carnii*, could be selected. These agents will be selected for specificity for the target enzyme, and can be shown not to activate the enzyme of the natural host by employing the screening assay described herein. The control cellular constructs would contain the corresponding normal human enzyme, in order to show lack of toxicity when only the normal human enzyme is present.

Figures 2A and 2B show how one may conduct at least one embodiment of this aspect of the invention. A foreign gene, e.g., a human gene encoding TS, is inserted into the host cell such that human TS is expressed. The "control cell" does not express the

target enzyme. In some embodiments it may be necessary to supplement the culture media with the protein product of the target enzyme.

The candidate prodrug can be directly added to the cell culture media and the target cell or the culture media is then assayed for the amount of label released from the candidate prodrug if the prodrug contains a detectable label. Alternatively, cellular uptake may be enhanced by packaging the prodrug into liposomes using the method described in Lasic, D.D. (1996) or combined with cytofectins as described in Lewis, J.G. et al. (1996).

An alternative embodiment for a prodrug taking advantage of TS overexpression in tumor cells is a deoxyuridine phosphoramidate, or other modifications (cited herein) conjugated with a therapeutic radionuclide. An example of a therapeutic radionuclide is rhenium 188. The isotope can be synthesized essentially as described by Callahan, et al. (1989). Alternatively, it can be obtained commercially, for example from Mallicrodt Medical BV, The Netherlands. The therapeutic radionuclide can be conjugated with deoxyuridine, or deoxyuridine 5'-phosphoramidate, or other derivative, by standard methods for example, as described by Lin, W.Y. et al. (1997). The radionuclide-containing deoxyuridine phosphoramidate will be preferentially taken up into the DNA of tumor cells overexpressing thymidylate synthase and cause their death via concentrated emission of beta and gamma radiation. Alternative radionuclides include rhenium-186, and others. (Troutner, D.A. (1987)).

The compounds are useful to predict whether a subject will be suitably treated by a prodrug of this invention by delivering to a sample containing the cell to be treated a prodrug and assaying for cell death or inhibition of cell proliferation. Applicants provide kits for determining whether a pathological cell or a patient will be suitably treated by this therapy by providing at least one prodrug of this invention and instructions for use.

This invention also provides a method for inhibiting the proliferation of a pathological or target cell *in vitro* or *in vivo* by delivering to the cell an effective amount of a prodrug of this invention. When practiced *in vivo*, the method is useful to treat a pathology characterized by target cells in a subject by delivering to the subject an effective amount of a prodrug of this invention.

When the target cell is target cell is resistant to a chemotherapeutic drug, the method can be further modified by contacting or administering to the cell or patient an effective amount of the drug to which the cell has developed resistance. Because the prodrugs of this invention can reverse resistance to the prior therapy, subsequent to

successful treatment with a prodrug of this invention, administration of the previous therapy can again inhibit growth or metastasis of tumors. Examples where this may occur include, but are not limited to when the target cell expresses an enzyme that is amplified as a result of selection *in vivo* by chemotherapy or when the target enzyme is an endogenous intracellular enzyme that is overexpressed in the target cell. An example of such an enzyme is thymidylate synthase which has been shown to be overexpressed as a result of prior chemotherapy and confers a drug resistant phenotype on the cell to the prior drug.

The prodrugs of this invention can also be combined with other known therapies to enhance or synergize the therapeutic effects of either or both prior therapies or the therapeutic effect of the prodrug. Such prior therapies include, but are not limited to cancer chemotherapy, radiation therapy and surgery.

When delivered to an animal, the method also is useful to further confirm efficacy of the prodrug. As an example of an animal model, groups of nude mice (Balb/c NCR nu/nu female, Simonsen, Gilroy, CA) are each subcutaneously inoculated with about $10^5$ to about $10^9$ hyperproliferative, cancer or target cells as defined herein. When the tumor is established, the prodrug is administered, for example, by intraperitoneal or intravenous routes. Tumor measurements to determine reduction of tumor size are made in two dimensions using venier calipers twice a week. Other animal models may also be employed as appropriate. (Lovejoy et al. (1997); Clarke, R. (1996); and Pegram, M. D. et al. (1997)).

Administration *in vivo* can be effected in one dose, continuously or intermittently throughout the course of treatment. Methods of determining the most effective means and dosage of administration are well known to those of skill in the art and will vary with the composition used for therapy, the purpose of the therapy, the target cell being treated, and the subject being treated. Single or multiple administrations can be carried out with the dose level and pattern being selected by the treating physician. Suitable dosage formulations and methods of administering the agents can be found below.

The prodrug, combinations of drugs, or compositions containing either can be used in the manufacture for medicaments for the treatment of humans and other animals by administration in accordance with conventional procedures, such as an active ingredient in pharmaceutical compositions.

The pharmaceutical compositions can be administered orally, intranasally, parenterally or by inhalation therapy, and may take the form of tablets, lozenges, granules, capsules, pills, ampoules, suppositories or aerosol form. They may also take the form of suspensions, solutions and emulsions of the active ingredient in aqueous or nonaqueous diluents, syrups, granulates or powders. In addition to a compound of the present invention, the pharmaceutical compositions can also contain other pharmaceutically active compounds or a plurality of compounds of the invention.

More particularly, a compound of the formula of the present invention also referred to herein as the active ingredient, may be administered for therapy by any suitable route including oral, rectal, nasal, topical (including transdermal, aerosol, buccal and sublingual), vaginal, parental (including subcutaneous, intramuscular, intravenous and intradermal) and pulmonary. It will also be appreciated that the preferred route will vary with the condition and age of the recipient, and the disease being treated.

In general, a suitable dose for each of the above-named compounds, is in the range of about 1 to about 100 mg per kilogram body weight of the recipient per day, preferably in the range of about 1 to about 50 mg per kilogram body weight per day and most preferably in the range of about 1 to about 25 mg per kilogram body weight per day. Unless otherwise indicated, all weights of active ingredient are calculated as the parent compound of the formula of the present invention for salts or esters thereof, the weights would be increased proportionately. The desired dose is preferably presented as two, three, four, five, six or more sub-doses administered at appropriate intervals throughout the day. These sub-doses may be administered in unit dosage forms, for example, containing about 1 to about 100 mg, preferably about 1 to above about 25 mg, and most preferably about 5 to above about 25 mg of active ingredient per unit dosage form. It will be appreciated that appropriate dosages of the compounds and compositions of the invention may depend on the type and severity and stage of the disease and can vary from patient to patient. Determining the optimal dosage will generally involve the balancing of the level of therapeutic benefit against any risk or deleterious side effects of the treatments of the present invention.

Ideally, the prodrug should be administered to achieve peak concentrations of the active compound at sites of disease. This may be achieved, for example, by the intravenous injection of the prodrug, optionally in saline, or orally administered, for

example, as a tablet, capsule or syrup containing the active ingredient. Desirable blood levels of the prodrug may be maintained by a continuous infusion to provide a therapeutic amount of the active ingredient within disease tissue. The use of operative combinations is contemplated to provide therapeutic combinations requiring a lower total dosage of each component antiviral agent than may be required when each individual therapeutic compound or drug is used alone, thereby reducing adverse effects.

While it is possible for the prodrug ingredient to be administered alone, it is preferable to present it as a pharmaceutical formulation comprising at least one active ingredient, as defined above, together with one or more pharmaceutically acceptable carriers therefor and optionally other therapeutic agents. Each carrier must be "acceptable" in the sense of being compatible with the other ingredients of the formulation and not injurious to the patient.

Formulations of the present invention suitable for oral administration may be presented as discrete units such as capsules, cachets or tablets, each containing a predetermined amount of the active ingredient; as a powder or granules; as a solution or suspension in an aqueous or non-aqueous liquid; or as an oil-in-water liquid emulsion or a water-in-oil liquid emulsion. The active ingredient may also be presented a bolus, electuary or paste.

A tablet may be made by compression or molding, optionally with one or more accessory ingredients. Compressed tablets may be prepared by compressing in a suitable machine the active ingredient in a free-flowing form such as a powder or granules, optionally mixed with a binder (e.g., povidone, gelatin, hydroxypropylmethyl cellulose), lubricant, inert diluent, preservative, disintegrant (e.g., sodium starch glycolate, cross-linked povidone, cross-linked sodium carboxymethyl cellulose) surface-active or dispersing agent. Molded tablets may be made by molding in a suitable machine a mixture of the powdered compound moistened with an inert liquid diluent. The tablets may optionally be coated or scored and may be formulated so as to provide slow or controlled release of the active ingredient therein using, for example, hydroxypropylmethyl cellulose in varying proportions to provide the desired release profile. Tablets may optionally be provided with an enteric coating, to provide release in parts of the gut other than the stomach.

Formulations suitable for topical administration in the mouth include lozenges comprising the active ingredient in a flavored basis, usually sucrose and acacia or tragacanth; pastilles comprising the active ingredient in an inert basis such as gelatin and glycerin, or sucrose and acacia; and mouthwashes comprising the active ingredient in a suitable liquid carrier.

Pharmaceutical compositions for topical administration according to the present invention may be formulated as an ointment, cream, suspension, lotion, powder, solution, past, gel, spray, aerosol or oil. Alternatively, a formulation may comprise a patch or a dressing such as a bandage or adhesive plaster impregnated with active ingredients and optionally one or more excipients or diluents.

For diseases of the eye or other external tissues, e.g., mouth and skin, the formulations are preferably applied as a topical ointment or cream containing the active ingredient in an amount of, for example, about 0.075 to about 20% w/w, preferably about 0.2 to about 25% w/w and most preferably about 0.5 to about 10% w/w. When formulated in an ointment, the prodrug may be employed with either a paraffinic or a water-miscible ointment base. Alternatively, the prodrug ingredients may be formulated in a cream with an oil-in-water cream base.

If desired, the aqueous phase of the cream base may include, for example, at least about 30% w/w of a polyhydric alcohol, i.e., an alcohol having two or more hydroxyl groups such as propylene glycol, butane-1,3-diol, mannitol, sorbitol, glycerol and polyethylene glycol and mixtures thereof. The topical formulations may desirably include a compound that enhances absorption or penetration of the prodrug ingredient through the skin or other affected areas. Examples of such dermal penetration enhancers include dimethylsulfoxide and related analogues.

The oily phase of the emulsions of this invention may be constituted from known ingredients in a known manner. While this phase may comprise merely an emulsifier (otherwise known as an emulgent), it desirably comprises a mixture of at lease one emulsifier with a fat or an oil or with both a fat and an oil. Preferably, a hydrophilic emulsifier is included together with a lipophilic emulsifier that acts as a stabilizer. It is also preferred to include both an oil and a fat. Together, the emulsifier(s) with or without stabilizer(s) make up the so-called emulsifying wax, and the wax together with the oil and/or fat make up the so-called emulsifying ointment base which forms the oily dispersed phase of the cream formulations.

Emulgents and emulsion stabilizers suitable for use in the formulation of the present invention include Tween 60, Span 80, cetostearyl alcohol, myristyl alcohol, glyceryl monostearate and sodium lauryl sulfate.

The choice of suitable oils or fats for the formulation is based on achieving the desired cosmetic properties, since the solubility of the active compound in most oils likely to be used in pharmaceutical emulsion formulations is very low. Thus the cream should preferably be a non-greasy, non-staining and washable product with suitable consistency to avoid leakage from tubes or other containers. Straight or branched chain, mono- or dibasic alkyl esters such as di-isoadipate, isocetyl stearate, propylene glycol diester of coconut fatty acids, isopropyl myristate, decyl oleate, isopropyl palmitate, butyl stearate, 2-ethylhexyl palmitate or a blend of branched chain esters known as Crodamol CAP may be used, the last three being preferred esters. These may be used alone or in combination depending on the properties required. Alternatively, high melting point lipids such as white soft paraffin and/or liquid paraffin or other mineral oils can be used.

Formulations suitable for topical administration to the eye also include eye drops wherein the active ingredient is dissolved or suspended in a suitable carrier, especially an aqueous solvent for the prodrug ingredient. The prodrug ingredient is preferably present in such formulation in a concentration of about 0.5 to about 20%, advantageously about 0.5 to about 10% particularly about 1.5% w/w.

Formulations for rectal administration may be presented as a suppository with a suitable base comprising, for example, cocoa butter or a salicylate.

Formulations suitable for vaginal administration may be presented as suppositories, tampons, creams, gels, pastes, foams or spray formulations containing in addition to the prodrug ingredient, such carriers as are known in the art to be appropriate.

Formulations suitable for nasal administration, wherein the carrier is a solid, include a coarse powder having a particle size, for example, in the range of about 20 to about 500 microns which is administered in the manner in which snuff is taken, i.e., by rapid inhalation through the nasal passage from a container of the powder held close up to the nose. Suitable formulations wherein the carrier is a liquid for administration as, for example, nasal spray, nasal drops, or by aerosol administration by nebulizer, include aqueous or oily solutions of the prodrug ingredient.

Formulations suitable for parenteral administration include aqueous and non-aqueous isotonic sterile injection solutions which may contain anti-oxidants, buffers, bacteriostats and solutes which render the formulation isotonic with the blood of the intended recipient; and aqueous and non-aqueous sterile suspensions which may include suspending agents and thickening agents, and liposomes or other microparticulate systems which are designed to target the compound to blood components or one or more organs. The formulations may be presented in unit-dose or multi-dose sealed containers, for example, ampoules and vials, and may be stored in a freeze-dried (lyophilized) condition requiring only the addition of the sterile liquid carrier, for example water for injections, immediately prior to use. Extemporaneous injection solutions and suspensions may be prepared from sterile powders, granules and tablets of the kind previously described.

It should be understood that in addition to the ingredients particularly mentioned above, the formulations of this invention may include other agents conventional in the art having regard to the type of formulation in question, for example, those suitable of oral administration may include such further agents as sweeteners, thickeners and flavoring agents.

Prodrugs and compositions of the formula of the present invention may also be presented for the use in the form of veterinary formulations, which may be prepared by methods that are conventional in the art.

The following examples are intended to illustrate, but not limit the invention.

*Materials and Methods*

*Synthesis of Nucleoside ECTA Compounds*

Synthesis of the above noted 5-substituted pyrimidine nucleosides can be accomplished by methods that are well-known in the art and briefly described above.

In one embodiment, the present invention involves four classes of compounds. Each class is defined by the structure of the uricil base, or modified uricil base present. These classes are ECTA compounds where: 1) the base is a furano-pyrimidinone derivative of uracil; 2) the base is 6-fluoro uracil; 3) the base is 4-hydrazone substituted uracil derivative; 4) the base is uracil. The uracil or modified uracil derived base is used to synthesize compounds substituted with toxic leaving groups at the 5 position,

attached by an electron conduit tether at this 5 position, and including an appropriate spacer moiety between the electron conduit and the toxic leaving group. The ECTA compounds can be unphosphorylated, 5' monophosphate, 5' phosphodiester, or 5' protected ("masked") deoxyuridines or comparable derivatives of alternative carbohydrate moieties, as described below. Protected 5-substituted deoxyuridine monophosphate derivatives are those in which the phosphate moiety has been blocked through the attachment of suitable chemical protecting groups. Protection of 5-substituted deoxyuridine monophosphate derivatives can improve solubility, facilitate cellular penetration, facilitate passage across the blood-brain barrier, and prevent action of cellular or extracellular phosphatases, which might otherwise result in loss of the phosphate group. In another embodiment, 5-substituted uracil or uridine derivatives are administered to cells containing nucleoside kinase activity, wherein the 5-substituted uracil/uridine derivative is converted to a 5-substituted uridine monophosphate derivative. Uridine derivatives may also be modified to increase their solubility, cell penetration, and/or ability to cross the blood-brain barrier.

Action of thymidylate synthase upon 5-substituted uridine monophosphate derivatives can release the substituent attached to the 5-position ("leaving group") of the pyrimidine ring. The released substituent is then capable, either inherently or following reaction with another cellular component, of acting as a toxin or an inhibitor of cellular proliferation.

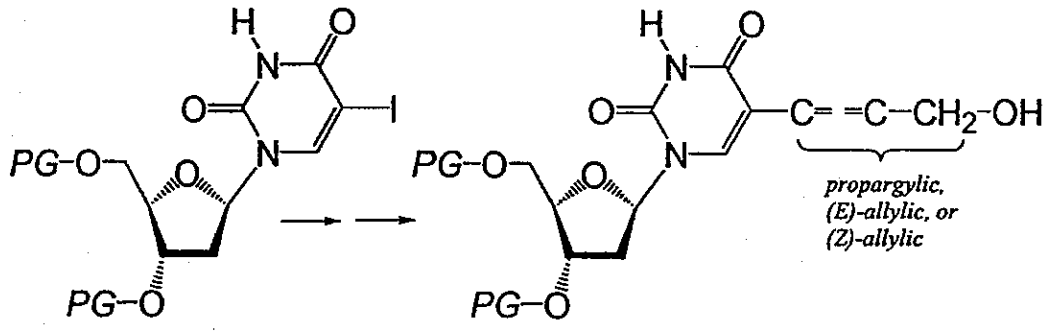
***Synthesis of ECTA compounds with propargyl tethers***

The synthesis of propargylic and allylic alcohol-equipped 2'-deoxyuridines is straightforward. Many of these and their close derivatives are reported in the literature, and some have even been studied in connection with TS. For example, 5-alkynyl-dUMPs including the 5-(3-methoxy-1-propynyl) and 5-(3-hydroxy-1-propynyl) ones have been examined as TS inhibitors (Barr, and Robins (1981)) and some of these have been shown to become incorporated into the DNA of TS-deficient cancer cells (Balzarini, J. et al. (1985)).

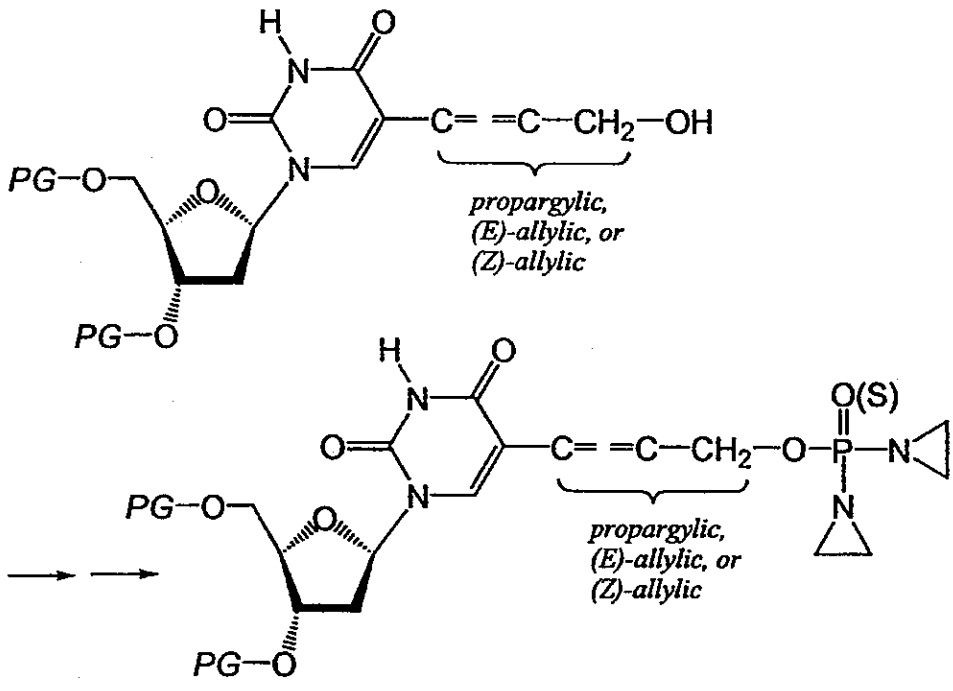
Both 5-mercuri- (Ruth, J. L. et al. (1978)) and 5-iodouridines (Robins, M. J. et al. (1981)) readily condense with alkenes and alkynes in the presence of a palladium catalyst to afford C5 tether-equipped uridines. The latter route is the more often employed (Robins, M. J. et al. (1982)), Asakura, J. et al. (1988) and (1990)). High-

yielding condensations of protected 5-iodo-2'-deoxyuridines with t-butyidimethylsilyl propargyl ether (Graham, et al. (1998); De Clercq, et al. (1983), methyl propargyl ether (Tolstikov, et al. (1997)) and even propargyl alcohol itself (Chaudhuri, et al. (1995) and Goodwin, et al. (1993)) have been achieved. The 3-hydroxy-l-propynyl substituent introduced by the latter reaction can also be accessed by DIBAL-H reduction of a methacrylate group (Cho, Y. M. et al. (1994)), itself arising from the same Heck reaction used in the synthesis of BVdU. These palladium-catalyzed reactions are so versatile that they can used to condense very long and elaborately-functionalized propargyl-based tethers to 5-iodo-2'-deoxyuridines. (Livak, et al. (1992) and Hobbs (1989)). (Z)-Allyl-based tethers are generated by the partial hydrogenation of a propargylic precursor over Undiar catalyst (Robins, M. J. et al. (1983)) whereas the (E)-allyl-based ones are best prepared by Heck coupling of an (E)-tributylstannylated ethylene (Crisp, (1989)).

Closely following the literature procedures, a t-butyldimethylsilyl propargyl ether-equipped 3', 5'-di-O-protected 2'-deoxyuridine (Graham, et al. (1998), and De Clercq, et al. (1983)) is prepared and a portion of it, converted to the corresponding (Z)-allyl ether, (Robins and Barr (1983)) is reduced. Because the TBAF-mediated removal of a TBDMS group generates an oxyanion that can be functionalized *in situ*, these TBDMS-protected propargyl- and (Z)-allytic-tethered nucleosides will serve as convenient precursors to some of the toxophore-equipped targets. For the (E)-allyl alcohol equipped nucleoside, the known O-tetrahydropyranyl ether derivative is prepared by the literature Heck coupling of an (E)-tributylstannylated ethylene (Crisp (1989)).

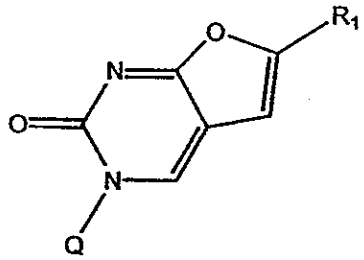


Using a two step literature protocol (Phelps et al. (1980) and Hsiao and Bardos (1981)), the propargylic and (E) and (Z)-allylic alcohols are converted to their corresponding bis-aziridinyl phosphoramidates or thiophosphoramidates so that TS processing of the 5'-mononucleotide versions will release an active metabolite of the cytostatic drugs TEPA or ThioTEPA (Dirven, et al. (1995)), respectively.



***Synthesis of furano-pyrimidinones***

Synthesis of furano-pyrimidinones begins with synthesis of a C5 propargylic - alcohol-equipped 2'-deoxyuridine. Furano-pyrimidinone compounds are then be formed from the O-tetrahydropyranyl ether derivative described above. Synthesis proceeds by reaction of the second carbon of the propargyl bond with the oxygen attached to the C4 position of the pyrimidine ring to yield a fluorescent furano-pyrimidinone which can be readily separated from the reaction mix. Such compounds provide an additional basis for synthesis of ECTA compounds through various combinations of specific electron conduits, spacers and toxic leaving groups.




The furo[2,3-d]pyrimidinone nucleosides were prepared by condensing 2',3'-di-O-p-toluoyl or 2',3'-di-O-acetyl-5-iodo-2'-deoxyuridine with 1-(tetrahydropyranyloxy)-2-propyne ("New methods of synthesis of δ-aminoethylpyrazoles" Jones, R. G. and Mann, M. J. (1953)) under conditions known to promote the formation of these fluorescent compounds (Robins, M. J. et al.(1983)). Base-catalyzed removal of the carbohydrate protecting groups gave the 6-(tetrahydropyran-2-yloxymethyl)-substituted bicyclic nucleoside which was either subjected to standard acidic THP group hydrolysis (TFA in $CH_2Cl_2$) or was regioselectively 5'-phosphoramidated by the same procedure used to prepare BVdU-PA and 5FUdR-PA. After the phosphoramidation, the THP group could be removed by acidic hydrolysis, as shown in Figure 4.

***TS ECTA compounds based on furano-pyrimidinones***

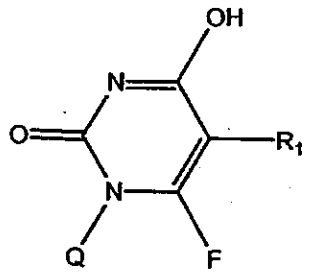
Toxic $R^4$ leaving groups can be attached to the furan-2 methyl alcohol using methods similar to those employed to attach toxic leaving groups to the hydroxyl on the C5 propargyl uridine compound, as explained with the synthesis of the TEPA and

ThioTEPA derivatives described above. A variety of alternative toxic leaving groups, apparent to one skilled in the art, are envisioned. In addition, modifications to the length and composition of the $R^2$ electron conduit component and of the composition of the $R^3$ spacer element are also envisioned.

TS ECTA compounds based on furano-pyrimidinones can also consist of variously modified "Q" moeities. Many 5-substituted 2'-deoxyuridines are not substrates for human TK, but interestingly 5-(4-hydroxy-l-butynyl)-2'-deoxyuridine was found to be an exception (Barr, P. J. et al. (1981)). The ECTA compounds can have a free 5' hydroxyl, a 5' monophosphate, or a 5' phosphoramidate group attached to alternative carbohydrate groups. A novel method for synthesis of such phosphoramidate compounds is accomplished by reacting a 2-deoxy 3'-hydroxy, 5'-hydroxy unprotected nucleotide with a phosphochloridate in the presence of an HCl scavenger. In a preferred embodiment, the phosphochloridate comprises a phosphorus substituent which is derived from an amino acid such as alanine. For example, the phosphochloridate can be phenyl-L-methoxyalanine phosphorochloridate.

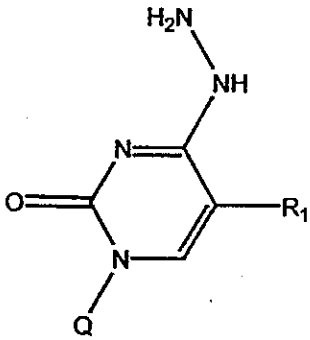
***C6 Fluoro uridine and C4 hydozone based compounds***

The neutral thiol addition to the pyrimidine C5-C6 double bond proceeds as an exothermic reaction (3-9 kcal per mol; see review by Les, A. et al. (1998) in the normal TS reaction with dUMP. Alternative substituents to the TS reactive hydrogen at the 6 position that can facilitate the formation of the sulfydryl bond with the enzyme, via the active human TS cysteine (homologous with cys-198 of *L. casei*), include fluorine. Such substituents at other positions in the pyrimidine ring can also facilitate the reaction between the substrate and TS. For instance, a 4-hydrazone substitution on the uracil (as described by Les, A. et al. (1998) facilitates formation of the thiol with TS. It is important that the resulting nucleotide-thiol (TS) intermediate rearranges in such a way as to release the altered nucleotide which can be accomplished passively via hydrolysis.




The introduction of fluorine at the C6 position has not been previously reported, but it can be synthesized by following the synthetic descriptions of Krajewskas and Shugar (1982), who describe the synthesis of a number of 6 substituted uracil and uridine analogues.

Chemistry facilitating substitutions at the C4 position of the pyrimidine base are well known by those skilled in the art. Examples of literature descriptions include Wallis et al. (1999); Negishi, et al. (1996), Barbato et al. (1991), Barbato, et al. (1989) and Holy et al. (1999). These synthetic techniques also enable combinations of substitutions, for instance at the C4 and C5 positions of the pyrimidine ring (Pluta, et al. 1999) or the C2 and C4 positions of the pyrimidine ring (Zeid, et al. (1999)).

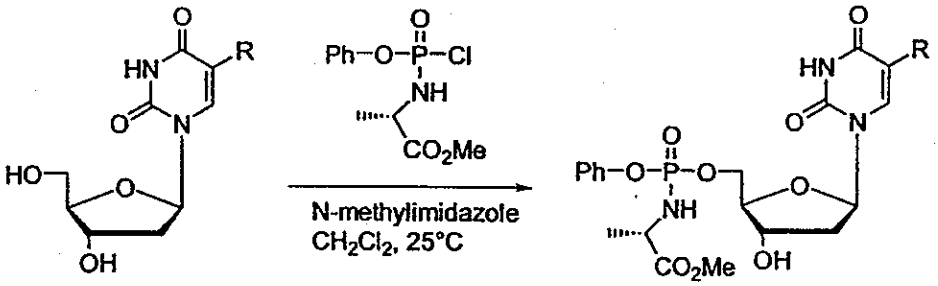



In another embodiment of the invention, ECTA compounds are synthesized by addition of alternative electron conduits, spacer moieties and toxic leaving groups to either the C6 fluoro-uridine base or the C4 hydrazone modified pyrimidine. Methods described above for synthesis of 2, deoxyuridine based ECTA compounds can again be employed for synthesis of such molecules.

***Synthesis of Nucleoside Phenyl Methoxyalaninyl Phosphoramidates***

The use of phosphoramidates as phosphate prodrugs for nucleotides was first reported by McGuigan, C. et al. (1993) and McGuigan, C. et al. (1994). These authors showed that phosphoramidate derivatives of antiviral 2',3'-dideoxynucleoside derivatives such as d4T retain their antiviral activities in thymidine-kinase deficient cells. Further studies showed that the phosphoramidate group was hydrolyzed to the phosphate group inside cells (McGuigan, C. et al. (1996); Balzarini, J. et al. (1996); and Saboulard, et al. (1999)). The phospharamidates were synthesized by reacting 2',3'-dideoxynucleosides with phenyl methoxyalaninyl phosphorochloridate (PMPC).

Since only one hydroxyl group is present, these reactions usually proceed smoothly. In compounds where more than one hydroxyl group is present, the appropriately protected nucleoside might be required. Since the 5'-OH group of 2'-deoxynucleosides is much less hindered than the 3'-OH group, selective phosphoramidation with PMPC is possible under carefully controlled conditions. Both BVdU and 5FUdR condensed with PMPC in the presence of N-methylimidazole in anhydrous $CH_2Cl_2$ to give the corresponding phosphoramidates. In both cases, the desired product was readily separable from the starting material using column chromatography on silica gel. The synthetic scheme is summarized below.




The following examples are intended to illustrate, but not limit the invention.

**Examples 1 and 2**

**Synthesis of ECTA compounds with propargyl tethers**

Using the general synthetic procedure described *supra*, **bis-aziridin-1-yl-phosphinic acid 3-[2-deoxyuridin-5-yl]-prop-2-ynyl ester** was synthesized and analyzed by $^1H$ NMR to yield the following result: $^1H$ NMR ($(CD_3)_2SO$) complicated

due to noise. Salient features: δ 8.28 (d, 1, H6), 6.10 (pseudo-t, 1, H1'), 5.26 (m, exchanges with $D_2O$, 1, 3'-OH), 5.13 (m, exchanges with $D_2O$, 1, 5'-OH), 4.81 (q or dd, 2, propargyl-$CH_2$), 4.24 (m, 1, H3'), 3.57 (m, 2, 5'-$CH_2$), 2.15-2.0 (m, 8, aziridine-$CH_2$).

**Bis-aziridin-1-yl-phosphinothioic acid 3-[2-deoxyuridin-5-yl]-prop-2-ynyl ester** was also synthesized and analyzed by $^1H$ NMR to yield the following result: $^1H$ NMR ($(CD_3)_2SO$) complicated due to noise. Salient features: δ 8.29 (d, 1, H6), 6.10 (pseudo-t, 1, H1'), 5.22 (m, exchanges with $D_2O$, 1, 3'-OH), 5.10 (m, exchanges with $D_2O$, 1, 5'-OH), 4.88 (q or dd, 2, propargyl-$CH_2$), 4.31 (m, 1, H3'), 3.52 (m, 2, 5'-$CH_2$), 2.15-2.0 (m, 8, aziridine-$CH_2$).

**Examples 3 to 8**

**Synthesis of furano-pyrimidinones**

Using the general synthetic procedure described *supra*, the following compounds were prepared.

**Example 3**

**3-(2-Deoxy-β-D-ribofuranosyl)-6-(tetrahydropyran-2-yloxymethyl)furo[2,3-*d*]pyrimidin-2(3H)-one.** $^1H$ NMR ($(CD_3)_2SO$) δ 8.80 (s, 1, H4), 6.74 (s, 1, H5), 6.16 (pseudo-t, 1, H1'), 5.27 (d, exchanges with $D_2O$, 1, 3'-OH), 5.12 (t, exchanges with $D_2O$, 1, 5'-OH), 4.72 (m, 1, THP-H2), 4.56 (q, 2, $CH_2OTHP$), 3.92 (m, 1, H4'), 3.64 (m, 2, 5'-$CH_2$), 2.40 (m, 1, H2'a), 2.03 (m, 1, H2'b), 1.68 and 1.50 (m, 8, THP). Low-resolution mass spectrum (DCI-$NH_3$) on bis-TMS derivative, *m/z* 323 (B+TMS+$H^+$), 511 ($MH^+$), 583 (M+$TMS^+$).

**Example 4**

**3-(2-Deoxy-β-D-ribofuranosyl)-6-(hydroxymethyl)furo[2,3-*d*]pyrimidin-2(3H)-one.** $^1H$ NMR ($(CD_3)_2SO$) δ 12.0 (bs, 1, OH), 8.24 (s, 1, H4), 6.53 (s, 1, H5), 5.51 (pseudo-t, 1, H1'), 4.42 (m, 2, $CH_2OH$). Low-resolution mass spectrum (DCI-$NH_3$), *m/z* 167 (B+$2H^+$), 184 (B+$NH4^+$).

**Example 5**

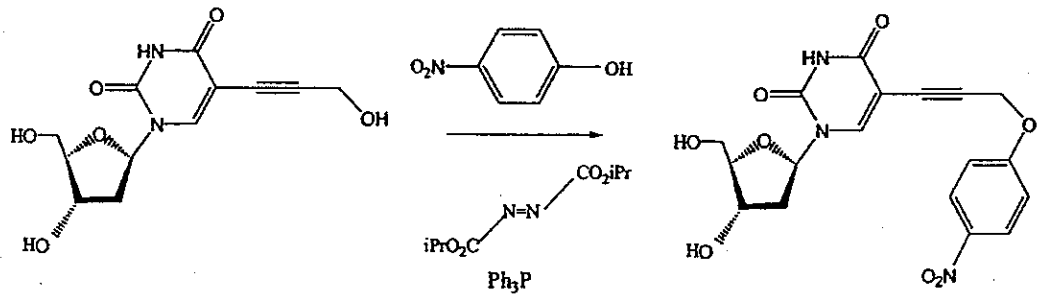
**1-[6-(Tetrahydropyran-2-yloxymethyl)furo[2,3-d]pyrimidin-2(3H)-on-3-yl]-2-deoxy-β-D-ribofuranos-5-yl phenyl methoxy-L-alaninylphosphoramidate.** 1H NMR ((CD3)2SO) complicated due to presence of diastereomers. Salient features: δ 8.62 and 8.59 (each s, each 1, H4), 7.4-7.1 (m, 5, PhO), 6.61 and 6.60 (each s, each 1, H5), 6.25 (m, 1, H1'), 4.56 (q, 2, propargyl-CH2), 3.56 and 3.54 (each s, each 3, CO2Me), 2.0 (m, 1, H2'b), 1.22 (m, 3, alaninyl-α-Me). Low-resolution mass spectrum (DCI-NH3), m/z 167 ($B+2H^+$), 184 ($B+H^++NH4+$-THP).

**Example 6**

**1-[6-(Hydroxymethyl)furo[2,3-*d*]pyrimidin-2(3H)-on-3-yl]-2-deoxy-β-D-ribofuranos-5-yl phenyl methoxy-L-alaninylphosphoramidate.** $^1H$ NMR ($CDCl_3$) complicated due to presence of diastereomers. Salient features: δ 8.5 (s, 1, H4), 7.4-7.1 (m, 5, PhO), 6.36 and 6.30 (each s, each 1, H5), 6.23 (m, 1, H1'), 3.67 and 3.65 (each s, each 3, $CO_2Me$), 2.69 (m, 1, H2'a), 2.10 (m, 1, H2'b), 1.35 (m, 3, alaninyl-α-Me). Low-resolution mass spectrum (DCI-$NH_3$), *m/z* 525 ($MH^+$), 595 ($MNH_4^+$).

**Example 7**

The 4-nitrophenyl ether derivative of 5-(3-hydroxy-1-propynyl)-2'-deoxyuridine was prepared according to standard ether synthesis as shown below.



**Example 8**

**5-[3-(4-Nitrophenoxy)-1-propynyl]-2'-deoxyuridine.** A solution of pre-dried 5-(3-hydroxy-1-propynyl)-2'-deoxyuridine Robins, M. J. et al., (1983) (565 mg, 2 mmol) in 40 mL of anhydrous THF under argon was treated with 4-nitrophenol (696 mg, 5 mmol), triphenylphosphine (787 mg, 3 mmol), and diisopropyl azodicarboxylate (590 L, 3 mmol), and the reaction mixture heated at 60 °C until the solution was clear, and then 1 h longer. The mixture was allowed to cool to 23 °C and then it was evaporated onto $SiO_2$ and purified by chromatography using $MeOH/CH_2Cl_2$ as eluent to afford 107 mg (13%) of the desired ether product: mp 112-118 °C. $^1H$ NMR ($(CD_3)_2SO$) δ 11.65 (s, exchanges with $D_2O$, 1, NH), 8.29 (s, 1, H6), 8.24 (d, $J = 9.3$ Hz, 2, *m*-ArH), 7.23 (d, $J = 9.3$ Hz, 2, *o*-ArH), 6.09 (pseudo-t, 1, H1'), 5.17 (s, 2, propargyl-$CH_2$), 4.22 (m, 1, H3'), 3.80 (m, 1, H4'), 3.59 (m, 2, 5'-$CH_2$), 2.13 (pseudo-t, 2, 2'-$CH_2$). Low-resolution mass spectrum (DCI-$NH_3$) on *per*-trimethylsilyated material, *m/z* 547 [$M(TMS)_2H^+$], 565 [$M(TMS)_2NH_4^+$], 620 [$M(TMS)_3H^+$].

**Example 9**

**5-(4-Carbethoxy-1,3-butadienyl)-2'-dexoyuridine**

**(a) 5-(Carbomethoxyvinyl)-2'-deoxyuridine-3',5'-bis(tetrahydro-2*H*-pyran-2-yl)ether (I)**

A slurry of 5-(carbomethoxyvinyl)-2'-deoxyuridine (3.0 g, 9.6 mmol), 3,4-dihydro-2*H*-pyran (22 mL, 21.3 mmol) and pyridinium *p*-toluenesulfonate (PPTS, 0.242 g, 0.96 mmol) in dimethylformamide (DMF, 5 mL) was stirred at 50°C for 18 h. The resulting solution was concentrated *in vacuo* (bath temperature 45°C) to give a thick, pale yellow oil. The oil was dissolved in EtOAc and the solid was filtered. The solution was again concentrated. The oil obtained was purified by column chromatography on silica gel using 50-75% EtOAc/hexane as eluent to give 3.81 g (85%) of pure product as a colorless oil.

**(b) 5-(3-Hydroxyprop-1-enyl)-2'-deoxyuridine-3',5'-bis(tetrahydro-2*H*-pyran-2-yl)ether (II)**

A solution of (I) (3.5 g, 7.27 mmol) in $CH_2Cl_2$ (14 mL) was cooled to -78°C in a dry ice/acetone bath. Diisobutylaluminum hydride (DIBAL-H) in toluene (1.0 M, 24 mL, 24.0 mmol) was added dropwise over 2 h while the temperature was maintained at -78°C. The solution was stirred at -78°C for an additional 2 h and MeOH (2.5 mL) was added dropwise to destroy any excess DIBAL-H. The reaction mixture was cannulated into a mixture of 30% citric acid solution (50 mL), ice (25 g) and EtOAc (30 mL) over ca. 20 min. The phases were separated and the aqueous phase was extracted with EtOAc (2 × 25 mL). The combined organic phase was washed with saturated $NaHCO_3$ (20 mL) and brine (20 mL), dried over $MgSO_4$ and concentrated to give 3.288 g (100%) of colorless oil

**(c) 5-(3-Oxoprop-1-enyl)-2'-dexoyuridine-3',5'-bis(tetrahydro-2*H*-pyran-2-yl)ether (III)**

To a solution of crude (II) obtained from above (1.988 g, 4.4 mmol) in $CH_2Cl_2$ (9 mL) was added solid pyridinium dichromate (PDC; 1.82 g, 4.8 mmol) with water cooling. The suspension was stirred while acetic acid (0.4 mL) was added dropwise. The water bath was removed and the reaction was stirred at room temperature for 1 h. The crude product was filtered through a pad of florisil (2 × 2.5 cm) and the florisil washed with 35 mL EtOAc. The brown solution obtained was filtered through another column of florisil (3.5 cm diam × 2.5 cm height). The filtrate was concentrated to give 1.273 g (64% yield) of very light brown oil.

**(d) 5-(4-Carbethoxy-1,3-butadienyl)-2'-dexoyuridine-3',5'-bis(tetrahydro-2*H*-pyran-2-yl)ether (IV)**

(Carbethoxymethylene)triphenylphosphorane (0.32 mg, 0.92 mmol) was added to a solution of the crude aldehyde (III) (0.344 g, 0.77 mmol). The solution darkened and turned rust color. After 1 h, (III) was completely consumed as judged by thin layer chromatography. The solvent was evaporated and the crude product was purified by

column chromatography on silica gel using 35-45% EtOAc/hexane as eluent. The pure product (0.310 g, 78% yield) was obtained as colorless oil.

**(e) 5-(4-Carbethoxy-1,3-butadienyl)-2'-dexoyuridine (V)**

5-(4-Carbethoxy-1,3-butadienyl)-2'-dexoyuridine-3',5'-bis(tetrahydro-2*H*-pyran-2-yl)ether (IV) (0.637 g, 1.22 mmol) was dissolved in MeOH (1.5 mL) and PPTS (0.049 g, 0.16 mmol) was added. The solution was stirred at 50°C for 7.5 h and left at room temperature overnight. A white precipitate was formed. The reaction mixture was cooled to 0°C and filtered to give pure (V) as a white solid (0.188 g). The filtrate was concentrated and chromatographed on silica gel using 50-100% EtOAc/hexane as eluent to give a further 0.180 g product. The total yield of the product was 0.368 g (86%).

$^1$H NMR (DMSO-$d_6$): 1.22 (3H, t, J = 7 Hz), 2.17 (2H, br t, J = 5.5 Hz), 3.55-3.75 (2H, m), 3.81 (1H, m), 4.12 (2H, q, J = 7 Hz), 4.25-4.28 (1H, m), 5.19 (1H, t, J = 4.8 Hz), 5.27 (1H, d, J = 4.1 Hz), 5.98 (1H, d, J = 14.5 Hz), 6.14 (1H, t, J = 6.3 Hz), 6.75 (1H, d, J = 14.5 Hz), 7.18-7.30 (2H, m), 8.30 (1H, s), 11.56 (1H, s).

**Example 10**

**5-(4-Carbomethoxy-1,3-butadienyl)-2'-dexoyuridine (Va)**

A solution of triethylamine (3.9 mL, 28.2 mmol) in dioxane (12 mL) was deareated by bubbling nitrogen through for 15 min. Palladium acetate (0.60 g, 0.26 mmol) and triphenylphosphine (0.183 g, 0.70 mmol) were added and the solution was heated at 70°C for 20 min to give a dark brown solution. 5-Iodo-3'-deoxyuridine (5.0 g, 14.1 mmol) and methyl 2,4-pentadienoate (2.5 g, 22.3 mmol) was added and the mixture was heated under reflux for 15h. The solvent and volatile components were evaporated in vacuo and the residue was partitioned between water (15 mL) and EtOAc (15 mL). The phases were separated and the aqueous phase was extracted twice with EtOAc (10 mL each). The combined organic phase was washed with brine and concentrated. The residue was dissolved in MeOH (15 mL) and allowed to cool to room temperature. The solid formed was collected by filtration, washed with a small quantity of MeOH and dried in vacuo to give 0.38 g brown powder.

$^1$H NMR (DMSO-$d_6$): 2.17 (2H, t, J = 6.4 Hz), 3.55-3.70 (2H, m), 3.66 (3H, s), 3.82 (1H, q, J = 3.6 Hz), 4.27 (1H, m), 5.18 (1H, t, J = 4.9 Hz), 5.26 (1H, d, J = 4.5 Hz),

5.99 (1H, d, J = 14.4 Hz), 6.14 (1H, d, J = 6.4 Hz), 6.74 (1H, d, J = 14.8 Hz), 7.20-7.35 (2H, m), 8.30 (1H, s), 11.56 (1H, s).

The filtrate from above was concentrated and chromatographed on silica gel using 60-100% EtOAc/hexanes as eluent to give another 0.70 g of product as a brown foam. The combined yield was 1.08 g (22.6%).

**Example 11**

**5-(4-Carboxy-1,3-butadienyl)-2'-dexoyuridine (VI)**

**Method I**

5-(4-Carbethoxy-1,3-butadienyl)-2'-dexoyuridine (V, from Example 1) (0.449 g, 1.28 mmol) was dissolved in 2N NaOH (3 mL) and stirred at 25°C. After 20 min, a precipitate was formed and TLC showed that the starting material was completely consumed. The mixture was cooled to 0°C and acidified to pH 1 with 2N HCl. The resulting off-white solid was filtered off, washed with water and dried *in vacuo* to give 0.225 g (54%) product.

$^1$H NMR (DMSO-$d_6$): 2.12-2.19 (2H, m), 3.50-3.70 (2H, m), 3.75-3.85 (1H, m), 4.24-4.29 (1H, m), 5.19 (1H, t, J = 4.8 Hz), 5.27 (1H, d, J = 4.2 Hz), 5.80-5.95 (1H, m), 6.14 (1H, t, J = 6.4 Hz), 6.60-6.75 (1H, m), 7.15-7.25 (2H, m), 8.26 (1H, s), 11.56 (1H, s), 12.16 (1H, br s).

The filtrate and washings were combined and evaporated to dryness. The resulting sticky yellow solid was dissolved in MeOH from which a white precipitate was formed. The solid was filtered off to give an additional 0.200 g of product.

**Method II**

The title compound can also be prepared from 5-(4-carbomethoxy-1,3-butadienyl)-2'-dexoyuridine (**Va**) (prepared according to the method in Example 2) in comparable yield as mentioned above.

**Example 12**

**5-(4-Bromo-1*E*,3*E*-butadienyl)-2'-dexoyuridine (VIIa) and**

**5-(4-Bromo-1*E*,3*Z*-butadienyl)-2'-dexoyuridine (VIIb)**

To a solution of 5-(4-carboxy-1,3-butadienyl)-2'-dexoyuridine (VI) (0.200 g, 0.62 mmol) in DMF (1 mL) was added $KHCO_3$ (0.185 g, 1.84 mmol) and the mixture

was stirred for 20 min at 25°C. A solution of *N*-bromosuccinimide (0.117 g, 0.65 mmol) in DMF (0.3 mL) was added dropwise. Smooth gas evolution ($CO_2$) occurred throughout the addition. The resulting brown suspension was stirred for 2 h at 25°C at which time TLC showed that (VI) was completely consumed. Water (10 mL) was added to the suspension and the resulting solution was extracted with EtOAc (2 × 15 mL). The extract was dried over $MgSO_4$ and the solvent was evaporated *in vacuo* to give a yellow solid (178 mg, 80% yield) consisting of a mixture of two isomers as shown by $^1$H NMR. The crude product was separated by semi-preparative HPLC (reversed phase C18 column) using 20% acetonitrile in water as the mobile phase to give the following isomers:

**5-(4-Bromo-1*E*,3*Z*-butadienyl)-2'-dexoyuridine:** retention time 10.5 minutes; $^1$H NMR: (DMSO-$d_6$): 2.11-2.18 (2H, m), 3.50-3.70 (2H, m), 3.80 (1H, distorted q, J = 3.5 Hz), 4.25 (1H, br s), 5.08 (1H, br s), 5.25 (1H, br s), 6.15 (1H, t, J = 6.5 Hz), 6.40 (1H, d, J = 7 Hz), 6.53 (1H, d, J = 15.6 Hz), 6.83 (1H, dd, J = 7, 10 Hz), 7.39 (1H, dd, J = 10, 15.6 Hz).

**5-(4-Bromo-1*E*,3*E*-butadienyl)-2'-dexoyuridine:** retention time 15.1 minutes; $^1$H NMR (DMSO-$d_6$): 2.12-2.16 (2H, m), 3.50-3.70 (2H, m), 3.80 (1H, q, J = 3.2 Hz), 4.26 (1H, m), 5.13 (1H, br s), 5.25 (1H, br s), 6.14 (1H, t, J = 6.5 Hz), 6.36 (1H, d, J = 15.6 Hz), 6.67 (1H, d, J = 13.1 Hz), 6.84 (1H, dd, J = 11, 13.1 Hz), 7.04 (1H, dd, J = 11, 15.6 Hz).

**Example 13**

Using the procedures mentioned in Example 11, Method II, the following compounds can be obtained in a similar fashion: 5-(4-chloro-1,3-butadienyl)-2'-dexoyuridine (using *N*-chlorosuccinimide in place of *N*-bromosuccinimide in Step B); 5-(4-iodo-1,3-butadienyl)-2'-dexoyuridine (using iodine in sodium idodide in place of *N*-bromosuccinimide).

## Example 14

### 5-(2-Bromovinyl)-2'-Deoxyuridine Phenyl *N*-Methoxy-L-alaninyl Phosphoramidate

#### Phenyl *N*-Methoxy-*L*-alaninyl Phosphorochloridate

*L*-alanine methyl ester hydrochloride (245.8 g; 1.76 mol) was placed in a 12 L three-neck round bottom flask (equipped with a mechanical stirrer and thermometer) followed by 4.0 L of dichloromethane. The mixture was stirred for 15 minutes at room temperature. Phenyl phosphodichloridate (370.0 g; 1.76 mol) was added to the mixture and stirring was continued for 15 min at room temperature. The flask was placed in the bath with dry ice and the stirring was continued for 20 minutes until a uniform suspension was formed.

Freshly distilled tri-n-butylamine (626.5 g; 3.38 mol) was added dropwise (~90 min) with vigorous stirring to the reaction mixture so that the temperature inside the flask was held at ~0°C. The bath was removed and the stirring was continued for 6 hours at room temperature. The solution was concentrated to ~2.84 L by evaporating several portions of the mixture on a rotary evaporator and the mixture was sealed under argon and stored at -20°C. The product was 85% pure by phosphorus NMR to give an estimated concentration of phenylmethoxyalaninyl phosphochloridate of ~0.5 M.

## Example 15

### 5-(2-Bromovinyl)-2'-Deoxyuridine Phenyl *N*-Methoxy-*L*-alaninyl Phosphoramidate (NB1011)

The reaction was performed under argon atmosphere. 5-(2-bromovinyl)-2'-deoxyuridine (BVdU) (204 g; 612 mmol) was placed in three-neck 3 L round bottom flask equipped with mechanical stirrer. The flask was placed in ice-water bath and 1600 mL (~ 800 mmol) of phenylmethoxyalaninyl phosphochloridate reagent were added using an addition funnel over 15 min with vigorous stirring of the reaction mixture, followed by the addition of 100 mL of N-methylimidazole over 5 minutes using syringe. After 5 minutes the mixture became clear and after 10 minutes the ice-water bath was removed to allow the mixture to warm up to room temperature while stirring was continued. The reaction was monitored by reversed phase HPLC and was complete in 3 hours. The reaction was quenched by the addition of 100 mL of methanol and the mixture was evaporated to an oil, re-dissolved in 6 L of dichloromethane and

passed through 800 g of silica gel. The major portion of BVdU-PA referred to herein as NB1011, was passed through the column during the loading and finally the elution of NB1011 was completed by passing 5 L of 5% methanol in dichloromethane. All fractions containing NB1011 were combined and evaporated to an oil, the residue was dissolved in 4 L of ethyl acetate and the mixture was extracted with water (2 x 2 L). The organic layer was dried with sodium sulfate, filtered, and washed with ethyl acetate (3 x 300 mL). The combined filtrate and washings were evaporated to produce a lightly colored white foam; total weight ~540 g.

The crude product was purified by two silica gel chromatography using 0-5% MeOH in $CH_2Cl_2$ and 10% MeOH in $CH_2Cl_2$, respectively, as eluent. The yield of product (>98% pure) was 64 g.

**Example 16**

Using the methods described in Example 15, the phenyl *N*-methoxy-*L*-alanyl phosphoramidates of the following nucleosides were prepared:

1. 5-(4,4-dibromo-1,3-butadienyl)-2'-deoxyuridine,
2. 5-(2-chlorovinyl)-2'-deoxyuridine,
3. 5-trifluoromethyl-2'-deoxyuridine,
4. 5-(4-carbethoxy-1,3-butadienyl)-2'-deoxyuridine,
5. 5-(4-carbomethoxy-1,3-butadienyl)-2'-dexoyuridine,
6. 5-(4-bromo-1*E*,3*E*-butadienyl)-2'-deoxyuridine,
7. 5-(4-bromo-1*E*,3*Z*-butadienyl)-2'-deoxyuridine,
8. 5-(trimethylsilylethynyl)-2'-deoxyuridine,
9. 5-(ethynyl)-2'-deoxyuridine,
10. 5-(1-decynyl)-2'-deoxyuridine,
11. 3-(2'-deoxy-$\beta$-*D*-ribofuranosyl)-2,3-dihydrofuro[2,3-*d*]pyrimidin-2-one
12. 3-(2'-deoxy-$\beta$-*D*-ribofuranosyl)-6-octyl-2,3-dihydrofuro[2,3-*d*]pyrimidin-2-one.

Derivatives

Salts, esters, and ethers of the above compounds disclosed herein are also within the scope of this invention. Salts of the prodrugs of the present invention may be

derived from inorganic or organic acids and bases. Examples of acids include hydrochloric, hydrobromic, sulfuric, nitric, perchloric, fumaric, maleic, phosphoric, glycollic, lactic, salicyclic, succinic, toluene-p-sulfonic, tartaric, acetic, citric, methanesulfonic, ethanesulfonic, formic, benzoic, malonic, naphthalene-2-sulfonic and benzenesulfonic acids. Other acids, such as oxalic, while not in themselves pharmaceutically acceptable, can be employed in the preparation of salts useful as intermediates in obtaining the compounds of the invention and their pharmaceutically acceptable acid addition salts. Examples of bases include alkali metal (e.g., sodium) hydroxides, alkaline earth metal (e.g., magnesium) hydroxides, ammonia, and compounds of formula $NW_4^+$, wherein W is $C_{1-4}$ alkyl.

Examples of salts include: acetate, adipate, alginate, aspartate, benzoate, benzenesulfonate, bisulfate, butyrate, citrate, camphorate, camphorsulfonate, cyclopentanepropionate, digluconate, dodecylsulfate, ethanesulfonate, fumarate, flucoheptanoate, glycerophosphate, hemisulfate, heptanoate, hexanoate, hydrochloride, hydrobromide, hydroiodide, 2-hydroxyethanesulfonate, lactate, maleate, methanesulfonate, 2-naphthalenesulfonate, nicotinate, oxalate, palmoate, pectinate, persulfate, phenylproprionate, picrate, pivalate, propionate, succinate, tartrate, thiocyanate, tosylate and undecanoate. Other examples of salts include anions of the compounds of the present invention compounded with a suitable cation such as $Na^+$, $NH_4^+$, and $NW_4^+$ (wherein W is a $C_{1-4}$ alkyl group).

For therapeutic use, salts of the compounds of the present invention will be pharmaceutically acceptable. However, salts of acids and bases which are non-pharmaceutically acceptable may also find use, for example, in the preparation or purification of a pharmaceutically acceptable compound.

Esters of the prodrugs or compounds identified by the method of this invention include carboxylic acid esters (i.e., -O-C(=O)R) obtained by esterification of the 2'-, 3'- and/or 5'-hydroxy groups, in which R is selected from (1) straight or branched chain alkyl (for example, n-propyl, t-butyl, or n-butyl), alkoxyalkyl (for example, methoxymethyl), aralkyl (for example, benzyl), aryloxyalkyl (for example, phenoxymethyl), aryl (for example, phenyl optionally substituted by, for example, halogen, $C_{1-4}$alkyl, or $C_{1-4}$alkoxy or amino); (2) sulfonate esters, such as alkylsulfonyl (for example, methanesulfonyl) or aralkylsulfonyl; (3) amino acid esters (for example, L-valyl or L-isoleucyl); (4) phosphonate esters and (5) mono-, di- or triphosphate esters.

The phosphate esters may be further esterified by, for example, a $C_{1-20}$ alcohol or reactive derivative thereof, or by a 2,3-di-($C_{6-24}$)acyl glycerol. In such esters, unless otherwise specified, any alkyl moiety present advantageously-contains from 1 to 18 carbon atoms, particularly from 1 to 6 carbon atoms, more particularly from 1 to 4 carbon atoms. Any cycloalkyl moiety present in such esters advantageously contains from 3 to 6 carbon atoms. Any aryl moiety present in such esters advantageously comprises a phenyl group. Examples of lyxo-furanosyl prodrug derivatives of the present invention include, for example, those with chemically protected hydroxyl groups (e.g., with O-acetyl groups), such as 2'-*O*-acetyl-lyxo-furanosyl; 3'-*O*-acetyl-lyxo-furanosyl; 5'-*O*-acetyl-lyxo-furanosyl; 2',3'-di-*O*-acetyl-lyxo-furanosyl and 2',3',5'-tri-*O*-acetyl-lyxo-furanosyl.

Ethers of the compounds of the present invention include methyl, ethyl, propyl, butyl, isobutyl, and sec-butyl ethers.

In a further embodiment, the substrate may not be chemically related to pyrimidines or folates, but rather synthesized based upon known parameters of rational drug design. See Dunn, W.J. et al. (1996).

Chemical assays for products, for example, where a reaction product is an anti-metabolite of the bromovinyl-derivatives of dUMP, are described in the Examples provided below or by Barr, P.J. et al. (1983).

Assays for Amount of Nucleoside and Monophosphate In Cells

This assay was performed with the compound NB 1011. However, it understood to those of skill in the art that the below method is easily modifed for application or use with the prodrugs of this invention.

The cell lines MCF7 (breast cancer cell line); MCF7TDX (Tomudex resistant breast cancer cell line) and H630R10 (5-FU resistant colon cancer cell line obtained from Dr. S. Copur, Yale University, see Copur, S. et al. (1995)) in RPMI medium supplemented with 10% FCS and antibiotics were plated in 100 mm Petri dishes at the amount of 750,000 cells per dish and were incubated overnight at 37°C. 100 μM of BVdU was added to each dish and the cells continued to grow for 2 days at 37°C. After that, the medium was aspirated and the cells were washed twice with phosphate buffered saline (PBS). Then 1 ml of PBS per dish was added and the dishes were

placed in a -80°C freezer. The cells were disrupted via freezing/thawing procedure, repeated twice. The cell debris in the resulting suspension were spun down (13,000 rpm, 10 minutes) and the supernatant was deproteinized by passing though a Centrifugal Filter Device (Centrifree, 30,000 cut-off, Amicon) by centrifugation at 5,000 rpm for 30 minutes on Sorvall Super T21 centrifuge (Rotor SL-50-T, 1,912 g) at 20°C. The pass-through was concentrated to the volume of 100 µl, and injected onto an HPLC column (Adsorbosphere HS, C18, 5 µm, 4.6 mm x 150 mm, Alltech). The samples were analyzed using acetonitrile gradient in water containing trifluoroacetic acid. The peaks were detected at 300 nm, the maximum of absorption of BVdU-derivatives, and the amounts of compounds were expressed as peak area at 300 nm. The identification of compounds was done by comparison of their retention times and spectra with those of authentic standards.

AlamarBlue Cell Proliferation Assay

This assay was performed with the compound NB1011 and the prodrugs of this invention. Cells growing exponentially were transferred to 384-well flat bottom tissue culture plates. All cell types were plated at a density of 500 cells per well in 25 µL of complete medium (RPMI 1640 + 10% fetal bovine serum + antibiotics/antimycotics). After 24 hours (day 0), 25 µL of complete medium containing the compounds over the dose range of $10^{-3}$ to $10^{-10}$ M were added in triplicate. Drug exposure time was 120 hours (day 5), after which growth inhibition was assayed. 5 µL of the redox indicator, alamarBlue, was added to each well (10% v/v). After 4 hours incubation at 37°C, fluorescence was monitored at 535 nm excitation and 595 nm emission.

Crystal Violet Cell Proliferation Inhibition Assay

This assay was performed with the compound NB1011 and the prodrugs of this invention. The ability of the test compounds to block proliferation of cells was determined by the crystal violet procedure. (Sugarman, B. J. et al. (1985); and Antelman, D. et al. (1995)).

Compounds were dissolved in dimethyl sulfoxide to a concentration of 1M. They were further diluted as necessary into DMEM cell culture medium, and subsequently into the first wells of the 96-well microtiter plate. Each concentration was

tested in triplicate on the target cell line. Compound concentrations from 1 μM to 3000 μM were tested. Cells were incubated with compound for 72 hours, the plates were washed, and the cells fixed with methanol and stained with crystal violet as described in Sugarman, B. J. et al. (1985); and Antelman, D. et al. (1995).

Western Blot Analysis of TK and TS Levels in Cell Lines

Western blot experiments were performed using human normal colon epithelium cell type CCD18co (obtained from ATCC, Manassas, VA), colon adenocarcinoma cell line H630R10 resistant to 5-FU (obtained from Dr. S. Copur, Yale University, see Copur, S. et al. (1995), and a HER2-transfected breast cancer cell lines (Pegram, M. D. et al. (1997)). Cells were lysed in RIPA buffer (50 mM Tris-HCl, pH 7.5,150 mM NaCl, 0.5% Triton X-100, 0.1% SDS and 0.5% deoxycholic acid, sodium salt and protease inhibitors). Protein concentrations were determined by using BCA-200 protein assay kit (obtained from Pierce, Rockford, IL). 10 μg of proteins from each cell line were resolved by 12% SDS-PAGE. The separated proteins were transferred onto PVDF membrane (obtained from Amersham, England), followed by immunoblot with human thymidylate synthase monoclonal primary antibody and anti-tubulin monoclonal antibody (manufactured by NeoMarkers, Fremont, CA). Horseradish peroxidase linked sheep anti-mouse Ig was used as secondary antibody (Amersham). The ECL plus kit (Amersham) was used for detection of immunoreactivity. The bands corresponding to thymidylate synthase were quantified and normalized to that of tubulin by image analysis (Molecular Dynamics Storm).

RT-PCR Analysis of TS mRNA in Cell Lines

Expression level of human thymidylate synthase transcripts in different cell lines were quantified by using RT-PCR. Oligonucleotide primers for amplification of the human thymidylate synthase and B-actin were designed as follows: Thymidylate synthase sense primer (SEQ ID NO:1) 5'-GGGCAGATCCAACACATCC-3' (corresponding to bases 208-226 of thymidylate synthase cDNA sequence, Genbank Accession No. X02308), antisense primer (SEQ ID NO:2) 5'-GGTCAACTCCCTGTCCTGAA-3' (corresponding to bases 564-583), β-actin sense primer (SEQ ID NO:3) 5'-GCCAACACAGTGCTGTCTG-3' (corresponding to bases

2643-2661 of β-actin gene sequence, Genbank Accession No. M10277) and antisense primer (SEQ ID NO:4) 5'-CTCCTGCTTGCTGATCCAC-3' (corresponding to bases 2937-2955).

Total RNAs were isolated from cells using Rneasy mini kit (obtained from Qiagen, Valencia, CA). To monitor for possible DNA contamination, the primers for amplification of β-actin were designed to span the exon4/intron5/exon5 junction. Genomic DNA template leads to a 313 bp β-actin fragment, and cDNA template generates a 210 bp product.

Reverse transcription reactions were performed, using SuperScript preamplification system (Gibco/BRL, Gaithersburg, MD). 3 μg total RNA was applied in a volume of 20 μl buffer to conduct reverse transcription reaction, followed manufacturer's protocol.

PCR reactions were performed in a volume of 48 μl, containing 3 μl of cDNA mixture from reverse transcription reaction, 3 mM $MgCl_2$, 50 mM KCl, 20 mM Tris-Cl, pH 8.4, 0.2 mM of each dNTP, 0.4 TM of thymidylate synthase sense and antisense primers and 3 units of Taq DNA polymerase (obtained from Promega, Madison, WI). The reaction mixtures were incubated at 94°C for 3 min, followed by 10 cycles of 1 min incubation at 94°C, 1 min incubation at 58°C, and then 1 min incubation at 72°C. After 10 cycles, human β-actin primers in 2 μl were added to achieve a final concentration of 0.2 μM, bringing the final reaction volume to 50 μl. PCR reaction was continued to a total of 28 cycles, followed by a 7 min incubation at 72°C.

5 μL of PCR products were resolved by electrophoresis in 2% agarose gel, followed by staining with SYBR Gold nucleic acid gel stain (obtained from Molecular Probes, Eugene, Oreg.). The DNA bands corresponding to thymidylate synthase were quantified and normalized to that of β-actin by Molecular Dynamics Storm.

Northern Blot Analysis

Northern blots were obtained from Invitrogen (Carlsbad, CA) and hybridized to a cloned TS cDNA probe. Hybridization signals were normalized vs. ribosomal protein S9 as a housekeeping transcript. A tumor was considered to overexpress TS mRNA if the normalized signal was enhanced at least 2-fold as compared to the normal tissue control.

Cloning, Expression and Purification of Human Thymidylate Synthase

For expression in *E. coli* the complete human TS ORF was subcloned into the T7 promoter expression vector pET28a (Novagen, Inc.). The resulting plasmid vector encodes the synthesis of human TS as a recombinant fusion protein with a six histidine followed by a thrombin cleavage site. This fusion protein adds a total of 20 extra amino acids to the amino terminus of human TS. To produce recombinant protein, the human TS expression vector was introduced into the *E. coli* strain BL21(DE3), a strain with the T7 RNA polymerase gene inserted into the chromosome under the control of the lac operator.

The human TS-poly-His fusion protein was purified using a metal chelating affinity resin (Novagen, Inc.). Protein purification was followed by electrophoresis on 10% SDS polyacrylamide gels. Protein concentrations were determined using the Pierce BCA protein assay. Approximately 10 mg of human TS fusion protein was recovered from a 500 ml culture of *E. coli*. When visualized with silver stain only the band corresponding to human TS was apparent. The identity of the human TS band was confirmed by performing a Western blot with the anti-TS monoclonal antibody TS106 (NeoMarkers).

Thymidylate Synthase (TS) Enzyme Activity Assay

TS activity was measured using the spectrophotometric assay of Wahba, et al. (1961). In this assay, TS activity is monitored by measuring the increase in absorbency at 340 nm that occurs when the co-factor 5,10 methylene tetrahydrofolte is oxidized to dihydrofolate as dUTP is converted to dTTP. Enzyme prepared by this method has a specific activity of 0.5 - 0.65 units/mg protein. One unit is defined as the production of one micromole of dTMP per minute. This value is similar to that reported by Pedersen-Lane, J. et al. (1997).

Determination of Intracellular Products of TS Prodrug Metabolism

It is important to determine the intracellular products of TS prodrug metabolism in order to substantiate proposed mechanism of activation and action and to define agents that are candidate therapeutics. One acceptable view of intracellular metabolism of aryl phosphodiester amidates involves enzymatic conversion into a carboxylic acid,

intramolecular rearrangement of the phosphomonoester amidate into to a 5'-monophosphoryl nucleoside. See, Valette et al. (1996). However, this mechanism is unlikely to describe intracellular processing of all phosphoramidate-based pronucleotides. For example, a different mechanism was proposed for aryl phosphomonoester amidate processing, one involving the simple direct conversion of the phosphoramidate to the monophosphate species by a phosphoramidate hydrolates. See, McIntee et al. (1997) and Fries et al. (1995). Regardless of the mechanism for unmasking the nucleoside monophosphate, this assay will detect products of TS conversion of the intracellular monophosphate to cytotoxic compounds within the cell.

Cells are incubated with an amount of prodrug compounds that induces 50% growth inhibition of high TS expressing cell lines (in the 72H assay, *supra*). Both low and high TS expresser cells are used (e.g., CCD18co vs. H630R10). Time course studies are performed in which treated cells are processed according to the method described by McIntee, E. J. et al. (1997). Cells are lysed with 60% methanol in water at -20°C, and particulate residue removed by centrifugation. The supernatants are dried and stored at -20°C. The aliquots are evaluated initially by RP-HPLC and then by LC-MS to document the intracellular conversation of the phosphoramidate to the monophosphate and also the ensuring transformation of the monophosphate by thymidylate synthase.

Documentation of Substrate Activity Utilizing Purified Thymidylate Synthase

This assay determines the specific activities of future TS enzyme preparations, to assure the activity of such preparations over time, and to determine whether compounds screened for suitable activity do inactivate the TS enzyme under *in vitro* reaction conditions.

To determine what reaction products are produced when prodrug compounds are incubated together with purified recombinant TS enzyme, reaction conditions similar to those employed with the TS activity assay are used to generate reaction products *in vitro*. These reaction products will are then analyzed by GC-mass spectrometry to identify the actual molecules formed.

## Results

TS is highly expressed in several tumor cell lines and primary tumors

Thymidylate synthase expression levels in normal human brain, heart, kidney, spleen, liver, colon, lung, small intestine, stomach muscle, testis, ovary, uterus, prostate, thyroid gland, salivary gland, adrenal gland, skin, peripheral blood lymphocytes, bone marrow and from human colon and matched human normal tissues were determined using qualitative RT-PCR, as described above. Primers for β-actin were designed to span the exon4/intron5/exon5 junction to monitor for possible DNA contamination. Therefore, results reported in Figures 3 are shown as relative TS mRNA levels. Figure 3A shows relative TS mRNA in the multiple normal human tissues. Figure 3B is a comparison of average TS mRNA between normal and tumor human colon tissues.

Thymidylate synthase (TS) protein levels in various cultured normal and tumor cell lines were determined following procedures described above. Protein levels were measured by Western blot analysis; the results are shown below.

**Table 3.**
**TS protein level in human normal and tumor cell types**

| Cell strains | Description | TS level[1] |
|---|---|---|
| NHOST | Bone, osteoblast | 51 |
| NPRSC | Prostate, stroma | 98 |
| CCD18co | Colon epithelium, fibroblast | 100 |
| WI38 | Lung, embryonic | 150 |
| DET551 | Skin, fibroblast-like, embryonic | 177 |
| MRC9 | Lung, fibroblast-like, embryonic | 211 |
| NHDF | Skin, fibroblast | 224 |
| NHLF | Lung, fibroblast | 236 |
| Average | | 156±24[2] |

1. Thymidylate Synthase (TS) - TS levels were determined by using Western blot analysis, the quantified expression levels were expressed as values relative to that of cell strain CCD 18co.
2. Standard error

**Tumor Cells (≥ 4X TS)**

| Cell lines | Description | TS level[1] |
|---|---|---|
| H630/TDX | Colon, carcinoma, TDX[2] resistant | 671 |
| HCTC(+) | Colon, carcinoma, increased TS | 1276 |
| MCF7/TDX | Breast, adenocarcinoma, TDX[2] resistant | 1980 |
| H630R10 | Colon, carcinoma, 5-FU[3]resistant | 2305 |
| Average | | 1558±354[4] |

1. Thymidylate Synthase (TS) - TS levels were determined by using Western blot analysis, the quantified expression levels were expressed as values relative to that of cell strain CCD 18co.
2. Tomudex
3. 5-Fluorouracil
4. Standard Error

The Amounts of BVdUMP and BVdU Measured In Cells

The amounts of BVdUMP or BVdU present in lysates cell lines MCF7, MCF7TDX, and H630R10 each treated with BVdU, are expressed as an area of a corresponding HPLC peak at 300 nm. Note that extinction coefficients of these compounds at 300 nm are the same, therefore the comparison of the peak areas allows direct comparison of the amounts formed.

Table 4, below, shows the level of BVdUMP and BVdU measured inside the cells after 2 days treatment. Cells were incubated in complete media with 100 μm BVdU. Lysates were prepared and BVdUMP and BVdU levels were determined.

**Table 4.**

| Cell Lines | BVdUMP | BVdU | BvdUMP/(BVdUMP + BVdU) x100 |
|---|---|---|---|
| MCF7 | **63.7** | **90.8** | **44.2** |
| MCF7TDX | **10.9** | **39.0** | **21.2** |
| H630R10 | **<1** | **26.3** | **<4** |

The table shows that BVdU can penetrate all three cell types, but appears as BVdUMP most prominently in MCF7 and MCF7TDX cell lines. Little conversion occurs in the H630R10 cell line.

In a separate experiment, MCF7TDX and H630R10 cell lysates were used to catalyze BVdU phosphorylation in a cell free system with ATP added as a phosphate

donor method. This confirmed the much higher rate of this reaction in MCF7TDX cell lyzate as compared to H630R10 cell lysate. Tumor lysates prepared as described (Look, K.Y. et al. (1997)) can be prepared and used in this assay with BVdU (or other candidate nucleoside) to determine whether a given patient is a candidate for nucleoside versus phosphoramidate therapy.

These results show that in MCF7TDX cells BVdU can be phosphorylated to yield the corresponding monophosphate, a substrate for subsequent TS reaction whereas in H630R10 cells such phosphorylation goes on significantly slower than in MCF7TDX. It is important to mention that MCF7TDX is a breast tumor cell line. It has been reported that in breast tumor cells a TK with properties different from human TK from other tissue is found. (Madec, A. et al. (1988)). This TK might be responsible for BVdU phosphorylation. Based on the results presented above it is expected that nucleosides, such as BVdU, might be especially useful for treatment of certain types of cancer, including breast cancer, where BVdU can be phosphorylated. Moreover, the experimental procedure described above, allows *in vitro* determination of feasibility of using BVdU or other nucleosides for a given cancer type using the following approach. If a tissue sample is available the cells may be treated *in vitro* with BVdU and possible formation of BVdUMP can be monitored. Should BVdUMP formation be registered the tumor type must be considered as a candidate for successful nucleoside treatment as opposed to phosphoramidate derivatives.

**Table 5**

**Alamar Blue Cytotoxicity Assay of Normal and Tumor Cells**

| | MCF7TDX | IC50 (μM) H630R10 | HT1080 | Mean Tumor | IC50 (μM) CCd18co | Det551 | Mean Normal |
|---|---|---|---|---|---|---|---|
| NB1011 | 2 | 82 | 182 | 88.7 | 414 | 398 | 406 |
| BVDU | 0.02 | 201 | 719 | 306.7 | 1000 | ND | 1000 |
| NB1012 | 127 | 82 | - | 104.5 | ND | 110 | 110 |
| | | | | | | | |
| NB1013 | 26 | 92 | ND | 59.0 | ND | 570 | 570 |
| NB1020 | 0.48 | 326 | 1000 | 442.2 | 1000 | ND | 1000 |
| NB1014 | 396 | 287 | ND | 341.5 | ND | 239 | 239 |
| | | | | | | | |
| NB1016 | 877 | 337 | ND | 607.0 | ND | 338 | 338 |
| NB1021 | 637 | 141 | 1000 | 592.7 | 1000 | ND | 1000 |
| NB1017 | 55 | 52 | ND | 53.5 | ND | 137 | 137 |
| NB1024 | 4 | 14 | 258 | 92.0 | 1000 | ND | 1000 |
| NB1018 | 115 | 164 | ND | 139.5 | ND | 412 | 412 |
| NB1022 | 8 | 0.03 | 3 | 3.7 | 9 | ND | 9 |
| NB1019 | 251 | 95 | ND | 173.0 | ND | ND | - |
| NB1023 | 5 | 0.11 | 2 | 2.4 | 11 | ND | 11 |
| NB1026 | 1000 | 1000 | 1000 | 1000.0 | 1000 | ND | 1000 |
| NB1025 | 49 | 192 | 454 | 231.7 | 378 | ND | 378 |

**Table 6.**

**Crystal Violet Assay of Normal and Tumor Cells**

| | IC50 (uM) | | Mean | IC50 (uM) | | Mean |
|---|---|---|---|---|---|---|
| | H630R10 | HT1080 #12 | Tumor | CCD18co | Det551 | Normal |
| NB1011 | 130 | 1.2 | 65.6 | 408 | 356 | 382 |
| BVDU | 405 | 7 | 206.0 | 1000 | 625 | 812.5 |
| NB1017 | 111 | 17 | 64.0 | 206 | 253 | 229.5 |
| NB1024 | 92 | 3.3 | 47.7 | 784 | 460 | 622 |
| NB1018 | 248 | 20 | 134.0 | 254 | 431 | 342.5 |
| NB1022 | 3.8 | 0.3 | 2.1 | 0.7 | 3.6 | 2.15 |
| NB1019 | 220 | 24 | 122.0 | 162 | 824 | 493 |
| NB1023 | 2.7 | 0.2 | 1.5 | 0.9 | 3.3 | 2.1 |

Numbering in Tables 5 and 6 refer to the structures, shown infra. Results from testing using the alamarBlue assay are shown in Table 5, and results from crystal violet based assays are shown in Table 6. MCF7-TDX was derived from MCF7 breast tumor cells via selection in cell culture in the presence of Tomudex, a direct inhibitor of TS. Such selection often results in high intracellular levels of TS (Freemantle, S. J. et al. (1995)). Similarly, H630R10 is a 5FU (fluoropyrimidine) resistant colon cancer epithelial cell line derived from H630 colon cancer cells. See, Copur, S. et al. (1995). HT1080, #12, is a fibrosarcoma tumor cell line that expresses high levels of TS via a transgene introduced into HT1080 tumor cells. Normal cell lines used in these assays include CCD18co (normal colon epithelium) and Det551 (normal skin).

The data in both Tables 5 and 6 indicate that most compounds are active as phosphoramidates and nucleosides. One exception is NB1026™, which has little detectable activity as a phosphoramidate, but is active as a nucleoside (NB1025™). This result indicates that NB1026™ may not be activated similar to NB1011™. Cytotoxicity results with the nucleosides, especially BVdU, NB1020™ (ClVdU) and NB1024™, are surprising since the literature teaches that 5- substituted compounds like BVdU are not efficiently monophosphorylated by human cells unless they express viral

thymidine kinase. See, Balzarini, J. et al. (1985) and De Clerq, E. et al. (1997). These authors have proposed that once phosphorylated by the herpesvirus-encoded TK, the 5-substituted nucleotide derivative can bind to the TS enzyme and inactivate it. The results in Tables 4-6 demonstrate, for the first time, that at least some tumor cells may have an unusual thymidine kinase activity capable of phosphorylating 5-substituted uridine molecules similar to BVdU, and the other nucleosides shown herein. Alternatively, some tumor cells may have an unusually high amount, or form the normal TK enzyme (Suki, S. et al. (1995); and Romain, S. et al. (1995)), leading to a low, but sufficient activation of nucleosides to their corresponding monophosphates.

The compound NB1024™ as tested above consists of a mixture of two isomers, 5-(4-Bromo-1*E*,3*E*-butadienyl)-2'-dexoyuridine (VIIa), (Isomer 2), and 5-(4-Bromo-1*E*,3*Z*-butadienyl) -2'-dexoyuridine (VIIb), (Isomer 1). These two compounds were separated by semi-preparative HPLC (reversed phase C18 column) using 20% acetonitrile in water as the mobile phase as described above (Example 12). The ability of each isolated compound to block cell proliferation was then determined by the crystal violet procedure. Results are shown in Table 7. The two isomers display markedly different activities with the Z isomer, showing significantly greater growth inhibiting activity in comparison with the E isomer.

**Table 7. Cytotoxic Activity of NB1024 Isomers**

| | TS HT1080 #12 | MCF7TDX | CCD18co | Det551 |
|---|---|---|---|---|
| **NB1011** | 4.3 | 4.2 | 461 | 263 |
| **BVdU** | ND | <0.8 | >1000 | >1000 |
| **NB1024 Mixture** | 10.7 | 6.4 | >300 | >300 |
| **NB1024 Isomer 1** | 10.1 | 9.8 | >300 | >300 |
| **NB1024 Isomer 2** | >300 | >300 | >300 | >300 |

The first two cell lines, TS HT1080 #12 and MCF7TDX express high levels of TS, while the other two, CCD18co and Det551, are normal cells.

While the invention has been described in detail and with reference to specific embodiments thereof, it will be apparent to one skilled in the art that various changes

and modifications can be made therein without departing from the spirit and scope thereof.

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5

CLAIMS

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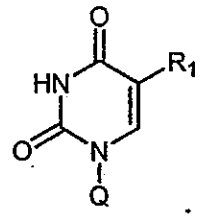
(i) compounds having the structure:

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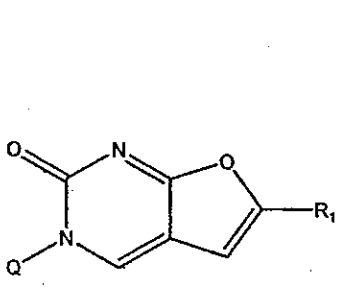


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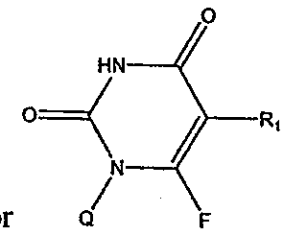
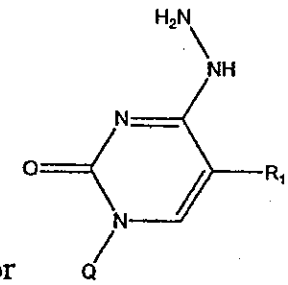
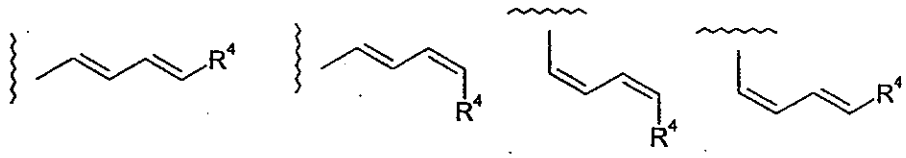



or



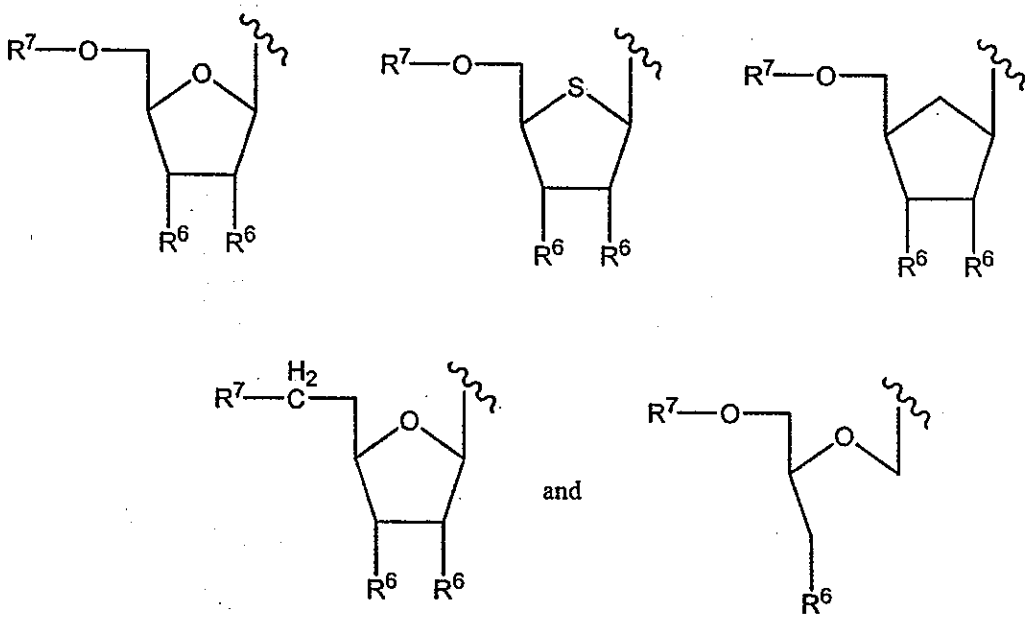

wherein:

$R^1$ is a group selected from:




$R^4$ is Cl, Br, I, or CN;

wherein Q is selected from:

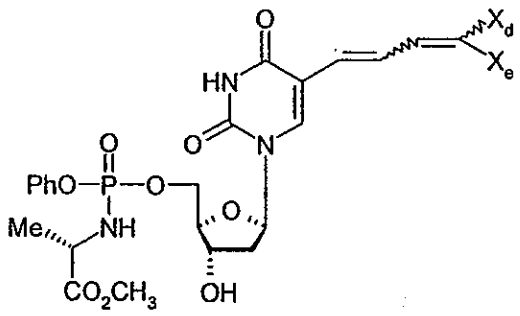


and

each $R^6$ is independently -H, -OH, $-OC(=O)CH_3$, or F; and,

$R^7$ is -H, a phosphate group, a phosphodiester group, a phosphoramidate group derived from an amino acid, or a phosphoramidate group attached to Q at the 5' position;

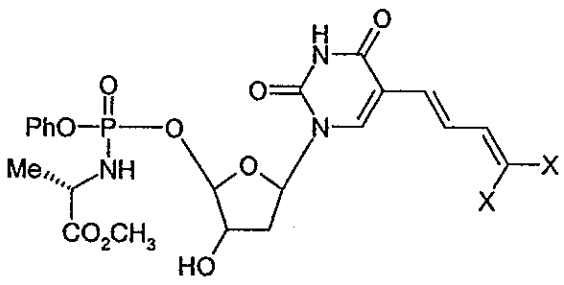
(ii) compounds having the structure:



wherein:

$X_d$ and $X_e$ are independently the same or different and are selected from the group consisting of Cl, Br, I, and CN;

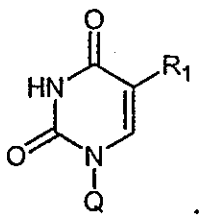
(iii) compounds having the structure:



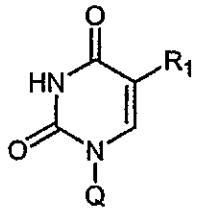

wherein each X is selected from Cl, Br, I, and CN;

wherein the compound may be in the form of a pharmaceutically acceptable salt or ester or any enantiomeric, diasteriomeric, or stereoisomeric form, including D-form, L-form, α-anomeric form, and β-anomeric form.

2. A compound according to claim 1, wherein the compound has the structure:

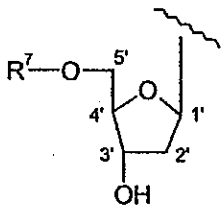


3. A compound according to claim 1 or 2, wherein the compound has the structure:

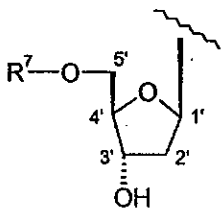



and wherein $R^7$ is a phosphoramidate group derived from the amino acid alanine or the amino acid tryptophane.

4. A compound according to any one of claims 1 to 3, wherein Q is:



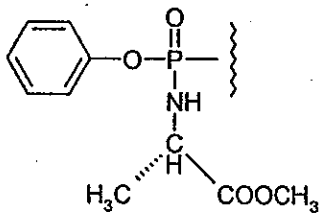

5. A compound according to any one of claims 1 to 3, wherein Q is:



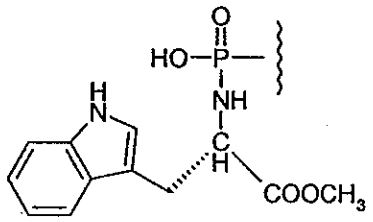

6. A compound according to any one of claims 1 to 5, wherein $R^7$ is -H.

7. A compound according to any one of claims 1 to 7, wherein $R^7$ is a phosphoramidate group derived from an amino acid.

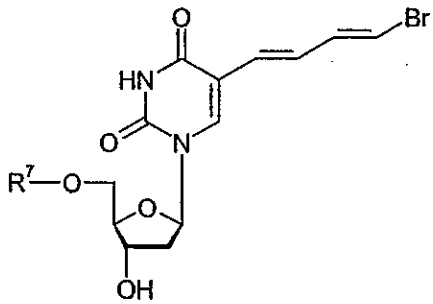
8. A compound according to any one of claims 1 to 5, wherein $R^7$ is:



9. A compound according to any one of claims 1 to 5, wherein $R^7$ is:

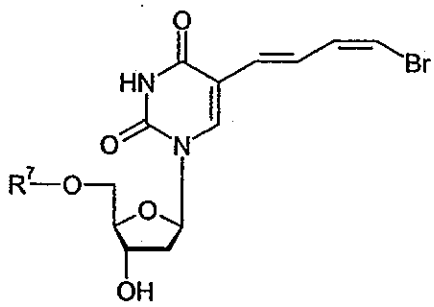


10. A compound according to claim 2, having the structure:



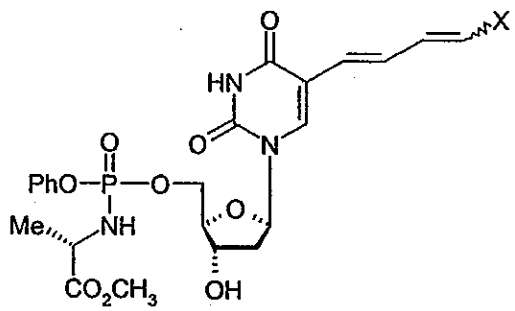
11. A compound according to claim 10, wherein $R^7$ is -H.

12. A compound according to claim 3, having the structure:



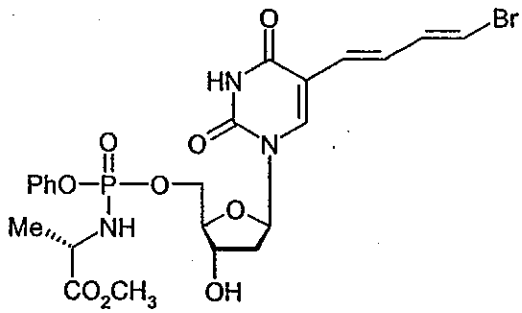

13. A compound according to claim 12, wherein $R^7$ is -H.

14. A compound according to claim 3, having the structure:

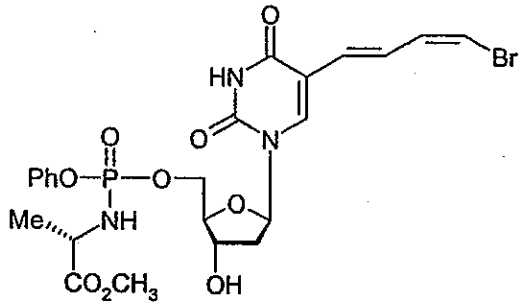



wherein X is selected from Cl and Br.

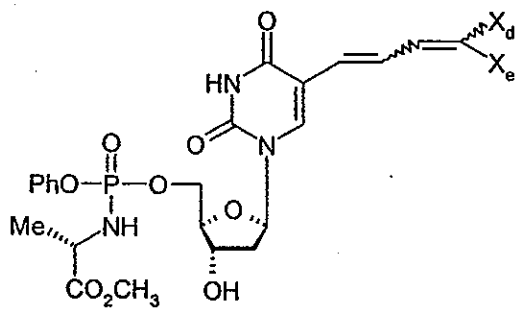
15. A compound according to claim 3, having the structure:



16. A compound according to claim 3, having the structure:



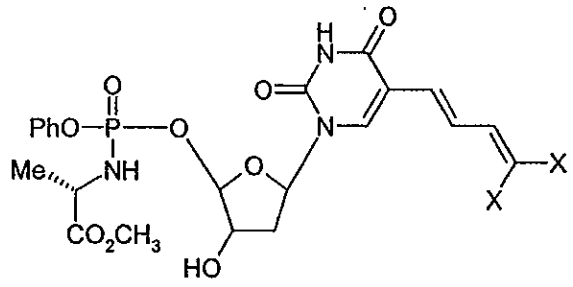

17. A compound according to claim 3, having the structure:




wherein $X_d$ and $X_e$ are independently the same or different and are selected from Cl, Br, I, and CN.

18. A compound according to claim 17, wherein $X_d$ is Cl or Br, and $X_e$ is H.

19. A compound according to claim 3, having the structure:




wherein each X is selected from Cl, Br, I, and CN.

20. A compound according to claim 19, wherein X is Cl or Br.

21. A compound according to claim 19, wherein X is Br.

22. A composition comprising a compound according of any one of claims 1 to 21, and a pharmaceutically acceptable carrier.

23. A composition of claim 21 or 22, further comprising an effective amount of (e)-5-(2-bromovinyl)-2'-deoxy-5-uridyl phenyl L-alaninylphosphoramidate.

24. Use of a substrate prodrug compound of any one of claims 1 to 21 or a metabolite thereof, or a composition of any one of claims 22 or 23, for the preparation of a medicament for selectively inhibiting the proliferation of pathological cells that overexpress an endogenous, intracellular activating enzyme and wherein the enzyme is not inactivated by a substrate prodrug compound.

25. The use of claim 24, wherein the activating enzyme is overexpressed as a result of prior chemotherapy.

26. The use of claim 25, wherein the prior chemotherapy is fluoropyrimidine or Tomudex.

27. The use of claim 24, wherein the pathological cell is a hyperproliferative or neoplastic cell.

28. The use of claim 27, wherein the neoplastic cell is selected from the group consisting of a gastric cancer cell, a breast cancer cell and a colon cancer cell.

29. The use of claim 24, wherein the activating enzyme is thymidylate synthase.

30. The use of claim 24, wherein the activating enzyme is selected from the group consisting of thymidylate synthase, tyrosine kinase or dihydrofolate reductase.

31. The use of claim 24, wherein the selective inhibition is *in vitro*, *ex vivo* and *in vivo*.

32. The use of claim 24, wherein the selective inhibition is *in vivo*.

33. The use of claim 24, wherein the activating enzyme is overexpressed at least 4 fold.

34. The use of claim 24, wherein the medicament is used in combination with an effective amount of a compound that diminishes intracellular thymidine or purine.

35. The use of claim 24, wherein the medicament is used in combination with an effective amount of 6-mercaptopurein, thioguanine, or 2'-deoxycoformycin.

36. The use of claims 34 or 35, wherein the medicaments are used sequentially.

37. The use of claims 34 or 35, wherein the medicaments are used simultaneously.

38. A method for screening for a therapeutic agent, comprising:

(a) contacting a sample containing a target cell with a compound according to any one of claims 1 to 21 or a composition of any one of claims 22 or 23;

(b) contacting a separate sample of the target cell with a potential therapeutic agent; and

(c) comparing the samples for inhibition of cellular proliferation or cell killing.

39. A method according to claim 38, wherein the target cell is characterized as resistant to a chemotherapeutic drug.

40. A method according to claim 38, wherein the target cell is characterized as expressing a target enzyme that is amplified as a result of selection *in vivo* by chemotherapy.

41. A method according to claim 38, wherein the target enzyme is an endogenous intracellular enzyme that is overexpressed in the target cell.