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A Novel Approach to Thymidylate Synthase as a Target for Cancer Chemotherapy

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ABSTRACT

Tumor cell resistance to fluoropyrimidines and other inhibitors of thymidylate synthase (TS) is a serious problem often associated with increased intracellular TS. Clinically, another problem that arises from the use of TS inhibitors is toxicity, which develops, in part, because normal cells may be adversely affected by doses of inhibitor that do not impact tumor cells. To circumvent this problem, we have devised a new strategy called enzyme-catalyzed therapeutic activation (ECTA), which takes advantage of overexpressed TS to enzymatically generate cytotoxic moieties preferentially in tumor cells. We show herein that tumor cells expressing elevated levels of TS are preferentially sensitive to NB1011, a phosphoramidate derivative of (*E*)-5-(2-bromovinyl)-2′-deoxyuridine. We find support for the proposed mechanism of NB1011 in the following results: 1) positive relationship between TS protein level and sensitivity to NB1011 in engineered HT1080 tumor cells, designed to express defined levels of TS protein; 2) NB1011 activity is enhanced on tumor cells which express endogenous elevated TS; 3) cytotoxicity of NB1011 is blocked by raltitrexed (Tomudex); 4) NB1011 selection of TS-overexpressing MCF7TDX tumor cells results in recovery of cell populations and clones with diminished TS levels (and restored sensitivity to raltitrexed). A preliminary comparison of TS mRNA levels in multiple normal tissues versus colon tumor samples suggests that selective tumor cytotoxicity of NB1011 may be possible in the clinical setting. Because NB1011 cytotoxicity is dependent upon activation by TS, its proposed mechanism of action is distinct from current TS-targeted drugs, which require inhibition of TS to be effective.

Thymidylate synthase (TS) is a key enzyme in the de novo synthesis of dTMP. Its structure and mechanism of action are well characterized (Carreras and Santi, 1995). One of the first rational approaches to pharmacological treatment of cancer was based upon fluoropyrimidine inhibitors of this enzyme (Heidelberger, 1957). There are several reasons why efforts to improve upon these initial efforts by devising more effective inhibitors have met with limited success. First among these is that increased expression of TS is common in cancer, resulting from lost tumor suppressor function (Bannerjee et al., 1998). This results in lower sensitivity of tumor cells to inhibitors compared with most healthy cells (Almasan et al., 1995; Li et al., 1995; Gorlick and Bertino, 1999). The genetic plasticity of tumor cells that have lost p53 function allows for genomic rearrangements and gene amplification, probably contributing to the malignant phenotype (Wahl et al., 1997; Agarwal et al., 1998). Data from tumor cells in vitro, and in vivo data from patient samples, show that exposure to TS inhibitors can lead to TS gene amplification and increased levels of TS protein (Copur et al., 1995; Lonn et al., 1996). Higher levels of TS protein in tumor cells correlates with lack of clinical response to fluoropyrimidines and more rapidly progressing disease (Johnston et al., 1995; Bathe et al., 1999).

Many variations have been made to the original fluoropyrimidine chemotherapeutic agent, including alterations in the pyrimidine ring and sugar moieties (Collins et al., 1999; Hughes and Calvert, 1999). One family of compounds, the halogen-substituted 5-vinyllic deoxyuridines, has been studied extensively as antiviral agents (De Clercq, 1997). These compounds have been characterized as requiring herpesvirus-encoded thymidine kinase for monophosphorylation in human cells; they are proposed to then react with TS, resulting in active site modification and inactivation of the enzyme (Balzarini et al., 1987). Despite this, it has been demonstrated that in cell-free conditions, with high concentrations of reducing agent, that *Lactobacillus casei* TS was capable of using (*E*)-5-(2-bromovinyl)-2-deoxyuridylate (BVdUMP) as a substrate, converting it into 5-(2–2-hydroxyethyl)thioethyldeoxyuridylate derivatives (Barr et al., 1983). Recent data relating to the structure of the human TS active site suggested that it may be significantly different from the bacterial enzyme (Schiffer et al., 1995) (P. Sayre and R. Stroud, personal communication, 1998). This new structural infor-

ABBREVIATIONS: TS, thymidylate synthase; BVdUMP, (*E*)-5-(2-bromovinyl)-2-deoxyuridylate; RT, reverse transcription; PCR, polymerase chain reaction; ECTA, enzyme-catalyzed therapeutic activation.

mation has led us to examine the interaction of BVdUMP with recombinant human TS. In addition, we speculated that if BVdUMP could be converted by human TS into cytotoxic metabolites, then the high TS phenotype that characterizes lack of clinical response to fluoropyrimidines could be used as a marker of susceptibility to BVdUMP. Because the charged nature of BVdUMP prevents efficient entry into cells, we prepared a phosphoramidate derivative, NB1011, to facilitate intracellular delivery of BVdUMP. We have shown that NB1011 can enter cells, be converted to the monophosphate and subsequently into cytotoxic products (Lackey et al., in press). We report herein that the cytotoxicity of NB1011 is more pronounced on high TS-expressing tumor cells. The cytotoxic activity of NB1011 is attenuated by inhibitors of TS enzyme activity, a profile opposite that of TS inhibitors. The activity of NB1011 is further distinguished from TS inhibitors by data showing that NB1011 selection of raltitrexed (Tomudex)-resistant breast cancer (high TS) cells results in recovery of clones with diminished TS levels, the result expected if NB1011-treatment selects against the high TS phenotype.

Experimental Procedures

Materials. Expression vector pcDNA3.1(−) was purchased from Invitrogen Inc. (Carlsbad, CA). General cell culture supplies and dialyzed fetal bovine serum were obtained from Life Technologies, Inc. (Grand Island, NY), G418 was obtained from Novagen Inc. (Madison, WI). 5-Fluorouracil was purchased from Sigma (St. Louis, MO). Raltitrexed was obtained from Zeneca Ltd. (Macclesfield, UK).

Synthetic Methodology. Synthesis of (*E*)-5-(2-Bromovinyl)-2′-deoxyuridine and its monophosphate and phosphoramidate derivatives is described by Lackey et al. (in press). Compounds were characterized by NMR and high-performance liquid chromatography analyses and were greater than 95% pure when used for cell-based assays. NB1011 is (*E*)-5-(2-bromovinyl)-2′-deoxyuridine-5′-(*l*-methylalaninyl)-phenylphosphoramidate ($C_{21}H_{24}N_3O_9PBr$).

Cell Culture. Cells were grown in RPMI 1640 medium supplemented with 10% (v/v) fetal bovine serum and the antibiotic/antimycotic Fungizone (Life Technologies) in a final concentration of 100 U/ml of penicillin, 100 μg/ml of streptomycin, and 0.25 μg/ml of amphotericin, in an atmosphere of 5% CO_2. CCD18co, Det551, WI38 cells, and HT1080 fibrosarcoma cell line were obtained from American Type Culture Collection (Manassas, VA). The H630P and H630R10 cell lines (Copur et al., 1995) were provided by Dr. E. Chu (NCI Navy Medical Oncology, Bethesda, MD); MCF7P and MCF7TDX cell lines (Drake et al., 1996) were provided by Dr. P. Johnston (Queens University of Belfast, Northern Ireland). Sublines of MCF7TDX, selected for resistance to NB1011 were obtained by continuous exposure of cells to media supplemented with 50 μM NB1011. The cell lines of CCD18co, H630R10, MCF7TDX, and HT1080 were sent to American Type Culture Collection quarterly for mycoplasma testing to ensure that they were mycoplasma-free.

Qualitative RT-PCR Analysis of TS mRNA in Human Tissues. Transcript levels of human thymidylate synthase in human tissues were quantified by using RT-PCR amplification. Oligonucleotide primers for amplification of the human TS and β-actin were designed as follows: TS forward primer 5′-GGGCAGATCCAACACATCC-3′ (corresponding to bases 208–226 of TS cDNA sequence, Genbank accession no. X02308); reverse primer 5′-GGTCAACTCCCTGTCCTGAA-3′ (corresponding to bases 564–583); β-actin forward primer 5′-GCCAACACAGTGCTGTCTG-3′ (corresponding to bases 2643–2661 of β-actin gene sequence, Genbank accession no. M10277); and reverse primer 5′-CTCCTGCTTGCTGATCCAC-3′ (corresponding to bases 2937–2955). 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-base-pair β-actin fragment and cDNA template generates a 210-base-pair product.

To evaluate the increased mRNA level in tumor tissues, human primary colon cancer tissues and matched normal tissues were obtained from Cooperative Human Tissue Network (Western Division, Cleveland, OH). Total RNAs were isolated using Tripure isolation reagent (obtained from Roche Diagnostics, Indianapolis, IN), following the manufacturer's protocol. Reverse transcriptions were performed, using SuperScript preamplification system (Life Technologies, Inc.). Following the manufacturer's protocol, 3 μg of total RNA was applied in a volume of 20 μl of buffer to conduct the reverse transcription reaction. PCR reactions were performed in a volume of 96 μl, containing 5 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 each dNTP, 0.3 μM each TS forward and reverse primer, and 5 U of *Taq* DNA polymerase (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 forward and reverse primers in 4 μl were added to achieve a final concentration of 0.2 μM each, bringing the final reaction volume to 100 μl. PCR reaction was continued to a total of 34 cycles, followed by a 7-min incubation at 72°C.

Transcript levels of thymidylate synthase in human normal tissues were investigated by qualitative RT-PCR amplification. Panels of cDNAs of human tissues were obtained from OriGene Technologics, Inc. (Rockville, MD). The mixtures of RNAs isolated from many specimens were used to generate cDNAs. PCR reactions were performed in a volume of 25 μl, containing cDNA (100×), 3 mM $MgCl_2$, 50 mM KCl, 20 mM Tris-Cl, pH 8.4, 0.2 mM each dNTP, 0.2 μM each TS forward and reverse primer, and 1.25 U of *Taq* DNA polymerase (Promega). The reaction mixtures were incubated at 94°C for 2 min, followed by 12 cycles of 40-s incubations at 94°C, 1-min incubation at 58°C, and then 1-min incubation at 72°C. A 25-μl reaction buffer containing 0.2 μM each β-actin forward and reverse primer, 0.2 μM each TS forward and reverse primer, 3 mM $MgCl_2$, 50 mM KCl, 20 mM Tris-HCl, pH 8.4, 0.2 mM each dNTP, and 1.25 units of *Taq* DNA polymerase were added to achieve a final concentration of 0.2 μM thymidylate synthase primers and 0.1 μM β-actin primers, bringing the reaction volume to 50 μl. PCR reaction was continued to a total of 36 cycles, followed by a 7-min incubation at 72°C.

Ten microliters of PCR products were resolved by electrophoresis in a 2% agarose gel, followed by staining with SYBR Gold nucleic acid gel stain (Molecular Probes, Eugene, OR). Quantification results indicated that amplification of TS and β-actin was linear between cycles 30 and 36. The DNA bands corresponding to TS were quantified and normalized to that of β-actin by Molecular Dynamics Storm (Sunnyvale, CA). The quantified expression levels were expressed as values of ratio between TS and β-actin.

Western Blot Analysis. Human normal and cancer cells were grown in 100-mm culture dishes with RPMI 1640 medium supplemented with 10% fetal bovine serum. Lysis was in 0.5 ml of radioimmunoprecipitation assay buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5% Triton X-100, 0.1% SDS, 0.5% deoxycholic acid, sodium salt, and protease inhibitors). Protein concentrations were determined with the use of the bicinchoninic acid-200 protein assay kit (Pierce, Rockford, IL). Total protein (15 μg) from each cell type was resolved by 12% SDS-PAGE followed by immunoblotting with human thymidylate synthase monoclonal primary antibody TS-106 (manufactured by NeoMarkers, Fremont, CA) and horseradish peroxidase-linked sheep anti-mouse Ig secondary antibody (Amersham Pharmacia Biotech, Piscataway, NJ). The enhanced chemiluminescence plus kit (Amersham) was used for detection of immunoreactivity. The bands corresponding to thymidylate synthase were quantified and normalized to that of tubulin by imaging (Molecular Dynamics Storm). The quantified expression levels were expressed as values relative to that of cell strain CCD18co.

Construction of TS Mammalian Expression Vector. The 5′ base pairs of TS cDNA was modified by decreasing the GC content without changing the amino acids they encoded, and additional DNA fragment was introduced to encode six histidines tagged to the N terminus of TS. The cDNA was subcloned into *Xho*I and *Hin*dIII sites of mammalian expression vector pcDNA3.1(−). The cDNA insert was confirmed by DNA sequencing.

Cell Transfection. HT1080 cells were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum, and transfected with TS expression vector. Forty-eight hours later, transfected cells were trypsinized and replated in culture medium containing 750 μg/ml G418. After selection with G418 for 2 weeks, surviving cells were cloned. Clones with different TS levels were selected based on Western blot analysis and expanded into cell lines. The stable HT1080 cells transfected with pcDNA3.1(−) only were used as control cells.

Growth Inhibition Studies. Cells growing exponentially were transferred to 384-well, flat-bottomed tissue culture plates. All cell types were plated at a density of 500 cells per well in 25 μL of complete medium. After 24 h, experimental compounds were added in triplicate wells. Drug exposure time was 120 h, after which growth inhibition was assayed using the Alamar blue signal reduction assay (Accu Med International, Inc., Chicago, IL). Correlation between Alamar blue and cell proliferation has been established previously (Goegan et al., 1995). In vitro cytotoxicity results have been confirmed using the crystal violet method (Sugarman et al., 1985; Pegram et al., 1999). Concentration versus relative fluorescence units was plotted, and sigmoid curves were fit using the Hill equation. The IC_{50}, indicated by the inflection point of the curve, is the concentration at which growth is inhibited by 50%. Each cytotoxicity assay was repeated at least three times.

Results

TS Levels in Tumor Samples Are Increased over Normal Tissue. Thymidylate synthase is a key enzyme in the de novo synthesis of dTMP. The elevated level of this enzyme in tumor cells might offer a therapeutic opportunity if it could be used to activate a relatively nontoxic substrate into toxic product(s). To confirm the frequency of overexpression of TS in human colon cancer, we obtained matched normal and tumor samples from the Cooperative Human Tissue Network. These samples were analyzed for TS mRNA level via quantitative RT-PCR, which is reported to give results similar to immunohistochemistry (Johnston et al., 1995). The result of the RT-PCR evaluation of the samples is shown in Fig. 1. 4-fold or more increased expression of TS in tumor was found in five of seven samples (Fig. 1A), and the average mRNA levels in tumor samples is 4.6-fold higher than that in matched normal tissues (Fig. 1B).

The TS expression level in normal human tissues was examined to determine whether some normal tissues may be targets for mechanized toxicity from TS activated agents. The relative TS mRNA levels in 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, and bone marrow tissues were determined by using quantitative RT-PCR. It has been shown that TS mRNA levels in most of these tissues were equal to or less than that in normal colon tissue, except in bone marrow (1.25-fold), ovary (1.38-fold), and testis tissues (2.13-fold) (Fig. 2). However, the average TS mRNA level in colon cancer samples was 4.6-fold greater than that in their matched normal colon tissue samples (Fig. 1).

Design and Synthesis of TS ECTA Prodrugs. Compounds that can be activated by TS for intracellular release of potentially chemotherapeutic cytotoxic agents were designed, synthesized, and characterized (Lackey et al., in press). The lead compound is NB1011, a 5′-phosphoramidate derivative of (*E*)-5-(2-bromovinyl)-2′-deoxyuridine (De Clercq

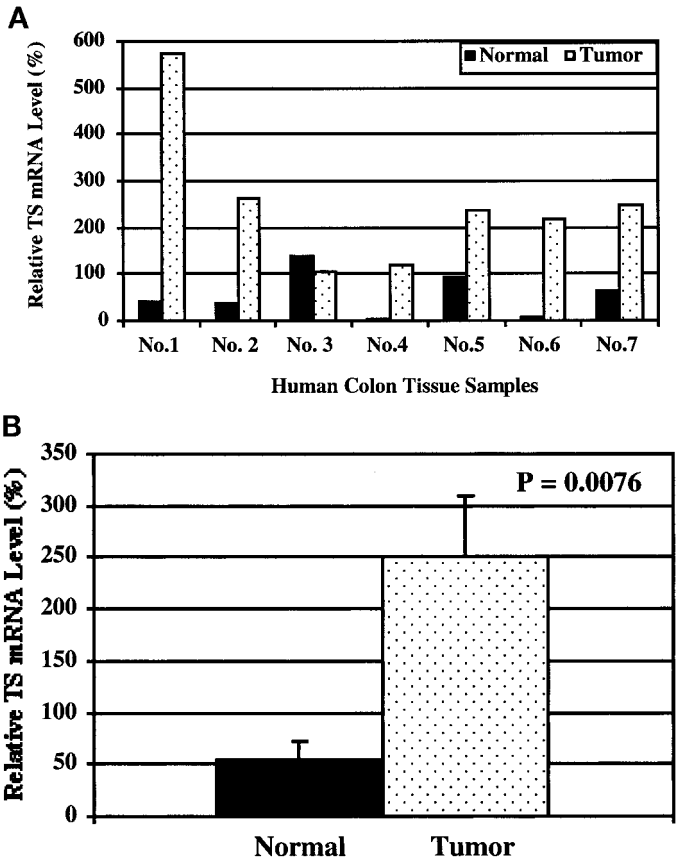

Fig. 1. Relative TS mRNA levels in human colon tumor and matched normal tissues. Total RNAs were isolated from human colon tumor and matched normal tissues. The TS mRNA levels were determined by quantitative RT-PCR. TS levels shown are relative to that of β-actin. A, comparison of TS mRNA level in human colon between tumor and matched normal tissues. B, comparison of average TS mRNA level between tumor and normal tissues (S.E.M. was shown).

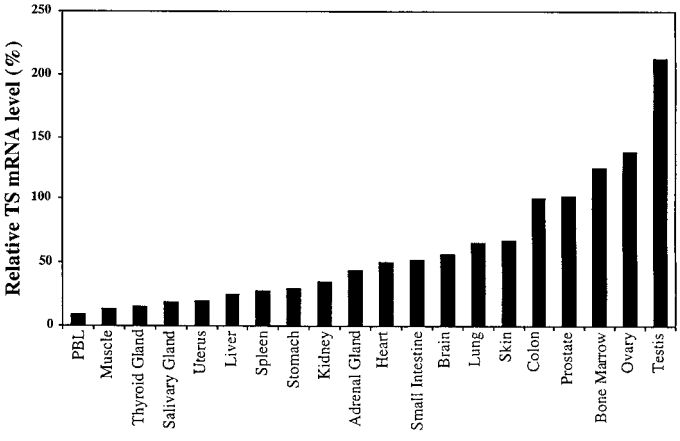

Fig. 2. Relative TS mRNA levels in multiple human tissues. cDNA derived from multiple human tissues were amplified for quantitative PCR analysis. The TS mRNA levels in different tissues were normalized to that of β-actin. The relative levels of TS mRNA are shown by comparison with that of normal colon, which is set at 100%

et al., 1979). Activation of NB1011 requires several steps. These include cell penetration, conversion to the nucleotide monophosphate, binding to TS, and subsequent toxic metabolism.

Increased Overexpression of TS in Engineered HT1080 Tumor Cells Enhances Their Sensitivity to NB1011. TS-dependent cytotoxicity of NB1011 was initially demonstrated by engineering HT1080 fibrosarcoma cells transfected with a plasmid encoding human TS, and subsequently isolating subclones that stably express defined levels of TS protein. Four of the cloned cell lines with TS increases of 2.38-, 2.71-, 3.86-, and 3.92-fold, compared with parental HT1080 cells, were used as models to characterize the biological activity of NB1011. The results of the cytotoxicity assay (Table 1) on these cell lines are particularly significant because they demonstrate, in a fairly uniform genetic background, that increasing TS levels predicts enhanced sensitivity to NB1011 (Spearman correlation coefficient of $R_S = -0.997$) (Fig. 3). This result is consistent with reports in the literature (Copur et al., 1995; Bannerjee et al., 1998; Shibata et al., 1998).

NB1011 Is Active against Tumor Cell Lines with Elevated TS Expression. The relationship between increased TS and increased sensitivity to NB1011 was further tested on normal cell types (CCD18co, WI38, and Det551 fibroblasts) and colon tumor cell lines H630P (colon adenocarcinoma), H630R10 (selected for resistance to 5-fluorouracil), H630TDX (selected for resistance to raltitrexed), MCF7P (breast adenocarcinoma), and MCF7TDX (selected for resistance to raltitrexed). All these cell types were characterized for TS protein levels. As expected, results of cytotoxicity assays (Table 2) indicated that increasing levels of TS protein in clinical drug-resistant cells is also associated with increasing sensitivity to NB1011 (Spearman correlation coefficient of $R_S = -0.78$).

Although tumor cells, especially drug-resistant tumor cells that express higher levels of TS, are usually more sensitive to NB1011, there is no linear relationship between TS level and sensitivity. This may be because of differences in permeability to NB1011 (Bannerjee et al., 1998), variability with respect to intracellular conversion of the phosphoramidate to the monophosphate (McGuigan et al., 1996), efficiency of nucleoside salvage pathways (Madec et al., 1988; Munch-Petersen, 1990), or other differences among cell types (Drake et al., 1996; Samsonoff et al., 1997).

Inhibition of NB1011 Activity by TS Inhibitor. Additional cell culture and genetic selection experiments also support the proposed mechanism of action of NB1011. If TS is an important intracellular target for NB1011, then inhibition of NB1011-mediated cytotoxicity was expected in the presence of increasing concentrations of raltitrexed, a direct inhibitor of TS enzyme activity (Danenberg et al., 1999). More than 80% inhibition of MCF7TDX cellular proliferation was observed when tumor cells were incubated in the presence of NB1011 alone (x-axis, Fig. 4A), whereas no inhibition was observed with raltitrexed alone at concentrations up to 10 μM (Z-axis). The surface features of Fig. 4A also show that increasing raltitrexed concentration antagonizes NB1011 cytotoxicity more than 50-fold (bold yellow, Fig. 4, A and B). Similar results have been obtained using 5-fluorodeoxyuridine as an antagonist for NB1011 (C. Boyer, Q. Li, H. M. Shepard, unpublished observations). Another approach to mechanism of NB1011 is defined by thymidine supplementation. Thymidine antagonizes the antiproliferative activity of TS inhibitors by providing a precursor for dTMP that can be used by cellular salvage pathways (Schultz et al., 1999). Assays carried out with CCD18co, a normal colon epithelial cell type, in dialyzed media ± 10 μM thymidine showed a thymidine rescue from raltitrexed of 15-fold (IC_{50} value changed from 6.5 nM to 95 nM), a rescue from 5-fluoro-2′-deoxyuridine greater than 590-fold (IC_{50} value increased

TABLE 1

NB1011 and raltitrexed sensitivity of TS-transfected HT1080 tumor cell lines

TS levels were determined by using Western blot analysis; the quantified expression levels were given as values relative to that of cell strain CCD18co (as values of 100). Values are expressed as mean ± S.E.M.

Cells	TS Level	IC_{50}	
		NB1011	Raltitrexed
		μM	nM
HT1080	173 ± 20	393 ± 62	73
HT1080/TS1	411 ± 29	258 ± 12	>1000
HT1080/TS2	468 ± 23	198 ± 73	>1000
HT1080/TS3	667 ± 4	62 ± 41	>1000
HT1080/TS4	678 ± 79	65 ± 16	>1000

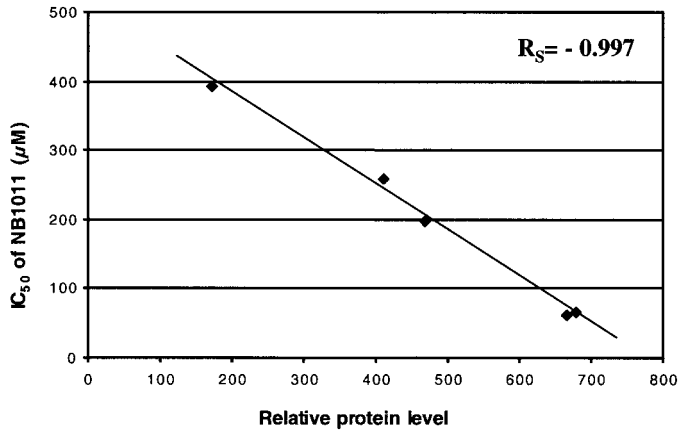

Fig. 3. The linear relation between IC_{50} of NB2001 and TS protein levels in engineered HT1080 cells. HT1080 cells were transfected with TS mammalian expression vector. Clones with different TS levels were selected based on Western blot analysis. The correlation coefficient between IC_{50} of NB2001 and TS protein levels of these clones (Table 1) were shown on the figure.

TABLE 2

NB1011 or raltitrexed cytotoxicity and level of TS expression

TS levels were determined by using Western blot analysis; the quantified expression levels were given as values relative to that of cell strain CCD18co (as values of 100). Values are given as mean ± S.E.M.

Cell type	TS protein level	IC_{50}[a]	
		NB1011	Raltitrexed
		μM	nM
CCD18co	100 ± 22	453 ± 99	20.2 ± 4.6
WI38	150 ± 20	335 ± 5	2.6 ± 0.3
Det551	177 ± 24	262 ± 44	2.6 ± 0.3
H630P	287 ± 35	193 ± 21	6.2 ± 1.2
H630TDX	671 ± 16	0.2 ± 0.1	>1000
H630R10	2405 ± 324	0.5 ± 0.3	330 ± 172
MCF7P	178 ± 45	205 ± 4	1.8 ± 0.1
MCF7TDX	1865 ± 78	0.9 ± 0.5	>1000
MCF7TDX/NB1011[b]	576 ± 54	278 ± 104	21 ± 15

[a] Dialyzed FBS was used.

[b] MCF7/TDX/NB1011 is an uncloned cell population selected from MCF7TDX in the presence of 50 μM NB1011.

from less than 0.01 μM to $>$ 5.9 μM), and little change in NB1011-mediated cytotoxicity (IC_{50} value decreased from 307 μM to 223 μM). These results suggest that full expression of NB1011 cytotoxicity requires TS activity.

Decreasing TS Level in Cells Selected for Resistance to NB1011. The data described above suggest that NB1011 must work, at least in part, via TS-mediated activation. If this is the case, then exposure of high TS expressing MCF7TDX cells to NB1011 should select for variants that express diminished TS activity. Cultures of MCF7TDX were maintained either in 50 μM NB1011 or without selection to control for possible change in TS phenotype in the absence of raltitrexed. A mixed population of NB1011-resistant tumor cells was recovered from this selection, as well as five independent clonal derivatives. Western blot analyses showed that both the population and the independent clones were characterized by diminished TS protein levels (Fig. 5). Because each of the five NB1011-resistant MCF7TDX cell clones share the property of diminished TS expression, it can be concluded that TS must be a critical intracellular target for NB1011. Importantly, the NB1011-selected cells demonstrated renewed sensitivity to raltitrexed (Table 2).

Discussion

We have combined recent structural data for human thymidylate synthase with known characteristics of its substrates and inhibitors to design compounds that are converted by the enzyme from relatively inactive prodrugs to active cytotoxic compounds. A prodrug activated in this way could provide a useful new mechanism of action for an anticancer agent because it would use an enzyme expressed at elevated levels in most cancers, especially in cancers pretreated with TS inhibitors, to preferentially generate cytotoxic compounds in tumor cells. The compound described here is NB1011, a phosphoramidate derivative of (*E*)-5-(2-bromovinyl)-2-deoxyuridylate (BVdUMP).

Enzyme catalyzed therapeutic activation (ECTA) is a novel prodrug strategy, the efficacy of which depends upon intracellular enzyme activation of the ECTA compound. The enzyme targets are chosen because they are well characterized, expressed at higher levels in a majority of cancers, and the overexpression can be related to a fundamental characteristic of the disease. This approach avoids many of the pitfalls of earlier prodrug strategies (Connors and Knox, 1995; Dubowchik and Walker, 1999).

The current understanding of thymidylate synthase and related biochemical pathways has been driven by the discovery and development of the first rationally designed cancer therapeutic, 5-fluorouracil (Heidelberger, 1957). Many variations of 5-fluorouracil are used clinically and the fluoropyrimidines as a class are important in the treatment of gastrointestinal and breast malignancies (Danenberg et al., 1999). Fluoropyrimidines have multiple intracellular targets, including nucleic acid metabolism and inhibition of thymidylate synthase. Folate analog inhibitors of thymidylate synthase, such as raltitrexed, have been developed more recently (Jackman et al., 1991; Danenberg et al., 1999). These chemotherapeutics share thymidylate synthase as a common target and overexpression of thymidylate synthase as a common resistance mechanism (Copur et al., 1995; Freemantle et al., 1995; Farrugia et al., 1998).

The expression of thymidylate synthase is subject to complex regulation, including transcriptional modulation by tumor suppressor elements (Bannerjee et al., 1998). The enzyme is often expressed at elevated levels in tumor cells, probably as a result of tumor suppressor loss of function, gene amplification, and other mechanisms (Copur et al., 1995; Li et al., 1995; Kitchens et al., 1999). The higher level of expression of thymidylate synthase in cancer cells is associated with lack of response to TS inhibitors and more aggressive disease (Johnston et al., 1995; Bathe et al., 1999). These properties define thymidylate synthase as a critical enzyme in the molecular pathogenesis of cancer, and provide

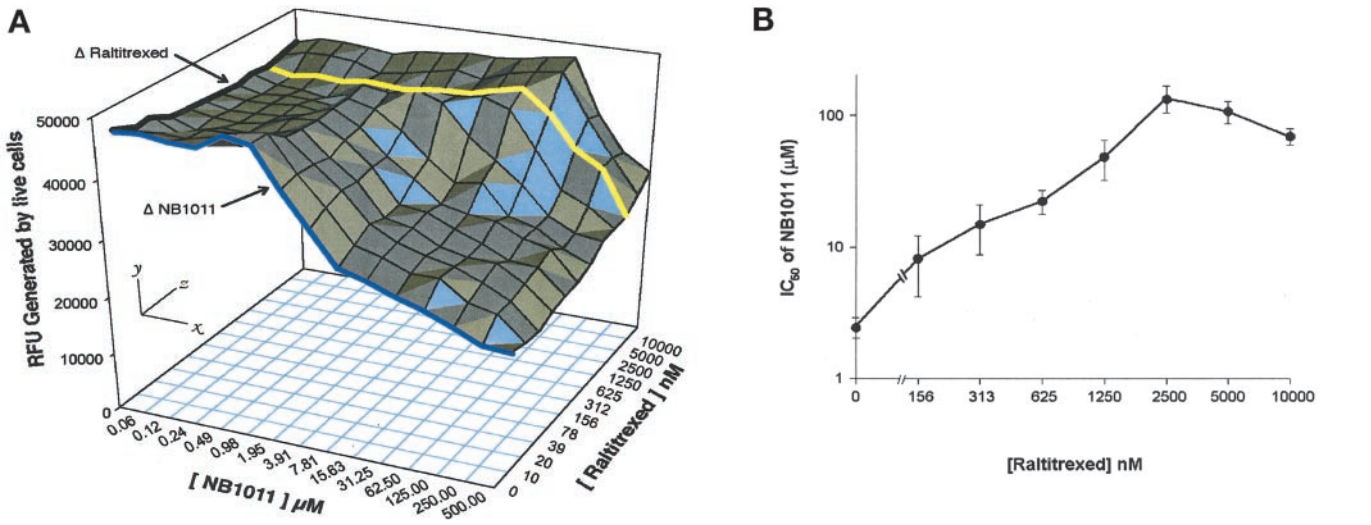

Fig. 4. Cytotoxicity of NB1011 and raltitrexed in MCF7TDX breast cancer cells in vitro. A, response surface of Alamar blue-generated fluorescence by MCF7TDX cells after treatment with NB1011 and raltitrexed in multiple dose combinations. The bold blue line (*x*-*y* plane) represents the Hill equation curve fit for NB1011 alone (IC_{50} = 2.5 μM). The bold black line (*y*-*z* plane) represents the flat dose-response curve for raltitrexed alone (IC_{50} > 10,000 nM). The bold yellow line (*x*-*y* parallel plane) represents the dose-response curve for NB1011 at a constant 2,500 nM raltitrexed concentration (IC_{50} = 135 μM). B, raltitrexed antagonizes NB1011-mediated cytotoxicity. Cytotoxicity of NB1011 as a function of increasing raltitrexed concentrations.

the rationale for its use as a prototype target for development of ECTA.

Our results show that cytotoxicity of NB1011 is related to the level of expression of human thymidylate synthase, a sensitivity profile opposite to that of the TS inhibitors (Copur et al., 1995; Drake et al., 1996; Rooney et al., 1998). This result is surprising because it has been reported previously that BVdUMP, an important cellular metabolite of NB1011, interacted with and inactivated thymidylate synthase (Balzarini et al., 1987), a result that would have given a cytotoxicity pattern similar to that of the TS inhibitory chemotherapeutics. We do not have a ready explanation for the differences between the earlier observations and those reported here. However, Barr et al. (1983) showed that *L. casei* thymidylate synthase could convert BVdUMP into products similar to those we have observed in cells treated with NB1011 (Lackey et al., in press) We have recently synthesized one proposed product, and shown that it has nonspecific cytotoxicity to both normal and tumor cells (M. F. Chan and R. Castillo, unpublished observations), as expected for toxic nucleotide products of NB1011 metabolism. The thymidylate synthase-mediated mechanism of NB1011 cytotoxicity that we propose is supported by two additional lines of evidence. First, we have shown that NB1011 anticellular activity is attenuated by raltitrexed, a specific inhibitor of thymidylate synthase. Second, NB1011 selection of high thymidylate synthase expressing breast tumor cells, MCF7TDX, results in recovery of cell populations and clones characterized by diminished TS expression. A likely explanation of this result is that thymidylate synthase converts an intracellular metabolite of NB1011 into a cytotoxic moiety and that cells that express high levels of the enzyme are selected against when it is present in culture media. An important additional characteristic of these NB1011-selected MCF7TDX tumor cells is their re-established sensitivity to raltitrexed. This sensitivity-resistance-sensitivity cycle could allow for improved management of malignancy if it can be shown to work in vivo.

These results describe the ECTA approach to chemotherapy. This approach is based upon activation of prodrug compounds by intracellular enzymes related to drug resistance. The prototype compound described in this report is preferentially toxic to tumor cells expressing elevated levels of thymidylate synthase. Because NB1011 provides the possibility of a mechanism of action distinct from commonly used chemotherapeutic agents, it may represent an important new lead in the discovery of cancer chemotherapeutic agents with minimal toxicity to healthy cells and efficacy against malignancy.

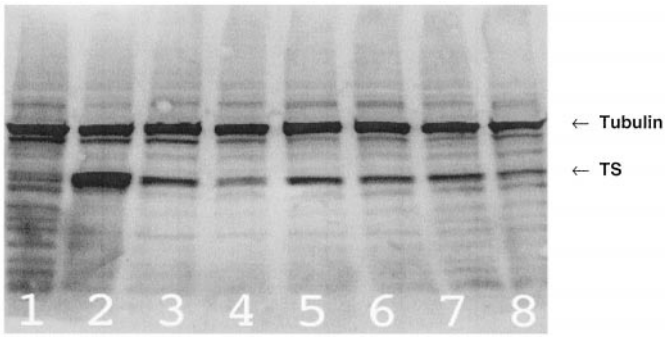

Fig. 5. MCF7TDX selection with NB1011 results in recovery of cells with diminished TS levels. Upper arrow indicates tubulin marker; lower arrow indicates thymidylate synthase. Lane 1, MCF7 parent cell line; lane 2, Cells maintained throughout the selection period, but without added drug; lane 3, NB1011-resistant MCF7TDX (uncloned); lanes 4 to 8, Independent clones of NB1011-resistant MCF7TDX.

References

Agarwal ML, Taylor WR, Chernov MV, Chernova OB and Stark GR (1998) The p53 network. *J Biol Chem* **273:**1–4.

Almasan A, Yin Y, Kelly RE, Lee Kelly, Bradley A, Li W, Bertino JR and Wahl GM (1995) Deficiency of retinoblastoma protein leads to inappropriate S-phase entry, activation of E2F-responsive genes, and apoptosis. *Proc Natl Acad Sci U S A* **92:**5436–5440.

Balzarini J, De Clercq E, Verbruggen A, Ayusawa D, Shimizu K and Seno T (1987) Thymidylate synthase is the principal target enzyme for the cytostatic activity of (*E*)-5-(2-bromovinyl)-2′-deoxyuridine against murine mammary carcinoma (FM3A) cells transformed with the herpes simplex virus type 1 or type 2 thymidine kinase gene. *Mol Pharmacol* **32:**410–416.

Bannerjee D, SB, Fu JZ, Adhikari D, Zhao S-C, Bertino JR (1998) Role of E2F-1 in chemosensitivity. *Cancer Res* **58:**4292–4296.

Barr PJ, Oppenheimer NJ and Santi DV (1983) Thymidylate synthetase-catalyzed conversions of E-5-(2-bromovinyl)-2′- deoxyuridylate. *J Biol Chem* **258:**13627–13631.

Bathe OF, Franceschi D, Livingstone AS, Moffat FL, Tian E and Ardalan B (1999) Increased thymidylate synthase gene expression in liver metastases from colorectal carcinoma: Implications for chemotherapeutic options and survival. *Cancer J Sci Am* **5:**34–40.

Carreras CW and Santi DV (1995) The catalytic mechanism and structure of thymidylate synthase. *Annu Rev Biochem* **64:**721–762.

Collins JM, Klecker RW and Katki AG (1999) Suicide prodrugs activated by thymidylate synthase: Rationale for treatment and noninvasive imaging of tumors with deoxyuridine analogues. *Clin Cancer Res* **5:**1976–1981.

Connors TA and Knox RJ (1995) Prodrugs in cancer chemotherapy. *Stem Cells (Dayton)* **13:**501–511.

Copur S, AK, Drake JC, Allegra CJ and Chu E (1995) Thymidylate synthase gene amplification in human colon cancer cell lines resistant to 5-fluorouracil. *Biochem Pharmacol* **49:**1419–1426.

Danenberg PV, Malli H and Swenson S (1999) Thymidylate synthase inhibitors. *Semin Oncol* **26:**621–631.

De Clercq E (1997) In search of a selective antiviral chemotherapy. *Clin Microbiol Rev* **10:**674–693.

De Clercq E, Descamps J, De Somer P, Barr PJ, Jones AS and Walker RT (1979) (E)-5-(2-Bromovinyl)-2′-deoxyuridine: A potent and selective anti- herpes agent. *Proc Natl Acad Sci USA* **76:**2947–2951.

Drake JC, Allegra CJ, Moran RG and Johnston PG (1996) Resistance to tomudex (ZD1694): Multifactorial in human breast and colon carcinoma cell lines. *Biochem Pharmacol* **51:**1349–1355.

Dubowchik GM and Walker MA (1999) Receptor-mediated and enzyme-dependent targeting of cytotoxic anticancer drugs. *Pharmacol Ther* **83:**67–123.

Farrugia DC, Norman AR and Cunningham D (1998) Single agent infusional 5-fluorouracil is not effective second-line therapy after raltitrexed (Tomudex) in advanced colorectal cancer. *Eur J Cancer* **34:**987–991.

Freemantle SJ, Jackman AL, Kelland LR, Calvert AH and Lunec J (1995) Molecular characterization of two cell lines selected for resistance to the folate-based thymidylate inhibitor, ZD1694. *Br J Cancer* **71:**925–930.

Goegan P, Johnson G and Vincent R (1995) Effects of serum protein and colloid on the alamar blue assay in cell cultures. *Toxicol In Vitro* **9:**257–266.

Gorlick R and Bertino JR (1999) Drug resistance in colon cancer. *Semin Oncol* **26:**606–611.

Heidelberger C (1957) Fluorinated pyrimidines and their nucleosides, in *Handbook of Experimental Pharmacology*, pp 193–231, Springer Verlag, New York.

Hughes A and Calvert H (1999) An update on thymidylate synthase inhibitors [editorial]. *Ann Oncol* **10:**1137–1139.

Jackman AL, Taylor GA, Gibson W, Kimbell R, Brown M, Calvert AH, Judson IR, Hughes LR (1991) ICI D1694, a quinazoline antifolate thymidylate synthase inhibitor that is a potent inhibitor of L1210 tumor cell growth in vitro and in vivo: A new agent for clinical study. *Cancer Res* **51:**5579–5586.

Johnston PG, Lenz HJ, Leichman CG, Danenberg KD, Allegra CJ, Danenberg PV and Leichman L (1995) Thymidylate synthase gene and protein expression correlate and are associated with response to 5-fluorouracil in human colorectal and gastric tumors. *Cancer Res* **55:**1407–1412.

Kitchens ME, Forsthoefel AM, Barbour KW, Spencer HT and Berger FG (1999) Mechanisms of acquired resistance to thymidylate synthase inhibitors: The role of enzyme stability. *Mol Pharmacol* **56:**1063–1070.

Lackey D, Groziak M, Sergeeva M, Malgorzata B, Boyer C, Stroud R, Sayre P, Park J, Johnstone P, Slamon D, et al. (2001) Enzyme-catalyzed therapeutic agent design: The anti- tumor ECTA compound NB1011 is activated by thymidylate synthase. *Biochem Pharmacol*, in press.

Li W, Fan J, Hochhauser D, Banerjee D, Zielinski Z, Almasan A, Yin Y, Kelly R, Wahl GM and Bertino JR (1995) Lack of functional retinoblastoma protein mediates increased resistance to antimetabolites in human sarcoma cell lines. *Proc Natl Acad Sci USA* **92:**10436–10440.

Lonn U, Lonn S, Nilsson B and Stenkvist B (1996) Higher frequency of gene amplification in breast cancer patients who received adjuvant chemotherapy. *Cancer* **77:**107–112.

Madec A, Javre JL, Haras D, Samperez S and Jouan P (1988) Some characteristics of fetal and adult isoenzymes of thymidine kinase in human breast cancers. *Bull Cancer* **75:**187–194.

McGuigan C, Cahard D, Sheeka HM, De Clercq E and Balzarini J (1996) Aryl

phosphoramidate derivatives of d4T have improved anti-HIV efficacy in tissue culture and may act by the generation of a novel intracellular metabolite. *J Med Chem* **39:**1748–1753.

Munch-Petersen B (1990) Thymidine kinase in human leukemia—expression of three isoenzyme variants in six patients with chronic myelocytic leukemia. *Leuk Res* **14:**39–45.

Pegram M, Hsu S, Lewis G, Pietras R, Beryt M, Sliwkowski M, Coombs D, Baly D, Kabbinavar F and Slamon D (1999) Inhibitory effects of combinations of HER-2/neu antibody and chemotherapeutic agents used for treatment of human breast cancers. *Oncogene* **18:**2241–2251.

Rooney PH, SD, Marsh S, Johnston PG, Haites NE, Cassidy J, McLeod HL (1998) Comparative genomic hybridization analysis of chromosomal alterations induced by the development of resistance to thymidylate synthase inhibitors. *Cancer Resh* **58:**5042–5045.

Samsonoff WA, Reston J, McKee M, O'Connor B, Galivan J, Maley G, Maley F (1997) Intracellular location of thymidylate synthase and its state of phosphorylation. *J Biol Chem* **272:**13281–13285.

Schiffer CA, Clifton IJ, Davisson VJ, Santi DV and Stroud RM (1995) Crystal structure of human thymidylate synthase: A structural mechanism for guiding substrates into the active site. *Biochemistry* **34:**16279–16287.

Schultz RM, Patel VF, Worzalla JF and Shih C (1999) Role of thymidylate synthase in the antitumor activity of the multitargeted antifolate, LY231514. *Anticancer Res* **19:**437–443.

Shibata J, Aiba K, Shibata H, Minowa S and Horikoshi N (1998) Detection and quantitation of thymidylate synthase mRNA in human colon adenocarcinoma cell line resistant to 5-fluorouracil by competitive PCR. *Anticancer Res* **18:**1457–1463.

Sugarman BJ, AB, Hass PE, Figari IS, Palladino MA, Shepard HM (1985) Recombinant human tumor necrosis factor-a: Effects on proliferation of normal and transformed cells in vitro. *Science (Wash DC)* **230:**943–945.

Wahl GM, Linke SP, Paulson TG and Huang LC (1997) Maintaining genetic stability through TP53 mediated checkpoint control. *Cancer Surv* **29:**183–219.

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