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-deoxyuridylate. 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 MCF7/DTX 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 (Bannetjee 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-hydroxyethyl)thioethyl-2-deoxyuridylate 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-
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-methyllalaninyl)-phenylphosphoramidate (C21H24N3O9PBr).

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% CO2. CCD18co, Det551, WI38 tissues.

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 premultiplication 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 MgCl2, 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 Technologies, 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 MgCl2, 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 MgCl2, 50 mM KCl, 20 mM Tris-Cl, 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 20 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 by using 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 XhoI and HindIII 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 IC₅₀, 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 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 Clerq 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 (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; Bannerejje 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), and 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 mM (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

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### TABLE 1

<table>
<thead>
<tr>
<th>Cells</th>
<th>TS Level</th>
<th>IC$_{50}^{	ext{NB1011}}$</th>
<th>IC$_{50}^{	ext{Raltitrexed}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>µM</td>
<td>nm</td>
<td></td>
</tr>
<tr>
<td>HT1080</td>
<td>173 ± 20</td>
<td>393 ± 62</td>
<td>73</td>
</tr>
<tr>
<td>HT1080/TS1</td>
<td>411 ± 22</td>
<td>258 ± 12</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>HT1080/TS2</td>
<td>468 ± 23</td>
<td>198 ± 73</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>HT1080/TS3</td>
<td>667 ± 4</td>
<td>62 ± 41</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>HT1080/TS4</td>
<td>678 ± 79</td>
<td>65 ± 16</td>
<td>&gt;1000</td>
</tr>
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</table>

**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.

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### TABLE 2

<table>
<thead>
<tr>
<th>Cell type</th>
<th>TS protein level</th>
<th>IC$_{50}^{	ext{NB1011}}$</th>
<th>IC$_{50}^{	ext{Raltitrexed}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>µM</td>
<td>nm</td>
<td></td>
</tr>
<tr>
<td>CCD18co</td>
<td>100 ± 22</td>
<td>453 ± 99</td>
<td>20.2 ± 4.6</td>
</tr>
<tr>
<td>WI38</td>
<td>150 ± 20</td>
<td>335 ± 5</td>
<td>2.6 ± 0.3</td>
</tr>
<tr>
<td>Det551</td>
<td>177 ± 24</td>
<td>262 ± 44</td>
<td>2.6 ± 0.3</td>
</tr>
<tr>
<td>H630P</td>
<td>287 ± 35</td>
<td>193 ± 21</td>
<td>6.2 ± 1.2</td>
</tr>
<tr>
<td>H630TDX</td>
<td>671 ± 16</td>
<td>0.2 ± 0.1</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>H630R10</td>
<td>2405 ± 324</td>
<td>0.5 ± 0.3</td>
<td>330 ± 172</td>
</tr>
<tr>
<td>MCF7P</td>
<td>178 ± 45</td>
<td>205 ± 4</td>
<td>1.8 ± 0.1</td>
</tr>
<tr>
<td>MCF7TDX</td>
<td>1865 ± 78</td>
<td>0.9 ± 0.5</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>MCF7TDX/NB1011$^a$</td>
<td>576 ± 54</td>
<td>278 ± 104</td>
<td>21 ± 15</td>
</tr>
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</table>

$^a$ Dialyzed FBS was used.

$^b$ MCF7TDX/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 (IC₅₀ 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 over-expression 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
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 activated 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 anticonvulsant 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.

References

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.
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.


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