Available online at www.sciencedirect.com
SCIENCE DIRECT®
Biochemical Pharmacology 65 (2003) 823–831
Biochemical Pharmacology
www.elsevier.com/locate/biochempharm

Cellular transformation of the investigational new anticancer drug NB1011, a phosphoramidate of 5-(2-bromovinyl)-2′-deoxyuridine, results in modification of cellular proteins not DNA

Maria V. Sergeeva*, Brian E. Cathers

NewBiotics, Inc., 4939 Directors Place, San Diego, CA 92121, USA

Received 27 December 2001; accepted 29 July 2002

Abstract

NB1011 [*E*-5-(2-bromovinyl)-2′-deoxyuridine-5′-(L-methylalaninyl)-phenylphosphoramidate], a phosphoramidate prodrug of *E*-5-(2-bromovinyl)-2′-deoxyuridine-5′-monophosphate (BVdUMP), is an investigational new anticancer drug. NB1011 targets thymidylate synthase (TS), which catalyzes the transformation of BVdUMP into cytotoxic reaction products. Due to the elevated levels of TS expression in tumor cells compared to normal cells, these cytotoxic products are preferentially generated inside tumor cells, and, as expected, NB1011 is more toxic to cells with higher levels of TS expression. Therefore, NB1011 therapy should kill tumor cells without severely damaging normal cells. Radiolabeled NB1011 was used to determine the intracellular fate of NB1011 reaction products and, possibly, the mechanism of action of this investigational new drug. We found significant incorporation of the radiolabel into cellular macromolecules. In contrast to our expectations that NB1011 product(s) would be incorporated into DNA, we discovered that cellular proteins were the labeled macromolecular fraction. Herein, we report that the intracellular transformation of NB1011 involves formation of the corresponding monophosphate, TS-dependent transformation into highly reactive intermediates, and subsequent incorporation into cellular proteins. TS itself appears to escape irreversible inactivation. Our data suggest that protein modification not DNA incorporation accounts for the therapeutic effect of NB1011. The proposed mechanism is rather unexpected for a nucleotide analogue and could lead to the discovery of new cellular protein targets for future drug design.

Keywords: Cancer; Chemotherapy; Thymidylate synthase; Nucleotide analog; Bromovinyldeoxyuridine (BVdU); Protein modification

1. Introduction

Cancer chemotherapeutics are typically DNA alkylators or inhibitors of specific enzymes critical for cell survival. For example, fluoropyrimidines have been used as chemotherapeutic agents for several decades [1]. Fluoropyrimidines inhibit TS, an enzyme that catalyzes the *de novo* synthesis of thymidine and is therefore crucial for DNA synthesis. With agents such as these, a favorable therapeutic index results from the increased metabolic demand of rapidly proliferating tumors. However, traditional chemotherapeutic agents—including TS inhibitors—also affect normal cells, leading to toxic side-effects. With slow growing, poorly vascularized, or resistant tumors, the therapeutic index for traditional chemotherapeutic agents becomes very low or is often inverted.

There are different mechanisms by which tumor cells become resistant to chemotherapy [2] including expression of P-glycoproteins (multidrug resistant-pump), overexpression of drug-detoxifying enzymes, or, as is commonly seen with TS inhibitors, overexpression of the target enzyme. TS expression in tumor cells is typically higher than in normal cells [3,4], and chemotherapy with TS inhibitors often selects for cancer cells that express even higher levels of the enzyme. As a consequence, it is often impossible to reach an inhibitor concentration high enough to eradicate cancer cells without severely damaging normal cells. For decades, the focus of drug discovery has been directed towards finding more potent TS inhibitors [5]. However, better TS inhibition results in even higher levels of TS

* Corresponding author. Tel.: +1-858-200-1848; fax: +1-858-200-1804.
E-mail address: msergeeva@newbiotics.com (M.V. Sergeeva).

Abbreviations: TS, thymidylate synthase; ECTA, enzyme-catalyzed therapeutic activation; dUMP, 2′-deoxyuridine 5′-monophosphate; BVdUMP, (*E*)-5-(2-bromovinyl)-2′-deoxyuridine 5′-monophosphate; Ala-BVdUMP, (*E*)-5-(2-bromovinyl)-2′-deoxyuridine-5′-(L-alaninyl)-monophosphate; BVdU, (*E*)-5-(2-bromovinyl)-2′-deoxyuridine; GIT, guanidinium isothiocyanate; FBS, fetal bovine serum; TCA, trichloroacetic acid.

0006-2952/03/$ – see front matter © 2003 Elsevier Science Inc. All rights reserved.
doi:10.1016/S0006-2952(02)01649-0

expression in tumor cells [6], which further erodes an already poor therapeutic index. To improve the efficacy of treatment and overcome drug resistance, a novel approach to drug design has been developed recently [7,8]. ECTA exploits the overexpression of TS in tumor cells. TS ECTA compounds are substrates, not inactivators of TS. They are designed to undergo a TS-catalyzed transformation, which generates cytotoxic reaction products. Due to elevated levels of TS in tumor cells relative to normal cells, the cytotoxic reaction products are preferentially generated inside tumor cells, thus achieving a favorable therapeutic index.

The natural substrate of TS is the 5′-monophosphate of dUMP. TS ECTA compounds are designed to mimic dUMP. The monophosphate moiety is important for enzyme binding; therefore, it must be preserved in TS ECTA molecules. Since monophosphates are negatively charged, they are unable to passively diffuse across cell membranes. There are two methods commonly employed for supplying nucleotide monophosphates to cells. One method is delivering the corresponding uncharged nucleoside and relying on intracellular kinases to form monophosphates. However, it is known that substituted uridyl nucleosides are poor substrates for human thymidine kinase [9,10], and the likelihood of successfully supplying the monophosphate via this route is low. An alternative approach for supplying monophosphates to the cell is by using "masked" monophosphates, such as esters or other derivatives that do not have a formal negative charge [11]. Phosphoramidates are examples of "masked" monophophates that are able to passively diffuse through the cell membrane and be transformed into monophosphates by other cellular enzymes, thus bypassing thymidine kinase [12,13].

NB1011 [*E*-5-(2-bromovinyl)-2′-deoxyuridine-5′-(L-methylalaninyl)-phenylphosphoramidate], a TS ECTA compound recently approved for clinical trials, is a phosphoramidate of *E*-5-(2-bromovinyl)-2′-deoxyuridine (Fig. 1). Previously, the cytotoxicity of NB1011 on cell lines with different levels of TS expression was studied [8,14]. Unlike TS inhibitors such as 5-fluorouracil (5-FU) or Tomudex, NB1011 has been demonstrated to be more toxic to cell lines having higher levels of TS expression [8,14]. This direct correlation between NB1011 cytotoxicity and levels of TS expression suggests that TS is involved in the activation of NB1011 and that TS itself is not irreversibly inactivated by BVdUMP. To gain a better understanding of cancer cell biology and to perhaps design still better drugs, it is important to understand the mechanism of action of this new kind of chemotherapeutic.

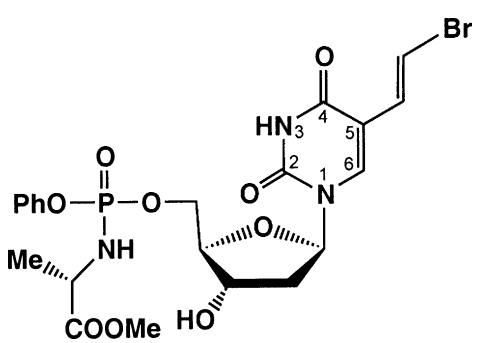

Fig. 1. Structure of NB1011 (Me, methyl; Ph, phenyl).

Herein, we demonstrate by use of a ^{14}C-labeled analogue of NB1011 that the intracellular transformation of NB1011 involves formation of the corresponding monophosphate and subsequent TS-dependent incorporation into the protein fraction of the cell. Our data suggest that modification of an as yet unidentified protein, or proteins, and not incorporation into nucleic acids, as would be expected for a nucleotide analogue, correlates with the therapeutic effect of NB1011.

2. Material and methods

2.1. Chemicals

NB1011 and HPLC standards, BVdUMP and Ala-BVdUMP, were obtained from TriLink, Inc. BVdU was purchased from the Sigma Chemical Co.

NB1011 labeled with ^{14}C in position 2 of the pyrimidine ring ([^{14}C]NB1011) with a specific activity of 50 mCi/mmol was synthesized at TriLink, Inc. using the radiolabeled nucleoside obtained from Moravek Biochemicals. The ^{3}H-labeled compounds [4,5-^{3}H]-L-leucine (specific activity 100 Ci/mmol; [^{3}H]Leu), [5-methyl-^{3}H]thymidine (specific activity 62 Ci/mmol; [^{3}H]Thy), and [5-^{3}H]uridine (specific activity 27 Ci/mmol; [^{3}H]Urd) were from Moravek Biochemicals. The medium for cell growth was obtained from Gibco BRL Life Technologies. Other compounds were purchased from Sigma or VWR and were of the highest purity available.

2.2. Cell lines

MCF7 and SW527, breast adenocarcinoma cell lines, and RKO, a colon carcinoma cell line, were obtained from the ATCC. MCF7TDX, SW527TDX, and RKOTDX cells resistant to 2 μM Tomudex [15] served as a model for high TS expressing human cancer cells. All cells were cultured under conditions of 37°, 95% humidified air, 5% CO_2 in RPMI 1640 culture medium containing 10% FBS (Life Technologies) and 1× penicillin/streptomycin/fungizone™ (Life Technologies). Tomudex-resistant cell lines were maintained continuously in 2 μM Tomudex before NB1011 was added.

2.3. Cell growth experiments and HPLC analysis of the cell lysates

MCF7 and MCF7TDX cells were plated in 100-mm diameter petri dishes (10 mL of RPMI 1640 + 10% FBS + antibiotics, 1 million cells per dish) and allowed

to attach/grow for ~16–40 hr in standard culture conditions. Then either 0–500 μM NB1011 or 30 μM (1.5 μCi/mL) [^{14}C]NB1011 was added, and the cells were incubated for 24–48 hr. The medium was aspirated, cells were rinsed briefly with PBS (twice), 1 mL of PBS per dish was added, and the cells were disrupted by two successive freeze–thaw cycles (−80° to room temperature). Cell debris was removed by centrifugation (16,000 *g*, 10 min at 4°), the supernatant was deproteinized by passing it through a 30,000 molecular weight cut-off membrane filter (Centrifree Centrifugal Filter Devices, Millipore), and the resulting solution was freeze-dried. Solid material was redissolved in 120 μL of water and analyzed using a Hewlett Packard (HP) model 1100 liquid chromatograph with a Photo Diode Array (PDA) or a flow scintillation detector (Packard). A C_{18} column (Adsorbosphere HS, 4.6 × 150 mm, 5 μm, from Alltech) was used with a flow rate of 1 mL/min of 0.025% (v/v) trifluoroacetic acid in HPLC grade water with a gradient of acetonitrile created according to the following scheme: an increase from 0 to 15% over 30 min, a further increase to 40% over the next 15 min, and constant at 40% for 5 min. The limit of detection of BVdU derivatives was 1 μM for UV detection and 0.2 μM for the flow scintillation detector with 100 μL injected.

2.4. Cell growth experiments and enzymatic treatment of the lysates to determine ^{14}C incorporation

Cells were plated in petri dishes as described above. The medium was then replaced by 10 mL of RPMI 1640 + antibiotics (without FBS), and 1.5 μCi/mL of [^{14}C]NB1011 was added. As a control, in separate experiments cells were also treated with the following compounds: 25 μCi/mL of [^{3}H]Leu, 2.5 μCi/mL of [^{3}H]Thy, and 2.5 μCi/mL of [^{3}H]Urd. Cells were incubated in the presence of each of the above compounds for ~3–24 hr. The medium was aspirated, and the cells were rinsed twice with PBS and lysed on a dish using 3 mL of 25 mM AcONa, pH 6, containing 4 M guanidinium isothiocyanate (GIT). Under these denaturing conditions, noncovalent interactions of proteins with small molecules should be disrupted.

From each cell lysate four 50-μL aliquots were removed and diluted 20-fold with TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 8.0). The first aliquot was left untreated. The second aliquot was treated with 500 μg/mL of RNase A for 1 hr at 37°. The third aliquot was treated with 500 μg/mL of DNase I in the presence of 20 mM $MgCl_2$ for 1 hr at 37°. The fourth aliquot was treated with 500 μg/mL of Proteinase K for 2 hr at 60°. After the enzymatic treatment, a TCA stock solution was added to each sample to achieve a final TCA concentration of 10%, and the samples were left on ice for 10 min. The resulting mixtures were filtered through GF/C filters (Whatman). The filters were washed with several milliliters of 95% ethanol and transferred into scintillation vials. To each vial 3 mL of scintillation fluid (ScintiSafe Plus^{TM} 50%, Fisher Scientific) was added, and ^{14}C or ^{3}H counts were determined for each sample using a 1450 Microbeta Trilux scintillation counter (Wallac).

2.5. Cell growth experiments and CsCl equilibrium centrifugation

MCF7 and MCF7TDX cells were grown as described above in the presence of [^{14}C]NB1011 or, as controls, [^{3}H]Leu and [^{3}H]Thy. After 3 hr (for [^{3}H]Thy) or 30 hr (for [^{14}C]NB1011 and [^{3}H]Leu) of incubation with the radiolabeled compounds, cells were lysed with GIT as described in the previous section. The lysates were layered carefully on top of a 7.5-mL CsCl cushion (5.7 M CsCl in 25 mM AcONa) in plastic tubes. The samples were centrifuged using a Beckman SW41 rotor at 130 000 *g* for 21 hr at 20°. After the centrifugation was complete, 500-μL fractions were removed carefully from each tube starting from the top of the gradient. To decrease the concentration of unincorporated radiolabeled compounds, GIT, and CsCl, the buffer in each fraction was exchanged with TE buffer using a Centricon 10,000 molecular weight cut-off membrane filtering device. To the resulting solutions, a TCA stock solution was added to achieve a final TCA concentration of 10%. TCA precipitates were treated as described above, and ^{14}C or ^{3}H counts were obtained for each fraction. Fraction 4 of the MCF7TDX/[^{14}C]NB1011 sample was treated in the following way: half of it was TCA-precipitated following the usual procedure; the other half was first treated with Proteinase K (500 μg/mL at 60° for 2 hr) and then was TCA-precipitated. The ^{14}C counts were obtained for both samples; they were multiplied by two and compared to those of the other fractions.

3. Results

3.1. Intracellular transformation of NB1011 into BVdUMP

We followed the route of intracellular transformation of NB1011 by treating MCF7 and MCF7TDX cells growing *in vitro* with different concentrations of the drug and analyzing the cell lysates for BVdU and other derivatives (see Section 2.3 for details). Using HPLC with UV detection we identified only Compounds I and II and BVdUMP (Fig. 2), consistent with the route described elsewhere for the intracellular processing of phosphoramidates [13]. No low molecular weight compounds downstream of TS were found. Note that the detection limit of the UV detector would have allowed for observation of products formed with at least 1% yield.

Because NB1011 is a deoxyribonucleotide analogue, it was logical to expect that NB1011, or its ultimate product, would be incorporated into DNA and, therefore, not

detected using the above method. We isolated DNA from MCF7 and MCF7TDX cells treated with NB1011, digested the DNA, and analyzed the resulting mixture for the presence of any non-natural nucleosides. In contrast to control experiments using 5-bromodeoxyuridine, no NB1011-related deoxyribonucleosides were found (data not shown). This observation is consistent with results reported by Balzarini *et al.* [16], who was studying BVdU in a herpes thymidine kinase (TK) model system. In the TK model system, BVdU is converted to BVdUMP by the overexpressed herpes TK. Once formed, BVdUMP was expected to impart a cytotoxic effect, like many other nucleotide analogues, through incorporation into DNA. Balzarini and colleagues observed no DNA incorporation of BVdU [16].

To improve the limit of detection for an NB1011 product(s) downstream of TS, a radiolabeled analogue of the drug having ^{14}C in position 2 of the pyrimidine ring was used (see Fig. 1). We chose to label the pyrimidine portion of the molecule and not the sugar to eliminate any radiolabel distribution to natural nucleotides resulting from sugar exchange. MCF7 and MCF7TDX cells were treated with [^{14}C]NB1011, and cell lysates were prepared in the same manner as the lysates of the cells treated with unlabeled NB1011. The cell lysates were analyzed by HPLC fitted with a flow scintillation detector. No radiolabeled compounds, other than the compounds shown in Fig. 2, were detected (detection of radioactivity allowed for the determination of the reaction product formed with at least 0.2% yield).

3.2. Identification of cellular macromolecules modified by an NB1011 reaction product(s) downstream of TS

3.2.1. TCA precipitation and fractionation by selective enzymatic digestion

The use of radiolabeled NB1011 allowed for the sensitive detection of incorporation of the radiolabel into macromolecular cellular components. MCF7 and MCF7TDX cells were treated with [^{14}C]NB1011 as described in Section 2.4. As a control, and in separate experiments, cells were treated with [^{3}H]Leu, [^{3}H]Thy, or [^{3}H]Urd. These compounds are incorporated into proteins, DNA, and RNA, respectively. The lysates were treated with Proteinase K, RNase, or DNase to remove protein, RNA, or DNA associated counts, respectively, from TCA-precipitable material. Experiments with [^{3}H]Leu, [^{3}H]Thy, and [^{3}H]Urd clearly indicated that treatment of lysates with the enzymes described above eliminates counts only in the expected macromolecular fraction (see Fig. 3B). As seen in Fig. 3A, after incubation with [^{14}C]NB1011 for 24 hr, there was significant incorporation of ^{14}C into TCA-precipitable material in the case of MCF7TDX cells (high TS expressor) but not in the case of MCF7 cells. We estimated that in MCF7TDX cells 10% of the ^{14}C label found inside the cells was associated with the macromolecular fraction; of the remaining 90% of the ^{14}C label, 95% was Ala-BVdUMP and the rest was NB1011, BVdUMP, and BVdU. For comparison, after 1 day of incubation of NB1011 in RPMI medium (with 10% FBS added and MCF7TDX cells present) more than 81% of the NB1011 remained. NB1011 metabolites Ala-BVdUMP, BVdUMP, and BVdU were present in the amounts of 12, 1, and 6%, respectively. Treatment with RNase or DNase did not affect the number of counts significantly, suggesting that RNA and DNA were not labeled with ^{14}C during the incubation with [^{14}C]NB1011. Treatment with Proteinase K, however, removed ^{14}C counts almost entirely from TCA-precipitable material. The same effect could be achieved using trypsin (data not shown), but with Proteinase K more complete digestion was accomplished. Fig. 3 is representative of at least four independent experiments. The most likely explanation for this result is that ^{14}C is incorporated into cellular proteins. We have also demonstrated in a pair of colon carcinoma cell lines (RKO/RKOTDX) that in the Tomudex-resistant (high TS expressing) cell line overall radiolabel incorporation after treatment with [^{14}C]NB1011 is higher, and that the radiolabel is incorporated predominantly into the protein fraction of the cell (Fig. 4). The ^{14}C counts from the MCF7TDX sample reflected an average incorporation of 1.2×10^8 molecules of the radioactive nucleotide derivative per cell. This is comparable to the

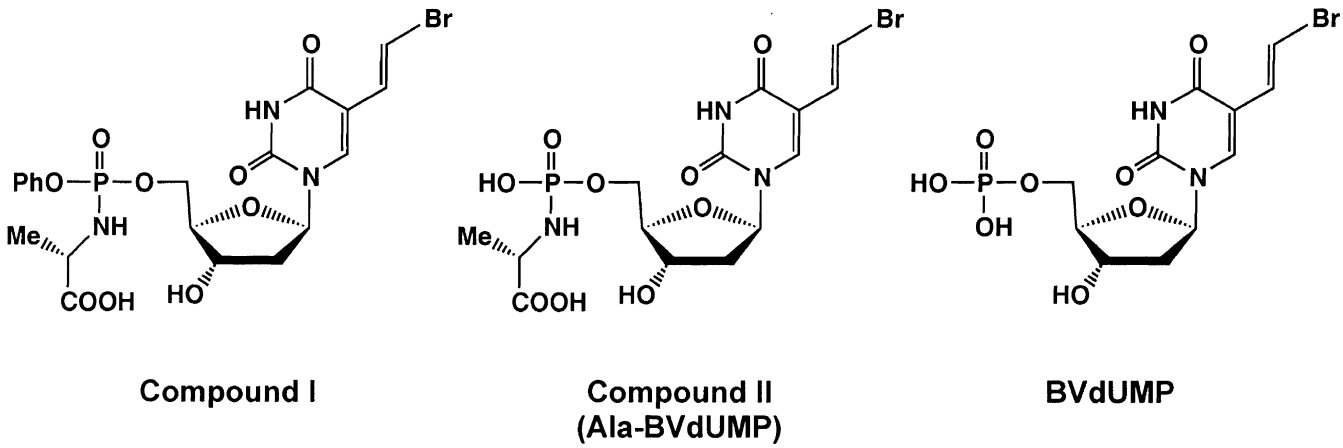

Fig. 2. Intracellular metabolites of NB1011 (Me, methyl; Ph, phenyl).

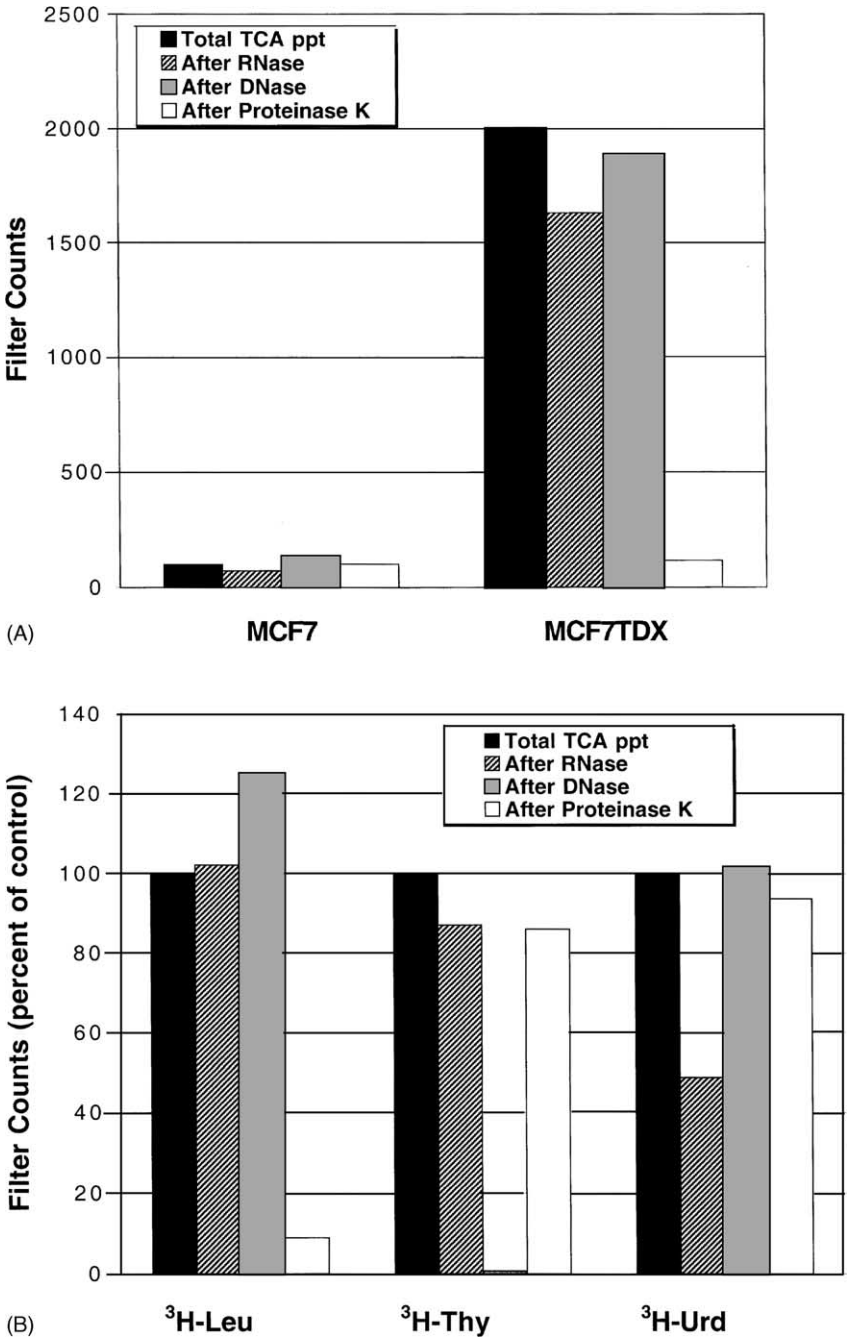

Fig. 3. Enzymatic treatment of cell lysates. (A) MCF7 or MCF7TDX cells treated with [^{14}C]NB1011 (filter counts in dpm normalized to the number of lysed cells); (B) MCF7TDX cells treated with [^{3}H]Leu, [^{3}H]Thy, or [^{3}H]Urd. Lysates of the cells treated with [^{14}C]NB1011 or [^{3}H]Leu, [^{3}H]Thy, or [^{3}H]Urd were prepared. From each lysate four aliquots were removed. The first aliquot was left untreated. TCA precipitation of this sample provides total counts in TCA-precipitable material. Other aliquots were treated with RNase A, DNase I, or Proteinase K. To the resulting solutions TCA was added, the solutions were filtered through GF/C filters, and ^{14}C or ^{3}H counts were determined (see Section 2 for details). The results are representative of at least four independent experiments.

amount of [^{3}H]Leu incorporated after a 24-hr incubation (3.8×10^{8}). However, the concentration of the radiolabeled L-leucine in the medium was 17-fold higher than that of NB1011.

3.2.2. CsCl equilibrium centrifugation

To confirm that [^{14}C]NB1011 treatment results in radiolabeling of cellular proteins, equilibrium centrifugation in CsCl was used. CsCl equilibrium centrifugation allows for the separation of proteins, DNA, and RNA based on their respective densities [17]. After equilibrium is achieved, proteins remain at the top of the CsCl gradient, RNA sinks to the bottom of the gradient, and DNA positions in the middle [17]. Lysates of MCF7 and MCF7TDX cells treated with [^{14}C]NB1011, [^{3}H]Leu, or [^{3}H]Thy were subjected to CsCl equilibrium centrifugation (see Section 2.5). A representative example of at least two experiments is presented in Fig. 5.

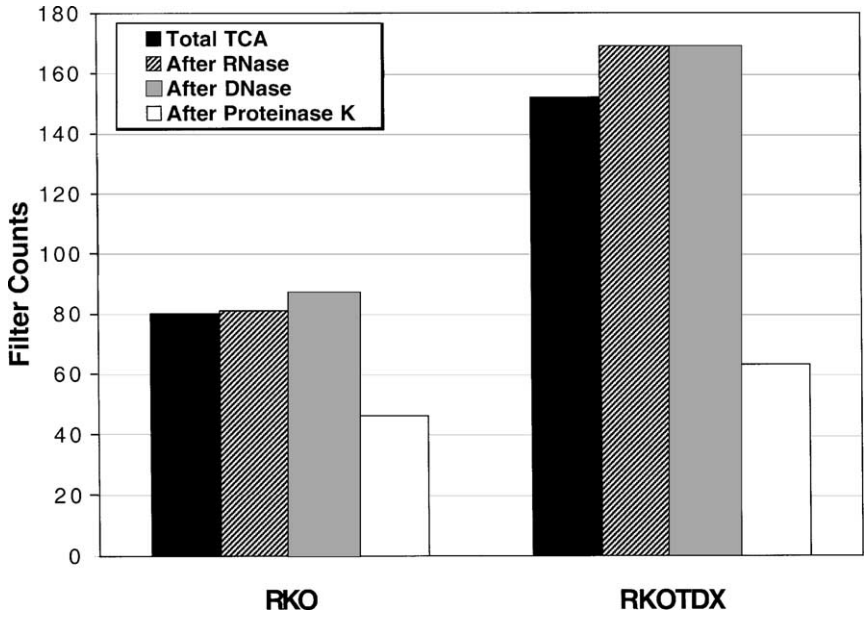

Fig. 4. Enzymatic treatment of cell lysates: RKO or RKOTDX cells treated with [^{14}C]NB1011 (filter counts in dpm normalized to the number of lysed cells). Lysates of the cells treated with [^{14}C]NB1011 were prepared. From each lysate four aliquots were removed. The first aliquot was left untreated. TCA precipitation of this sample provides total counts in TCA-precipitable material. Other aliquots were treated with RNase A, DNase I, or Proteinase K. To the resulting solutions TCA was added, the solutions were filtered through GF/C filters, and ^{14}C counts were determined (see Section 2 for details). The results are averages of two independent experiments.

As seen in Fig. 5B, proteins (exemplified by [^{3}H]Leu incorporation) were present in fractions 1–6, while DNA appeared in fractions 10–15 ([^{3}H]Thy incorporation). In the case of [^{14}C]NB1011-treated MCF7TDX cells (Fig. 5A), the highest counts were recorded in fraction 5 consistent with preferential incorporation of the radiolabel into proteins. To further confirm the identity of this peak, fraction 4 of the [^{14}C]NB1011 sample was treated with Proteinase K as described in Section 2.5. In previous experiments, fraction 4 or 5 of [^{14}C]NB1011-treated samples produced the highest counts. Based on those observations, fraction 4 was selected in the representative example (Fig. 5) for further confirmation of peak identity. In Fig. 5A, ^{14}C counts in fraction 4 after Proteinase K treatment are shown with a closed square. As expected, Proteinase K treatment removed ^{14}C counts from TCA-precipitable material, confirming the identity of the large peak as being protein.

4. Discussion

In cell-free experiments, we have shown that NB1011 and its corresponding nucleoside (BVdU) do not bind to purified human recombinant TS, whereas BVdUMP does and acts as a competitive inhibitor/substrate with respect to the TS-catalyzed transformation of dUMP into dTMP (data not shown). Therefore, before TS can recognize and activate NB1011 inside cells, NB1011 must be transformed into the corresponding monophosphate (BVdUMP). We have demonstrated that in cell cultures NB1011 is indeed transformed into the immediate precursor of BVdUMP, Ala-BVdUMP, and BVdUMP (Fig. 2). Furthermore, consistent with the data of Santi's group for *Lactobacillus casei* TS [18], we have shown that purified human recombinant TS catalyzes the transformation of BVdUMP into a reactive intermediate/product that is modified very rapidly by nucleophiles present in the reaction mixture. For example, in the presence of β-mercaptoethanol, the isolated products contain one or two β-mercaptoethanol molecules [8]. In cells, various other nucleophiles could substitute for β-mercaptoethanol in this reaction.

Since we failed to detect any low molecular weight adducts or non-natural nucleosides related to NB1011, it seemed unlikely that low molecular weight cellular nucleophiles were participating in the reaction or that traditional DNA incorporation of an NB1011-related compound was occurring. However, nucleophilic groups of macromolecules could also react with NB1011 reaction intermediates resulting in incorporation of the radiolabel into high molecular weight subcellular fractions (i.e. DNA, RNA, or proteins). Since our initial methods were designed to monitor small molecules, we could not detect NB1011-modified macromolecules. Therefore, we chose to look for [^{14}C]NB1011 incorporation specifically in the macromolecular fractions of the cell.

Two independent methods, (a) TCA precipitation and fractionation by selective enzymatic digestion (adapted from Herzig *et al.* [19]) and (b) CsCl equilibrium centrifugation, indicate that proteins are the labeled species following treatment of cells with [^{14}C]NB1011. The identity of the labeled fraction in CsCl equilibrium centrifugation as being a protein was determined by (a) comparison

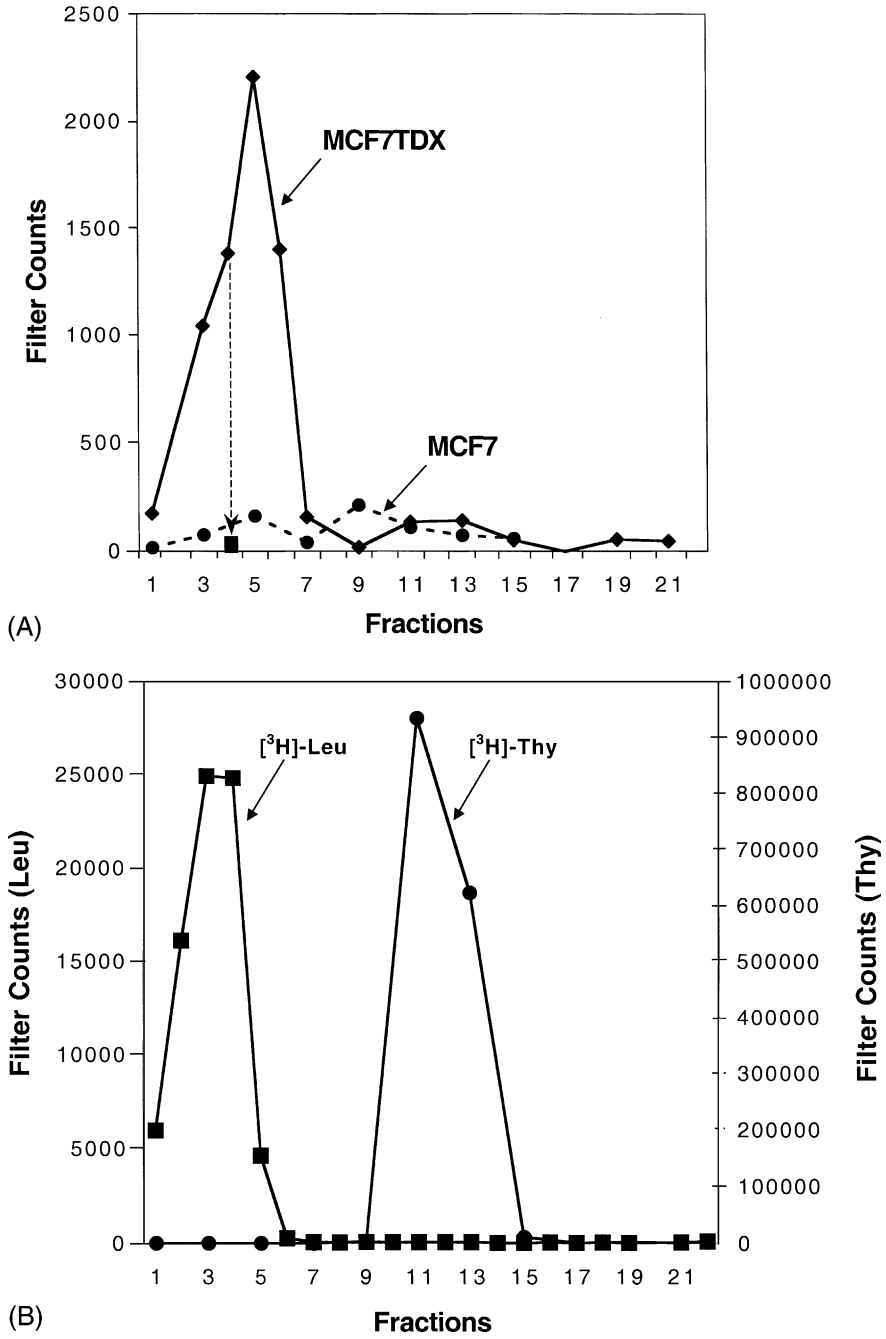

Fig. 5. CsCl centrifugation of cell lysates. (A) MCF7 or MCF7TDX cells treated with [^{14}C]NB1011 (filter counts in dpm in each fraction normalized to the number of lysed cells); (B) MCF7TDX cells treated with ^{3}H-labeled Leu or Thy. MCF7 and MCF7TDX cells were grown in the presence of [^{14}C]NB1011 or, as controls, in the presence of [^{3}H]Leu, or [^{3}H]Thy. The cells were lysed, and the lysates were layered on top of a CsCl cushion in plastic tubes and centrifuged. After the centrifugation was complete, fractions were removed carefully from each tube starting from the top of the mixture. Buffer was exchanged in each fraction, and macromolecules were precipitated from the resulting solutions with TCA. The TCA precipitates were filtered, through GF/C filters, and ^{14}C or ^{3}H counts were obtained for each fraction (see Section 2 for details). The results are representative of at least two independent experiments.

with a [^{3}H]Leu control, and (b) digestion of fraction 4 (Fig. 5) with Proteinase K. It would seem unlikely that metabolic breakdown of NB1011 could account for specific incorporation of the radiolabel into proteins because in the usual route of (deoxy)uridine and/or thymidine catabolism the carbon atom in position 2 of the pyrimidine ring is released in the form of CO_2, and this type of catabolism is thought to occur primarily in liver cells [20]. Further, MCF7TDX cells show much higher incorporation of the radiolabel from [^{14}C]NB1011 compared to the parental MCF7 cell line (Fig. 3A), and MCF7TDX cells differ mainly from MCF7 cells in that they have a much higher level of TS expression. An explanation most consistent with our observations is that a reactive product(s) resulting from TS activation of BVdUMP is responsible for the labeling of cellular proteins. It is important to mention

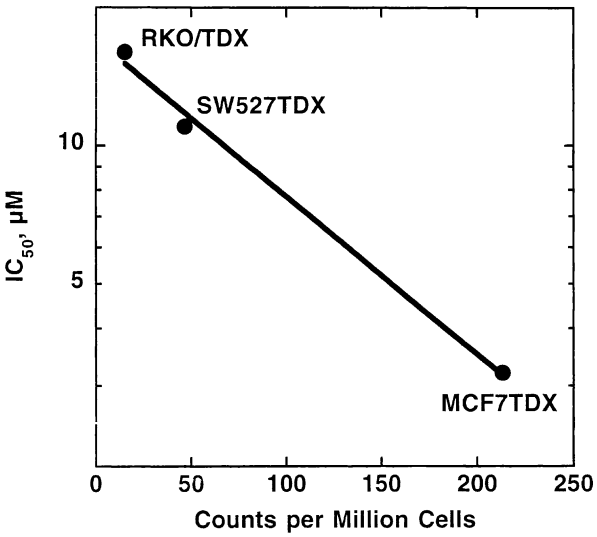

Fig. 6. Relationship of IC_{50} values of NB1011 in different cell lines with the radiolabel incorporation exemplified by the Tomudex selected TS overexpressing cell lines MCF7TDX, SW527TDX, and RKO/TDX.

that the level of incorporation of the radiolabel into the protein fraction of cellular macromolecules inversely correlates with the IC_{50} values of NB1011 in different cell lines. As seen in Fig. 6, lower IC_{50} values for NB1011 are found in Tomudex-selected high TS expressing cell lines that show higher levels of incorporation of the radiolabel. This result is consistent with a link between the modification of intracellular protein(s) and NB1011 efficacy. The logic behind this conclusion is not without precedent. The anticancer drug hydroxymethylacylfulvene (HMAF) has been shown to label proteins [19,21] via the formation of an electrophilic intermediate, which subsequently binds to nucleophilic side-chains of proteins. Furthermore, it was observed that HMAF-mediated protein alkylation is highly toxic to tumor cells [22].

It is important to note that treatment of cells with NB1011 and subsequent protein modification does not result in irreversible TS inactivation. We have shown that intracellular TS activity is transiently decreased in the presence of NB1011, but when NB1011 is removed from the cell culture medium, TS quickly regains its full catalytic activity [8]. In addition, cells do not respond to NB1011 in the same fashion as to TS inhibitors [14]. Cells initially insensitive to NB1011, but sensitive to TS inhibitors, can be made resistant to both 5-FU and Tomudex by culturing in the presence of Tomudex. The resulting resistant cells overexpress TS as compared to the parental cell line and, as expected, become sensitive to NB1011. By growing these cells in the presence of NB1011, a revertant cell line can be generated that expresses TS at a level comparable to that of the parental cell line, and that once again is sensitive to the TS inhibitors and insensitive to NB1011 [14].

The results presented in this manuscript suggest an unusual mechanism of action for a nucleotide-based anticancer drug that involves a TS-dependent modification of cellular proteins. Although the NB1011-labeled protein or proteins have yet to be identified, the data presented in this manuscript are consistent with the following mechanism of NB1011 action: inside cells, NB1011 is transformed into the corresponding monophosphate (BVdUMP); from cell-free BVdUMP studies with TS we infer that the monophosphate is activated by TS giving rise to a reactive intermediate or product; the reactive intermediate or product covalently modifies intracellular proteins, but TS itself appears to escape irreversible inactivation; the extent or amount of protein modification is dependent upon TS concentration or activity and correlates with the *in vitro* therapeutic effect of NB1011. This mechanism is rather unexpected for a nucleotide analogue and could lead to the discovery of new cellular protein targets for future drug design. We are currently attempting to identify the labeled protein targets.

Acknowledgments

We are grateful to Dr. H. Michael Shepard and Dr. Geoffrey M. Wahl for valuable discussion. We thank Ms. Patricia Karjian for providing us with the cell material and Dr. John Kolman for his help with CsCl centrifugation.

References

[1] van Laar JA, Rustum YM, Ackland SP, van Groeningen CJ, Peters GJ. Comparison of 5-fluoro-2′-deoxyuridine with 5-fluorouracil and their role in the treatment of colorectal cancer. Eur J Cancer 1998;34:296–306.
[2] Gorlick R, Bertino JR. Drug resistance in colon cancer. Semin Oncol 1999;26:606–11.
[3] Salonga D, Danenberg KD, Johnson M, Metzger R, Groshen S, Tsao-Wei DD, Lenz HJ, Leichman CG, Leichman L, Diasio RB, Danenberg PV. Colorectal tumors responding to 5-fluorouracil have low gene expression levels of dihydropyrimidine dehydrogenase, thymidylate synthase, and thymidine phosphorylase. Clin Cancer Res 2000;6:1322–7.
[4] Iqbal S, Lenz HJ. Determinants of prognosis and response to therapy in colorectal cancer. Curr Oncol Rep 2001;3:102–8.
[5] Adjei AA. A review of the pharmacology and clinical activity of new chemotherapy agents for the treatment of colorectal cancer. Br J Clin Pharmacol 1999;48:265–77.
[6] Copur S, Aiba K, Drake JC, Allegra CJ, Chu E. Thymidylate synthase gene amplification in human colon cancer cell lines resistant to 5-fluorouracil. Biochem Pharmacol 1995;49:1419–26.
[7] Shepard HM, Enzyme catalyzed therapeutic agents. US Patent No. US6,245,750 (2001).
[8] Lackey DB, Groziak MP, Sergeeva M, Beryt M, Boyer C, Stroud RM, Sayre P, Park JW, Johnston P, Slamon D, Shepard HM, Pegram M. Enzyme-catalyzed therapeutic agent (ECTA) design: activation of the antitumor ECTA compound NB1011 by thymidylate synthase. Biochem Pharmacol 2001;61:179–89.
[9] Bernaerts R, Desgranges C, De Clercq E. (*E*)-5-(2-Bromovinyl)uridine requires phosphorylation by the herpes simplex virus (type 1)-induced thymidine kinase to express its antiviral activity. Biochem Pharmacol 1989;38:1955–19561.

[10] Andrei G, Snoeck R, Reymen D, Liesnard C, Goubau P, Desmyter J, De Clercq E. Comparative activity of selected antiviral compounds against clinical isolates of varicella-zoster virus. Eur J Clin Microbiol Infect Dis 1995;14:318–29.

[11] Balzarini J, Karlsson A, Aquaro S, Perno CF, Cahard D, Naesens L, De Clercq E, McGuigan C. Mechanism of anti-HIV action of masked alaninyl d4T-MP derivatives. Proc Natl Acad Sci USA 1996;93: 7295–9.

[12] McGuigan C, Cahard D, Sheeka HM, De Clercq E, Balzarini J. 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 1996;39:1748–53.

[13] Saboulard D, Naesens L, Cahard D, Salgado A, Pathirana R, Velazquez S, McGuigan C, De Clercq E, Balzarini J. Characterization of the activation pathway of phosphoramidate triester prodrugs of stavudine and zidovudine. Mol Pharmacol 1999;56:693–704.

[14] Li Q, Boyer C, Lee JY, Shepard HM. A novel approach to thymidylate synthase as a target for cancer chemotherapy. Mol Pharmacol 2001; 59:446–52.

[15] Drake JC, Allegra CJ, Moran RG, Johnston PG. Resistance to Tomudex (ZD1694): multifactorial in human breast and colon carcinoma cell lines. Biochem Pharmacol 1996;51:1349–55.

[16] Balzarini J, De Clercq E, Verbruggen A, Ayusawa D, Shimizu K, Seno T. 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 1987;32:410–6.

[17] Sambrook J, Fritsch EF, Maniatis T. Molecular cloning: a laboratory manual. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press; 1989.

[18] Barr PJ, Oppenheimer NJ, Santi DV. Thymidylate synthetase-catalyzed conversions of *E*-5-(2-bromovinyl)-2′-deoxyuridylate. J Biol Chem 1983;258:13627–31.

[19] Herzig MC, Arnett B, MacDonald JR, Woynarowski JM. Drug uptake and cellular targets of hydroxymethylacylfulvene (HMAF). Biochem Pharmacol 1999;58:217–25.

[20] Zubay G. Biochemistry. New York: Macmillan Publishing Company; 1988.

[21] Woynarowski JM, Napier C, Koester SK, Chen SF, Troyer D, Chapman W, MacDonald JR. Effects on DNA integrity and apoptosis induction by a novel antitumor sesquiterpene drug, 6-hydroxymethylacylfulvene (HMAF, MGI 114). Biochem Pharmacol 1997;54: 1181–93.

[22] Woynarowska BA, Woynarowski JM, Herzig MC, Roberts K, Higdon AL, MacDonald JR. Differential cytotoxicity and induction of apoptosis in tumor and normal cells by hydroxymethylacylfulvene (HMAF). Biochem Pharmacol 2000;59:1217–26.