Inhibition of Cell Growth by NB1011 Requires High Thymidylate Synthase Levels and Correlates with p53, p21, Bax, and GADD45 Induction

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Abstract

NB1011, a phosphoramidate derivative of (*E*)-5-(2-bromovinyl)-2′-deoxyuridine, is a novel small molecule anticancer agent. NB1011 is selectively active against tumor cells expressing high levels of thymidylate synthase (TS), a critical enzyme in DNA biosynthesis. NB1011 is different from the current TS-targeted drugs, which require inhibition of TS to be effective, because NB1011 cytotoxicity depends upon activation by TS. Here we report a dose-dependent, antitumor activity of NB1011 against established Tomudex-resistant breast cancer (MCF7TDX) xenografts in athymic mice. Against 5-fluorouracil-resistant colon carcinoma (H630R10) xenografts, NB1011 was as efficacious as irinotecan, a drug recently approved for the treatment of 5-fluorouracil-resistant colon cancer. To gain insight into the mechanisms NB1011 uses to suppress cellular growth, we analyzed the downstream molecular events in the high TS-expressing MCF7TDX and RKOTDX cell lines upon NB1011 treatment. NB1011 treatment increased the mRNA levels of p21, Bax, and GADD45. Furthermore, NB1011 induced p53, p21, and Bax proteins specifically in high TS-expressing tumor cells, whereas no induction was observed in low TS-expressing tumor cells (MCF7) or normal cells (WI38). Cell cycle analysis demonstrated that NB1011 treatment of MCF7TDX and RKOTDX cells resulted in an accumulation of cells in the G_2-M phase of the cell cycle. Altogether, our data indicate that the induction of the p53 target genes *p21*, *bax,* and *GADD45,* with a concomitant deregulation of the cell cycle, may represent one of the mechanisms by which NB1011 exerts its growth-suppressive effects.

Introduction

TS[2] is a key enzyme in the *de novo* synthesis of dTMP and therefore plays a critical role in DNA synthesis and repair. TS catalyzes the reductive methylation of dUMP to dTMP using 5,10-methylenetetrahydrofolate as a cofactor (1). Because TS is an essential enzyme in proliferating cells, it has been an important chemotherapeutic target, and various anticancer agents focus on inhibiting TS function. Many tumor cells display increased levels of TS because of the loss of tumor suppressor gene function such as p53 or Rb. This increase in TS levels renders them less sensitive to TS inhibitors than normal cells (2, 3). Furthermore, *in vitro* data using tumor cell lines as well as analysis of tumor samples from patients have shown that exposure to TS inhibitors, such as 5-FU and Tomudex, can result in even higher intratumoral levels of TS, attributable to gene amplification and increased TS protein expression (4–6). Patients with tumors characterized with high TS expression show a poor response to fluoropyrimidine-based treatments and have poor overall survival (7–9). Drug-induced development of resistant tumor cells has become a major problem in the chemotherapeutic treatment of cancer. NB1011 is designed using an enzyme catalyzed therapeutic activation approach. The enzyme catalyzed therapeutic activation approach takes advantage of enzymes (in this case TS) that are specifically overexpressed in drug-resistant cancer cells by using their catalytic activity to generate cytotoxic products. We have shown previously that NB1011, a phosphoramidate derivative of (*E*)-5-(2-bromovinyl)-2′-deoxyuridine, is preferentially cytotoxic to tumor cells displaying elevated levels of TS, and furthermore, that the cytotoxicity of NB1011 is attenuated by Tomudex, a specific inhibitor of TS activity (10). In addition, we have shown a positive correlation between the intracellular TS protein levels and the sensitivity to NB1011 (10). This cytotoxicity profile of NB1011 is opposite to that of the classical TS inhibitors. These TS-targeted drugs require inhibition of TS to be effective, whereas NB1011 requires TS activity to become maximally cytotoxic. To gain insight into the mechanisms by which NB1011 suppresses cellular growth, we set out to study the downstream molecular events of the cellular response to NB1011. Here we report the effects of NB1011 treatment on gene expression, protein induction, and cell cycle regulation.

Materials and Methods

Cells and Culture Conditions. Normal human colon epithelial cells CCD18co, lung embryonic fibroblasts WI38, and breast adenocarcinoma cell lines MCF7 (p53wt), SW527(p53mu), and SKBR3 (p53mu) were obtained from American Type Culture Collection (Rockville, MD). The Tomudex-resistant breast cancer cell line MCF7TDX (11) and the Tomudex-resistant colon cancer cell line RKOTDX (p53wt) were provided by Dr. P. Johnston (Queens University of Belfast, Northern Ireland). The 5-FU-resistant H630R10

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[2] The abbreviations used are: TS, thymidylate synthase; 5-FU, 5-fluorouracil; BrdUrd, bromodeoxyuridine; 7-AAD, 7-amino-actinomycin D.

(p53wt) colon carcinoma cell line (4) was provided by Dr. E. Chu (National Cancer Institute-Navy Medical Oncology, Bethesda, MD). Tomudex-resistant SW527 cells were selected by culturing SW527 cells in medium supplemented with increasing concentrations of Tomudex up to a final concentration of 2 μM. SKBR3TS3 is a subclone of SKBR3 that stably expresses defined levels of TS protein and which was generated as described previously (10). Cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum and the antibiotic/antimycotic Fungizone (Life Technologies, Inc.) at 37°C in a humidified atmosphere containing 5% CO_2. MCF7TDX, SW527TDX, and RKOTDX cells were maintained continuously in the presence of 2 μM Tomudex, whereas the H630R10 cells were maintained in the presence of 10 μM 5-FU. Normal cells were passaged a maximum of 15 times to avoid senescence.

Cell Growth Inhibition Studies. Cells were seeded in 100 μl of medium in 96-well, flat-bottomed tissue culture plates. After 24 h, 100 μl of half-log serial dilutions of drug-containing medium were added in duplicate, and the cells were incubated for an additional 72 h. Cells were washed with PBS and stained with 0.5% crystal violet in methanol. Sorenson's buffer (0.025 M sodium citrate, 0.025 M citric acid in 50% ethanol) 0.1 ml was added, and the absorbance was monitored at the 535-nm wavelength (12). IC_{50}s were derived from sigmoid curves fit according to the Hill inhibitory Emax model and indicate the concentration at which growth is inhibited by 50%. Each cell growth inhibition assay was repeated at least three times. Cell growth inhibition assays using various times of drug exposure were performed as described above, with the exception that at 1, 6, 16, 24, and 48 h, the drug was washed out, and incubation of the cells continued for up to 72 h.

Mouse Xenograft Models. MCF7TDX or H630R10 cells (1.5×10^7) were injected s.c. in the mid-back region of female CD-1 (*nu/nu*) athymic mice (Charles River Laboratories, Wilmington, MA). Before cell injection, all mice were primed with 17β-estradiol (Innovative Research of America, Sarasota, FL) applied s.c. (1.7 mg estradiol/pellet) to promote tumor growth. Tumor volumes (length $\times$ width $\times$ depth) were monitored twice weekly by serial micrometer measurements. Six animals were randomly assigned to each treatment group. Single-factor ANOVA tests were performed to assure uniformity in starting tumor volumes between the various groups. All drugs were administered by i.p. injection once a day for 5 days on treatment, 5 days off treatment, and 5 days on treatment. Statistical analysis, single-factor ANOVA of the log-transformed tumor volume data, was performed as described by Pegram *et al.* (13).

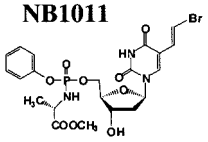
RNase Protection Assay. Cells were seeded in T225 tissue culture flasks and grown to a confluency of 40–50%. Medium was aspirated, and 25 ml of tissue culture medium containing 30 μM NB1011 were added to the cells. Twenty-five ml of tissue culture medium was added to control cells. After an additional 48- or 72-h incubation, NB1011-treated cells and control cells were harvested and counted, and 1×10^7 cells were snap frozen in liquid nitrogen and stored at −80°C. Total RNA was isolated using the RNeasy mini kit (Qiagen) according the manufacturer's protocol. Ten μg of each RNA were used by BD PharMingen for the RNase protection assay with the human Stress-1 template.

Western Blot Analysis. Cells were seeded in six-well plates at a density that results in 40–50% confluency after overnight incubation. Cells were left untreated or were treated with various concentrations of NB1011. To ensure that >50% of the cells are effected, concentrations higher than the IC_{50}s (10, 30, and 100 μM) were used for most of the MCF7TDX and RKOTDX experiments. For proper comparison, the MCF7 and WI38 cells were also treated with 10, 30, and 100 μM of NB1011. After 24, 48, or 72 h of incubation, cells were lysed at 4°C in lysis buffer containing 50 mM HEPES, 20 mM Tris-HCl (pH 7.5), 50 mM NaCl, 2.5 mM EDTA, 1% Triton X-100, 50 mM sodium fluoride, 10 mM sodium PP_i, 1 mM sodium orthovanadate, and Complete Protease Inhibitor Cocktail (Boehringer Mannheim). Insoluble material was cleared by centrifugation at 4°C, and protein concentrations were determined using BCA (Pierce Biochemicals, Rockford, IL). Protein lysates (25–50 μg) were separated by SDS-PAGE using 4–20% gels (Novex). Samples were transferred to Immobilon-P polyvinylidene difluoride membranes (Millipore Corp.), blocked in Blotto [5% nonfat dry milk in Tris-buffered saline with 0.05% Tween 20 (TBST, Sigma Chemical Co.)], and then incubated for 2 h with primary antibody diluted in Blotto. Membranes were washed (three times for 15 min each time) in TBST and incubated for 1 h with horseradish peroxidase-conjugated secondary antibody (Amersham Life Science) diluted in Blotto. After three 15-min washes in TBST, the bands were visualized with the ECL detection system (Amersham Life Science). A Storm Phosphorimager was used to quantitate protein levels using tubulin or β-actin as internal standards. The following mouse monoclonal primary antibodies were used: TS Ab-1 TS-106 (NeoMarkers), tubulin Ab-4 (NeoMarkers), WAF1 Ab-3 (Oncogene), anti-β-actin AC-15 (Sigma Chemical Co.), Bax Ab-3 (Oncogene), and p53 Ab-2 (Oncogene).

Cell Cycle Analysis. Cells were seeded in 10-cm plates at a density that results in 30–40% confluency after overnight incubation. Cells were left untreated or were treated with various concentrations of NB1011. To ensure that >50% of the cells are affected, concentrations up to 10 times the IC_{50}s were used for the experiments. For a proper comparison between MCF7TDX and MCF7 cells, the MCF7 cells were treated with the highest NB1011 dose (30 μM) that was used in the MCF7TDX experiments. After 72 h, cells were processed for cell cycle analysis according to the protocol provided with the BrdU Flow Kit #2354KK (BD PharMingen). Briefly, cells were pulsed with BrdUrd for 1 h, trypsinized, and counted. The cells were washed, fixed, and permeabilized. Incorporated BrdUrd was labeled with FITC-conjugated anti-BrdUrd antibody, and total DNA was stained with 7-AAD. At least 10,000 single cells were analyzed by flow cytometry using a FACScan (Becton Dickinson). Data analysis was done using the WinMDI 2.8 software, developed by Joseph Trotter at The Scripps Research Institute (La Jolla, CA). Three independent experiments were performed for the MCF7TDX and the RKOTDX cells. The MCF7 cell cycle analysis was done in duplicate.

Table 1 TS protein levels and sensitivity to NB1011

NB1011



Cell type	TS protein level	IC_{50} (μM)
SKBR3	0.5 ± 0.1	412 ± 23
CCD18co	1 ± 0.2	501 ± 29
WI38	1.5 ± 0.2	516 ± 43
MCF7	1.8 ± 0.2	168 ± 20
RKOTDX	4.6 ± 0.1	16 ± 6
SKBR3TS3	4.6 ± 0.5	13 ± 4
SW527TDX	10 ± 0.4	13 ± 3
MCF7TDX	20 ± 0.8	3 ± 0.3

TS protein levels were determined by Western blotting. The level of TS detected in lysates made from exponentially growing normal colon epithelial cells, CCD18co, is set at 1. TS protein levels are given as values relative to the TS level in CCD18co. IC_{50} is the concentration of NB1011 that inhibits cellular growth by 50%.

AnnexinV/PI Staining for Apoptosis. Cells, seeded in six-well plates, were either left untreated or treated with various concentrations of NB1011 for 24, 48, or 72 h. To ensure that >50% of the cells are affected, concentrations up to 10 times the IC_{50}s were used for the experiments. At the indicated time points, both floating and attached cells were collected. Cells were incubated with a staining solution containing Annexin V-fluorescein and propidium iodide (Annexin-V-Fluos staining kit; Boehringer Mannheim) according the manufacturer's protocol. The percentage of apoptotic cells was determined by flow cytometry using at least 30,000 single cells. Data analysis was done using the WinMDI 2.8 software, developed by Joseph Trotter at The Scripps Research Institute. Assays were repeated at least two times.

Results

TS Protein Levels and NB1011 Cytotoxicity. To determine the intracellular TS protein levels and the sensitivity of the various cell lines to NB1011, Western blot analysis and growth inhibition assays were performed. The TS protein level, detected in lysates made from exponentially growing normal colon epithelial cells, CCD18co, was set at 1. TS protein levels are given as values relative to the TS level in CCD18co. Table 1 shows that the TS protein levels in exponentially growing normal cell types, CCD18co and WI38, as well as in the MCF7 and SKBR3 breast tumor cell lines, are relatively low (1, 1.5, 1.8, and 0.5, respectively). The tumor cell lines RKOTDX, SW527TDX, and MCF7TDX, which are resistant to the direct TS inhibitor Tomudex, have elevated TS protein levels (4.6, 10, and 20, respectively). The TS protein level in SKBR3TS3 cells, which stably express recombinant human TS, is 4.6-fold higher than the TS level observed in the normal colon epithelial cells. The IC_{50}s of NB1011 against these various cell lines (Table 1) show that NB1011 is preferentially cytotoxic to tumor cell lines with high TS levels.

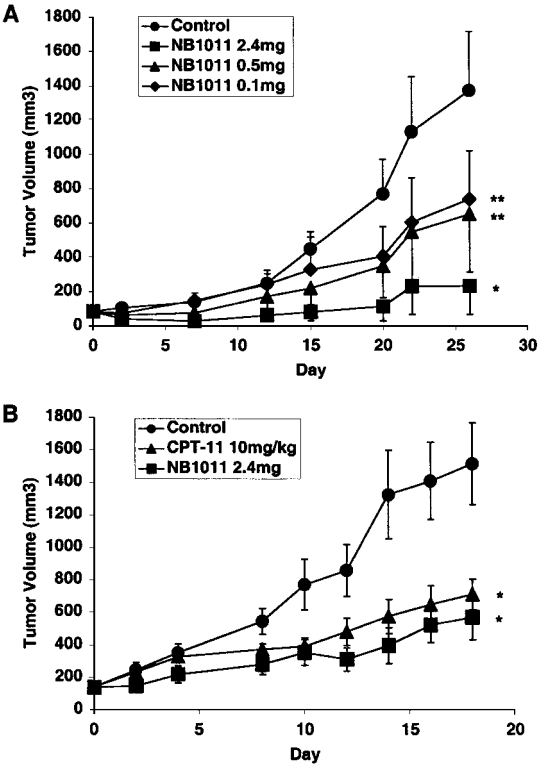


Fig. 1. Antitumor activity of NB1011 against human xenografts in athymic mice. *A,* dose-dependent efficacy of NB1011 against Tomudex-resistant MCF7TDX breast carcinoma. NB1011 was given at 0.1, 0.5, and 2.4 mg/day. *B,* efficacy comparison of NB1011 (2.4 mg/day) and CPT-11 (10 mg/kg/day) against 5-FU-resistant H630R10 colon carcinoma. Drugs were administered i.p. for 5 days on treatment, 5 days off treatment, and 5 days on treatment. Values are mean tumor volumes ± s.e.m.; $n = 6$. *, significantly different ($P < 0.05$) from excipient control. **, significantly different ($P < 0.05$) from the 2.4 mg/day NB1011 treated group.

NB1011 Has Antitumor Activity in Mouse Xenograft Models. To determine dose-dependent efficacy of NB1011 in a MCF7TDX mouse xenograft model, athymic mice bearing s.c. MCF7TDX tumors were administered i.p. three different doses of NB1011 (Fig. 1*A*). The highest dose of NB1011 (2.4 mg/day) resulted in a significant reduction ($P < 0.05$) in day 26 xenograft volume as compared with the excipient-treated control. Treatment with NB1011 at 2.4 mg/day was also significantly different ($P < 0.05$) at day 26 from treatment with NB1011 at 0.1 or 0.5 mg/day, but no statistically significant difference could be observed between mean xenograft volumes of animals treated with NB1011 at 0.1 or at 0.5 mg/day (Fig. 1*A*). In conclusion, these results show a dose-response between NB1011 and MCF7TDX xenograft volume with a significant difference ($P < 0.05$), maintained up to day 26, between treatment with NB1011 at 2.4 mg/day and NB1011 at 0.1 mg/day.

Our previous experiments showed that NB1011 is efficacious in 5-FU-resistant, high TS-expressing H630R10 colon xenografts in athymic mice (14). Irinotecan (also called

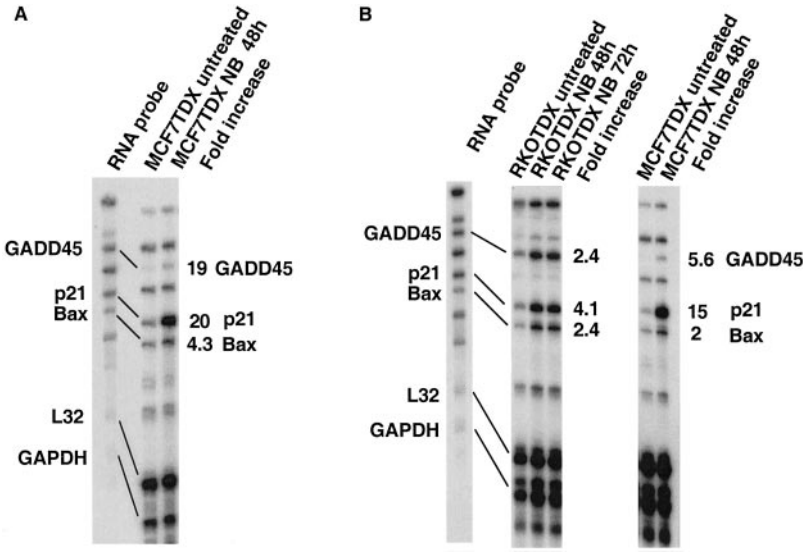


Fig. 2. NB1011 treatment of MCF7TDX and RKOTDX cells induces *p21*, *GADD45*, and *Bax* expression. MCF7TDX (*A* and *B*) or RKOTDX (*B*) cells were treated with 30 μM NB1011 or were left untreated. After 48 or 72 h, total RNA was isolated, and 10 μg of each RNA were used in RNA protection assays with the human Stress-1 template. *L32* and *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) genes serve as internal standards. The normalized fold increase in mRNA levels is indicated.

CPT11) is a recently approved drug for the treatment of 5-FU-resistant colon cancer. We therefore determined the relative efficacies of NB1011 and CPT11 in the H630R10 xenograft model. i.p. treatment with NB1011 at 2.4 mg/day was as efficacious as i.p. treatment with CPT11 at its maximum tolerated dose of 10 mg/kg (Fig. 1*B*). No statistically significant difference was observed in the mean xenograft volume between the NB1011- and CPT 11-treated groups. Both groups were statistically different ($P < 0.05$) from the excipient-treated control group (Fig. 1*B*).

NB1011 Treatment Induces p21, Bax, and GADD45 mRNA. To gain insight into the mechanism(s) by which NB1011 inhibits cellular growth, we investigated molecular events downstream of NB1011 treatment. We focused on the identification of cellular genes that are regulated upon treatment with NB1011. Tomudex-resistant breast carcinoma cells, MCF7TDX, which have high TS levels and are sensitive to NB1011 (Table 1), were left untreated or were treated with 30 μM NB1011 for 48 h. Cells were harvested, and total RNA was isolated. RNase protection assays using 10 μg of each RNA and the human Stress-1 template (BD PharMingen) showed a significant induction in p21 (20-fold), Bax (4.3-fold), and GADD45 (19-fold) mRNA levels in NB1011-treated MCF7TDX cells as compared with the untreated control (Fig. 2*A*). To confirm and extend these results, we analyzed MCF7TDX cells, and the Tomudex-resistant colon carcinoma cell line, RKOTDX, in the RNase protection assays. RKOTDX cells also express elevated levels of TS and are sensitive to NB1011 (Table 1). Results from this RNase protection assay (Fig. 2*B*) showed that in RKOTDX cells, NB1011 treatment leads to the induction of p21, Bax, and GADD45 mRNA, although to a lesser extent as seen in the MCF7TDX cells.

NB1011 Treatment Results in an Increase in the p21 and Bax Proteins. To determine whether the induction of mRNA in cells treated with NB1011 correlates with an increase in the corresponding proteins, we analyzed the endogenous p21 and Bax protein levels in MCF7TDX and RKOTDX cells. Results (Fig. 3*A*) show that NB1011 treatment of MCF7TDX cells leads to a 4–9-fold increase in the endogenous p21 protein levels as compared with the endogenous p21 protein levels observed in untreated MCF7TDX cells. A 2–2.4-fold increase in the endogenous Bax protein levels was detected upon NB1011 treatment (Fig. 3*A*). These data show that the increase in p21 and Bax mRNA in MCF7TDX cells upon NB1011 treatment correlates with an increase in the p21 and Bax proteins. Also in the RKOTDX cells the increase in Bax mRNA levels induced by NB1011 translates into an increase in the endogenous Bax protein levels (Fig. 3*B*). Endogenous p21 levels, however, could not be detected in the RKOTDX cells under the experimental conditions used (results not shown).

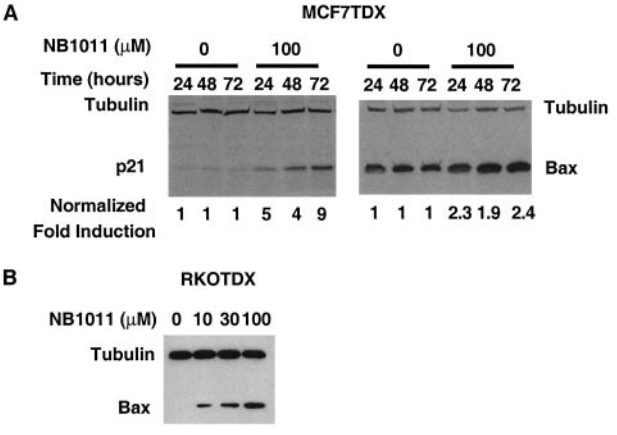
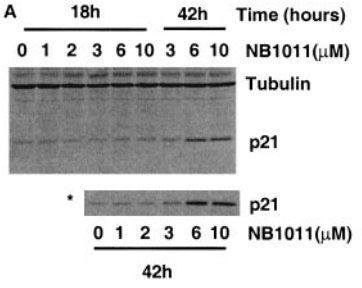


Fig. 3. NB1011 treatment increases the endogenous p21 and Bax protein levels. MCF7TDX (*A*) and RKOTDX (*B*) cells were treated with various concentrations of NB1011 as indicated. MCF7TDX cells were treated for 24, 48, or 72 h, and RKOTDX cells were treated for 48 h. At the indicated time points, total cell lysates were made, and the protein concentrations were determined using the BCA-200 protein assay. Endogenous p21, Bax, and tubulin protein levels were analyzed by Western blotting using 25 μg of the various cell lysates and the anti-p21, anti-Bax, and the anti-tubulin mouse monoclonal antibodies. The normalized fold increase in endogenous protein levels is indicated.



B

Time	NB1011 IC50 (μM)
1 hour	757
6 hours	180
16 hours	34
24 hours	20
48 hours	5
72 hours	3

Fig. 4. The cytotoxicity of NB1011 correlates with the induction of p21 in MCF7TDX cells. *A,* Western blot analysis of lysates (25 μg) made of MCF7TDX cells treated for 18 or 42 h with concentrations of NB1011 ranging from 0 to 10 μM. p21 and tubulin levels were determined by using the anti-p21 and anti-tubulin antibodies. *, the p21 protein levels in the complete set of lysates (0–10 μM NB1011) made at 42 h. *B,* IC_{50}s of NB1011 were determined using MCF7TDX cells and various times of drug exposure.

p21 Induction by NB1011 Is Dose and Time Dependent and Correlates with the Cytotoxicity of NB1011. To determine whether the induction of p21 is dependent upon the dose of NB1011 and on the time of drug exposure, we analyzed the endogenous p21 protein levels in MCF7TDX cells that were treated with concentrations of NB1011 ranging from 0 to 10 μM for 18 or 42 h. Fig. 4*A* shows that by Western blot analysis, no significant p21 induction can be detected in the MCF7TDX cells treated for 18 h with a concentration of NB1011 up to 10 μM. However, an increase in p21 protein levels can be detected in MCF7TDX cells treated with 6 or 10 μM NB1011 for 42 h. To correlate the induction of p21 with the cytotoxicity of NB1011, we determined the IC_{50} of NB1011 on MCF7TDX cells using various times of drug exposure (Fig. 4*B*). Our data show that the concentration of NB1011 that results in a noticeable induction of p21 (6 μM at 42 h) is similar to its IC_{50} (5 μM at 48 h).

NB1011 Induces p21 and Bax Proteins in MCF7TDX Cells but not in MCF7 or Normal WI38 Fibroblasts. We analyzed the induction of p21 and Bax by NB1011 in MCF7TDX cells, characterized by high TS protein levels, and in MCF7 tumor cells or in WI38 normal embryonic lung fibroblasts. Both these latter cell types express low levels of the TS protein (Table 1). Results show that the endogenous p21 and Bax proteins are induced 8–11-fold and 2–2.3-fold, respectively in the high TS-expressing MCF7TDX cells when treated with 10, 30, or 100 μM of NB1011. Upon NB1011 treatment of MCF7 cells, the p21 and Bax protein levels were induced only 1.6–1.8-fold and 1.3-fold, respectively (Fig. 5*A*). In the normal WI38 fibroblasts, NB1011 treatment (10 and 30 μM) did not result in the induction of the p21 protein, and only a 1.7-fold induction was observed when the cells were treated with 100 μM NB1011 (Fig. 5*B*).

Endogenous p53 Protein Levels Are Induced by NB1011. The *p21*, *bax* and *GADD45* genes are directly regulated by the tumor suppressor protein p53 (15–17). Because NB1011 treatment of MCF7TDX and RKOTDX cells, both of which are wild type for p53, results in the induction of p21, Bax, and GADD45 mRNAs (Fig. 2), we investigated whether NB1011 induces endogenous p53 protein levels. Fig. 5*C* shows that NB1011 treatment of MCF7TDX cells results in a 2–3-fold increase in the endogenous p53 protein levels as compared with the untreated control. Furthermore, the induction of p53 by NB1011 correlates well with the cytotoxicity of NB1011, because no p53 protein induction was observed in the NB1011-treated MCF7 cells.

Effects of NB1011 Treatment on the Regulation of the Cell Cycle. The p21 and GADD45 proteins have been reported to be involved in G_0-G_1 and G_2-M cell cycle regulation (18–20). Because NB1011 treatment results in an increase in p21 and GADD45, we investigated whether NB1011 has an effect on the regulation of the cell cycle. BrdUrd incorporation/proliferation assays were performed using asynchronous, exponentially growing MCF7TDX and RKOTDX cells, which were left untreated or were treated with various concentrations of NB1011 for 72 h. The total cellular DNA content (7-AAD staining) and the proportion of cells in S-phase (BrdUrd incorporation) were quantitated by FACS analysis. Results from a representative experiment (Fig. 6*A*) show that treatment of MCF7TDX and RKOTDX cells with NB1011 results in a decrease of cells in the S-phase (up to 14 and 19%, respectively) and an increase of cells in the G_2-M phase of the cell cycle (up to 26 and 15%, respectively) as compared with the untreated control cells. Representative results from cell cycle analysis of MCF7 cells (Fig. 6*A*) show that treatment with NB1011 did not affect the percentage of cells in the S-phase, whereas only a 5% increase in the percentage of cells in the G_2-M phase of the cell cycle was observed. Fig. 6*B* shows the total cell numbers for the untreated and NB1011-treated RKOTDX, MCFTDX, and MCF7 cells. Cells were counted before the addition of NB1011 and after 72 h of drug treatment. The cells were subsequently processed for cell cycle analysis. Total cell numbers are decreased 3–6-fold upon NB1011 treatment of the RKOTDX and MCF7TDX cells, respectively, whereas the total number of MCF7 cells showed only a 1.1-fold decrease. This correlates well with the cell cycle distribution (Fig. 6*A*) showing that both the RKOTDX and MCF7TDX cells are arrested in G_2-M, whereas the cell cycle distribution of the NB1011-treated MCF7 cells is similar to the untreated control.

Effects of NB1011 Treatment on the Induction of Apoptosis. Apart from p21 and GADD45, NB1011 treatment of both the MCF7TDX and RKOTDX cells induced the proapoptotic Bcl-2 family member, Bax. We used Annexin V combined with propidium iodide staining to determine whether this increase in Bax protein levels corresponded with an effect on the induction of apoptosis. After 72 h, NB1011 induced apoptosis in 12% of the RKOTDX cells as compared with 2% of apoptotic cells observed in the untreated control (data not shown). Also in the MCF7TDX cells, NB1011 treatment resulted in the induction of apoptosis in 10% of the cells as compared with 4% of apoptotic cells present in the untreated control (data not shown). This indicates that the increase in Bax protein levels observed upon NB1011 treatment is not sufficient to trigger the balance toward massive apoptosis.

Discussion

NB1011 is a novel anticancer agent that is specifically cytotoxic to human tumor cells with elevated TS protein levels

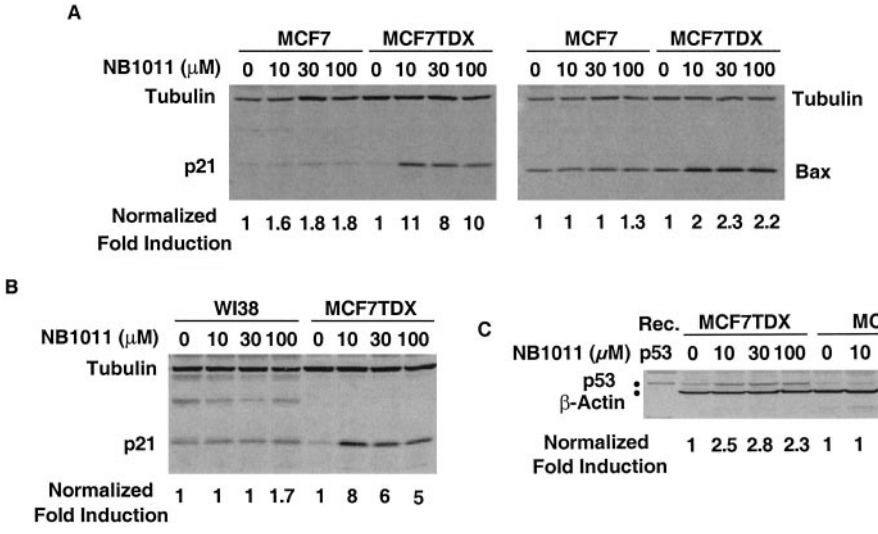


Fig. 5. NB1011 induces p21, Bax, and p53 proteins specifically in high TS-expressing tumor cells. *A,* p21 and Bax protein levels in MCF7TDX and MCF7 cell lysates (35 μg). *B,* p21 protein levels in WI38 and MCF7TDX cell lysates (35 μg). *C,* p53 protein levels in MCF7 and MCF7TDX cell lysates (50 μg). Cells were treated with 0, 10, 30, and 100 μM NB1011 and harvested after 48 h (*A* and *B*) or 72 h (*C*). Western blot analysis was performed to determine the endogenous protein levels using the anti-p21, anti-Bax, anti-Tubulin, anti-p53, and anti-β-actin antibodies. The normalized fold increase in protein levels is shown.

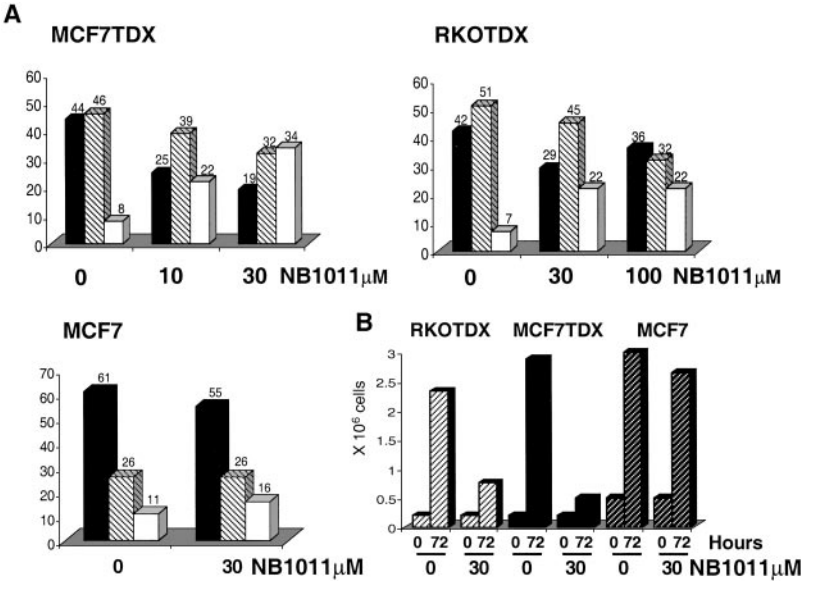


Fig. 6. Effects of NB1011 treatment on the cell cycle of MCF7TDX and RKOTDX cells. *A,* cells were treated with 10, 30, or 100 μM NB1011 or were left untreated (control). After 72 h, cells were analyzed by flow cytometry using BrdUrd incorporation and 7-AAD DNA staining to determine the cell cycle distribution of at least 10,000 cells. *Columns,* the percentages of cells in the G_0-G_1 ■, S ▨, or G_2-M □ phase of the cell cycle. *B,* cells were counted before the addition of NB1011 (*0 h*) and after 72 h of drug treatment. *Columns,* total cell numbers.

(Ref. 10); Table 1). The data presented in this study indicate that NB1011 has dose-related antitumor activity in high TS-expressing breast cancer xenografts and that its efficacy is comparable with that of CPT11 in 5-FU-resistant H630R10 colon carcinoma xenografts. To understand how NB1011 exerts its growth-suppressive effects, we delineated the downstream molecular events of the cellular response to NB1011. Our results show that NB1011 treatment of high TS-expressing MCF7TDX and RKOTDX cells leads to the induction of *p21*, *bax,* and *GADD45*, all of which are direct target genes of p53 (15–17). The p21 protein is an important cell cycle regulator that inhibits several cyclin/cyclin-dependent kinase complexes, which are essential for cell cycle progression (21). Although initially thought to be involved solely in the G_1 checkpoint control upon DNA damage, p21 has now been shown to play a crucial role in the G_2 DNA damage checkpoint (19, 22). The DNA damage-inducible gene *GADD45* has also been implicated in the activation of a G_2-M checkpoint (20, 23). Activation of the Cdc2/Cyclin B complex is required for mitotic entry in mammalian cells (24), and both p21 and GADD45 have been reported to inhibit the protein kinase activity of this Cdc2/Cyclin B complex (22, 25). MCF7TDX cells treated with NB1011 up-regulate p21 and GADD45 and accumulate in the G_2-M phase of the cell cycle. Additional preliminary experiments suggest that Cdc2 protein levels are down-regulated upon NB1011 treatment, and we are currently investigating whether NB1011 has an effect on the Cdc2 protein kinase activity. The induction of p53, p21, and GADD45, the down-regulation of Cdc2 protein levels, and consequently the accumulation of cells in the G_2-M phase of the cell cycle fit the profile of a classic DNA damage response (26). This suggests that NB1011 treatment results in DNA damage, although the mechanism of toxicity may not be limited to DNA damage. The detailed mechanism

by which NB1011 would produce DNA damage still needs to be elucidated, because experiments performed with radiolabeled NB1011 did not show incorporation into DNA.[3]

The induction of p21, Bax, and GADD45 by NB1011 is p53 dependent. NB1011 treatment of the high TS expressing SW527TDX and SKBR3TS3 cell lines, both of which are mutant for p53, did not result in a detectable increase in p21, GADD45, or Bax nor in the accumulation of cells in the G_2-M phase of the cell cycle (data not shown). Our results, showing that NB1011 is cytotoxic to high TS-expressing tumor cells with either wild-type p53 (MCF7TDX and RKOTDX) or mutant p53 (SW527TDX and SKBR3TS3), indicate that p53 is not required for the cytotoxicity of NB1011. Which particular genes become activated and which proteins will be induced upon NB1011 treatment of high TS-expressing, p53 mutant cell lines are intriguing questions that warrant further investigation. Apart from the important cell cycle regulators p21 and GADD45, NB1011 treatment of both the MCF7TDX and RKOTDX cells induced the proapoptotic Bcl-2 family member, Bax. However this increase in Bax protein levels did not shift the balance toward massive apoptosis, because NB1011 treatment for 72 h induced apoptosis in only 6–10% of the cells. It is known that the Bcl-2:Bax ratio or as reported recently for colon cancer cells, the Bcl-X_L:Bax ratio, rather than the Bax protein levels seems to be a key determinant in the decision of the cells to undergo apoptosis (27). High levels of Bcl-2 and Bcl-X_L have been reported for MCF7 cells (28). It will therefore be interesting to analyze the effects of the combination of NB1011 with Bcl-2 inhibitors on the induction of apoptosis in MCF7TDX cells.

NB1011 has a cytotoxicity profile that is clearly opposite to the classical TS inhibitors such as 5-FU and Tomudex, supporting a different mode of action of NB1011. NB1011 is preferentially cytotoxic to tumor cells with elevated TS protein levels, whereas normal cells with low TS levels are not (or less) sensitive to NB1011 treatment (Ref. 10; Table 1). 5-FU and Tomudex, on the other hand, are less effective against tumor cells that have increased TS protein levels; in fact, increasing the intracellular TS levels is one of the mechanisms tumor cells use to become resistant to these classical TS inhibitors (3). The accumulation of tumor cells in the G_2-M phase of the cell cycle upon NB1011 treatment as opposed to the G_1-S arrest induced by Tomudex and 5-FU (29, 30) also suggests that NB1011 treatment has consequences different from limiting the supply of thymidylate for DNA synthesis. In addition, the increase in cell size seen upon treatment of cells with 5-FU or Tomudex and characteristic for thymineless death (31, 32) is not observed when tumor cells are treated with NB1011.[4] The induction of p53, p21, and Bax by NB1011, the accumulation of cells in the G_2-M phase of the cell cycle, and the inhibition of cellular growth is observed in the high TS-expressing MCF7TDX and RKOTDX cells but is not detectable in NB1011-treated cells with low TS levels (*e.g.,* MCF7 and WI38). This correlation between high TS levels and p53 induction is consistent with the requirement for NB1011 to be activated by TS.

Significant antitumor activity of NB1011 was demonstrated in mouse xenograft models upon treatment with 2.4 mg/day (120 mg/kg). This dose of NB1011 corresponds with 360 mg/m^2. Toxicity studies using rats and dogs[5] have shown that doses as high as 2000 mg/m^2 (5.6 times the efficacious dose in mice) are well tolerated. Clinical trials (Phase I/II) to assess the safety and efficacy of NB1011 in patients with 5-FU-resistant colon cancer have been initiated using a NB1011 dose of 200 mg/m^2, which is just below the efficacious dose in mice. The minimal toxicity of NB1011 to normal cells with low TS levels and the favorable toxicity profiles in rats and dogs suggest that NB1011 represents a novel chemotherapeutic with the capacity to spare normal cells and preferentially target cancer cells with genetic changes that engender high TS levels.

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References

1. Carreras, C. W., and Santi, D. V. The catalytic mechanism and structure of thymidylate synthase. Annu. Rev. Biochem., *64:* 721–762, 1995.

2. Banerjee, D., Gorlick, R., Liefshitz, A., Danenberg, K., Danenberg, P. C., Danenberg, P. V., Klimstra, D., Jhanwar, S., Cordon-Cardo, C., Fong, Y., Kemeny, N., and Bertino, J. R. Levels of E2F-1 expression are higher in lung metastasis of colon cancer as compared with hepatic metastasis and correlate with levels of thymidylate synthase. Cancer Res., *60:* 2365–2367, 2000.

3. Gorlick, R., and Bertino, J. R. Drug resistance in colon cancer. Semin. Oncol., *26:* 606–611, 1999.

4. Copur, S., Aiba, K., Drake, J. C., Allegra, C. J., and Chu, E. Thymidylate synthase gene amplification in human colon cancer cell lines resistant to 5-fluorouracil. Biochem. Pharmacol., *49:* 1419–1426, 1995.

5. Rooney, P. H., Stevenson, D. A., Marsh, S., Johnston, P. G., Haites, N. E., Cassidy, J., and McLeod, H. L. Comparative genomic hybridization analysis of chromosomal alterations induced by the development of resistance to thymidylate synthase inhibitors. Cancer Res., *58:* 5042–5045, 1998.

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

7. Johnston, P. G., Lenz, H. J., Leichman, C. G., Danenberg, K. D., Allegra, C. J., Danenberg, P. V., and Leichman, L. 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, 1995.

8. Leichman, C. G., Lenz, H. J., Leichman, L., Danenberg, K., Baranda, J., Groshen, S., Boswell, W., Metzger, R., Tan, M., and Danenberg, P. V. Quantitation of intratumoral thymidylate synthase expression predicts for disseminated colorectal cancer response and resistance to protracted-infusion fluorouracil and weekly leucovorin. J. Clin. Oncol., *15:* 3223–3229, 1997.

9. Cascinu, S., Aschele, C., Barni, S., Debernardis, D., Baldo, C., Tunesi, G., Catalano, V., Staccioli, M. P., Brenna, A., Muretto, P., and Catalano, G. Thymidylate synthase protein expression in advanced colon cancer: correlation with the site of metastasis and the clinical response to leucovorin-modulated bolus 5-fluorouracil. Clin. Cancer Res., *5:* 1996–1999, 1999.

[3] M. Sergeeva and B. Cathers, unpublished data.
[4] S. T. C. Neuteboom, unpublished data.
[5] NewBiotics, Inc., internal documents.

10. Li, Q., Boyer, C., Lee, J. Y., and Shepard, H. M. A novel approach to thymidylate synthase as a target for cancer chemotherapy. Mol. Pharmacol., *59:* 446–452, 2001.

11. Drake, J. C., Allegra, C. J., Moran, R. G., and Johnston, P. G. Resistance to Tomudex (ZD1694): multifactorial in human breast and colon carcinoma cell lines. Biochem. Pharmacol., *51:* 1349–1355, 1996.

12. Flick, D. A., and Gifford, G. E. Comparison of *in vitro* cell cytotoxic assays for tumor necrosis factor. J. Immunol. Methods, *68:* 167–175, 1984.

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

14. Lackey, D. B., Groziak, M. P., Sergeeva, M., Beryt, M., Boyer, C., Stroud, R. M., Sayre, P., Park, J. W., Johnston, P., Slamon, D., Shepard, H. M., and Pegram, M. Enzyme-catalyzed therapeutic agent (ECTA) design: activation of the antitumor ECTA compound NB1011 by thymidylate synthase. Biochem. Pharmacol., *61:* 179–189, 2001.

15. el-Deiry, W. S., Tokino, T., Velculescu, V. E., Levy, D. B., Parsons, R., Trent, J. M., Lin, D., Mercer, W. E., Kinzler, K. W., and Vogelstein, B. WAF1, a potential mediator of p53 tumor suppression. Cell, *75:* 817–825, 1993.

16. Miyashita, T., and Reed, J. C. Tumor suppressor p53 is a direct transcriptional activator of the human *bax* gene. Cell, *80:* 293–299, 1995.

17. Kastan, M. B., Zhan, Q., el-Deiry, W. S., Carrier, F., Jacks, T., Walsh, W. V., Plunkett, B. S., Vogelstein, B., and Fornace, A. J., Jr. A mammalian cell cycle checkpoint pathway utilizing p53 and GADD45 is defective in ataxia-telangiectasia. Cell, *71:* 587–597, 1992.

18. el-Deiry, W. S., Harper, J. W., O'Connor, P. M., Velculescu, V. E., Canman, C. E., Jackman, J., Pietenpol, J. A., Burrell, M., Hill, D. E., Wang, Y., *et al.* WAF1/CIP1 is induced in p53-mediated G_1 arrest and apoptosis. Cancer Res., *54:* 1169–1174, 1994.

19. Bunz, F., Dutriaux, A., Lengauer, C., Waldman, T., Zhou, S., Brown, J. P., Sedivy, J. M., Kinzler, K. W., and Vogelstein, B. Requirement for p53 and p21 to sustain G2 arrest after DNA damage. Science (Wash. DC), *282:* 1497–1501, 1998.

20. Wang, X. W., Zhan, Q., Coursen, J. D., Khan, M. A., Kontny, H. U., Yu, L., Hollander, M. C., O'Connor, P. M., Fornace, A. J., Jr., and Harris, C. C. GADD45 induction of a G2/M cell cycle checkpoint. Proc. Natl. Acad. Sci. USA, *96:* 3706–3711, 1999.

21. Deng, C., Zhang, P., Harper, J. W., Elledge, S. J., and Leder, P. Mice lacking p21CIP1/WAF1 undergo normal development, but are defective in G1 checkpoint control. Cell, *82:* 675–684, 1995.

22. Smits, V. A., Klompmaker, R., Vallenius, T., Rijksen, G., Makela, T. P., and Medema, R. H. p21 inhibits Thr161 phosphorylation of Cdc2 to enforce the G2 DNA damage checkpoint. J. Biol. Chem., *275:* 30638–30643, 2000.

23. Jin, S., Antinore, M. J., Lung, F. D., Dong, X., Zhao, H., Fan, F., Colchagie, A. B., Blanck, P., Roller, P. P., Fornace, A. J., Jr., and Zhan, Q. The GADD45 inhibition of Cdc2 kinase correlates with GADD45-mediated growth suppression. J. Biol. Chem., *275:* 16602–16608, 2000.

24. Elledge, S. J. Cell cycle checkpoints: preventing an identity crisis. Science (Wash. DC), *274:* 1664–1672, 1996.

25. Zhan, Q., Antinore, M. J., Wang, X. W., Carrier, F., Smith, M. L., Harris, C. C., and Fornace, A. J., Jr. Association with Cdc2 and inhibition of Cdc2/Cyclin B1 kinase activity by the p53-regulated protein Gadd45. Oncogene, *18:* 2892–2900, 1999.

26. Taylor, W. R., and Stark, G. R. Regulation of the G2/M transition by p53. Oncogene, *20:* 1803–1815, 2001.

27. Nita, M. E., Nagawa, H., Tominaga, O., Tsuno, N., Fujii, S., Sasaki, S., Fu, C. G., Takenoue, T., Tsuruo, T., and Muto, T. 5-Fluorouracil induces apoptosis in human colon cancer cell lines with modulation of Bcl-2 family proteins. Br. J. Cancer, *78:* 986–992, 1998.

28. Wagener, C., Bargou, R. C., Daniel, P. T., Bommert, K., Mapara, M. Y., Royer, H. D., and Dorken, B. Induction of the death-promoting gene *bax-*α sensitizes cultured breast-cancer cells to drug-induced apoptosis. Int. J. Cancer, *67:* 138–141, 1996.

29. Inaba, M., and Mitsuhashi, J. Flow cytometric analysis of cell-killing actions of 5-fluorouracil in human colorectal cancer cells. Oncol. Res., *6:* 303–309, 1994.

30. Matsui, S. I., Arredondo, M. A., Wrzosek, C., and Rustum, Y. M. DNA damage and p53 induction do not cause ZD1694-induced cell cycle arrest in human colon carcinoma cells. Cancer Res., *56:* 4715–4723, 1996.

31. Welsh, S. J., Titley, J., Brunton, L., Valenti, M., Monaghan, P., Jackman, A. L., and Aherne, G. W. Comparison of thymidylate synthase (TS) protein up-regulation after exposure to TS inhibitors in normal and tumor cell lines and tissues. Clin. Cancer Res., *6:* 2538–2546, 2000.

32. Cohen, S. S. On the nature of thymineless death. Ann. NY Acad. Sci., *186:* 292–301, 1971.