Anti-Cancer Drugs 2002, **13,** pp. 29–36

Report

Nucleoside transport inhibitors, dipyridamole and *p*-nitrobenzylthioinosine, selectively potentiate the antitumor activity of NB1011

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NB1011, a novel anticancer agent, targets tumor cells expressing high levels of thymidylate synthase (TS). NB1011 is converted intracellularly to bromovinyldeoxyuridine monophosphate (BVdUMP) which competes with the natural substrate, deoxyuridine monophosphate, for binding to TS. Unlike inhibitors, NB1011 becomes a reversible substrate for TS catalysis. Thus, TS retains activity and converts BVdUMP into cytotoxic product(s). *In vitro* cytotoxicity studies demonstrate NB1011's preferential activity against tumor cells expressing elevated TS protein levels. Additionally, NB1011 has antitumor activity *in vivo*. To identify drugs which interact synergistically with NB1011, we screened 13 combinations of chemotherapeutic agents with NB1011 in human tumor and normal cells. Dipyridamole and *p*-nitrobenzylthioinosine (NBMPR), potent inhibitors of equilibrative nucleoside transport, synergized with NB1011 selectively against 5-fluorouracil (5-FU)-resistant H630R10 colon carcinoma cells [combination index (CI)=0.75 and 0.35] and Tomudex-resistant MCF7TDX breast carcinoma cells (CI=0.51 and 0.57), both TS overexpressing cell lines. These agents produced no synergy with NB1011 in Det551 and CCD18co normal cells (CI > 1.1) lacking TS overexpression. Dipyridamole potentiated NB1011's cytotoxicity in medium lacking nucleosides and bases, suggesting a non-salvage-dependent mechanism. We demonstrate that nucleoside transport inhibitors, dipyridamole and NBMPR, show promise for clinically efficacious combination with NB1011. [© 2002 Lippincott Williams & Wilkins.]

Key words: Dipyridamole, NB1011, nucleoside transport inhibitors, *p*-nitrobenzylthioinosine.

Introduction

Thymidylate synthase (TS) is a critical enzyme in the *de novo* biosynthesis of thymidine-5′-monophosphate (dTMP) and therefore essential for DNA synthesis. TS catalyzes the reductive methylation of 2′-deoxyuridine-5′-monophosphate (dUMP) to yield dTMP using 5,10-methylene tetrahydrofolate as a cofactor.[1] Since TS is an essential enzyme in proliferating cells and its increased expression, resulting from loss of tumor suppressor function, is common in cancer,[2–4] this enzyme has for many years been an important target in cancer chemotherapy.

The fluoropyrimidine, 5-fluorouracil (5-FU), and the quinazoline antifolate, Tomudex (TDX), are well-known examples of TS inhibitors. 5-FU is currently the most widely used drug for the treatment of colon cancer.[5] A common problem arising from the use of these TS inhibitors is the development of resistant tumor cells, which display increased intracellular TS expression due to gene amplification.[6–8] Intratumoral TS gene expression correlates with the lack of clinical response to fluoropyrimidines and with poor clinical prognosis in several cancers.[9–11]

To circumvent this problem of drug-induced resistance, we developed a novel approach called enzyme catalyzed therapeutic activation (ECTA). TS ECTA takes advantage of the overexpression of TS in tumor cells. The TS ECTA compound, NB1011, is a nucleotide analog phosphoramidate, (*E*)-5-(2-bromovinyl)-2′-deoxy-5′-uridyl phenyl L-alaninyl phosphoramidate, which upon entry into cells is converted to bromovinyldeoxyuridine monophosphate (BVdUMP).[12] Subsequently, during an enzymatic reaction catalyzed by TS, BVdUMP is converted into the proposed cytotoxic product(s).[12] Therefore, a high intracellular TS activity increases the sensitivity of a cell to NB1011 cytotoxicity. This differentiates NB1011 from classical TS inhibitors because NB1011

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requires TS activity to become maximally cytotoxic. Our previous work showed that NB1011 is preferentially cytotoxic to tumor cells displaying elevated TS levels as compared to normal cells with lower levels of TS.[13] Furthermore, NB1011 exhibited antitumor activity in colon and breast carcinoma xenografts in athymic mice.[12] Because very few anticancer compounds are optimally efficacious as single agents, we sought to determine whether the *in vitro* cytotoxic activity of NB1011 could be enhanced using subclasses of chemotherapeutic agents with well-characterized mechanisms of action. Nucleoside transport inhibitors were of particular interest since dipyridamole has been reported to enhance the cytotoxicity of various chemotherapeutic agents including 5-FU.[14]

Materials and methods

Cell lines and cell culture

Normal human colon epithelial cells (CCD18co) and skin fibroblasts (Det551) were purchased from ATCC (Rockville, MD). H630R10, a colon carcinoma cell line resistant to 10 μM 5-FU,[6] and MCF7TDX, a breast adenocarcinoma cell line resistant to 2 μM TDX,[15] served as models for high TS expressing human cancer cells. Both tumor cell lines express approximately 20-fold more TS protein than normal CCD18co and Det551 cells.[13] All cells were cultured under conditions of 37°C, 95% humidified air, 5% CO_2 in RPMI 1640 culture medium containing 10% fetal bovine serum (FBS) or 10% dialyzed FBS (Life Technologies, Carlsbad, CA) and penicillin/streptomycin/fungizone (Life Technologies). MCF7TDX cells were maintained continuously in 2 μM TDX and H630R10 cells were maintained continuously in 10 μM 5-FU. Normal cells were passaged a maximum of 15 times to avoid senescence.

Chemical reagents

Thirteen chemotherapeutic agents representative of 10 different mechanistic classes (Table 2) were analyzed: 5-FU, etoposide, vinblastine, taxol, cisplatin, doxorubicin and methotrexate (Sigma, St Louis, MO); oxaliplatin (Hande Tech, Houston, TX); TDX (Zeneca, Wilmington, DE); dipyridamole and *p*-nitrobenzylthioinosine (NBMPR; ICN Biomedicals, Aurora, OH); thiotepa (Lederle Laboratories, Pearl River, NY); irinotecan (Pharmacia & Upjohn, Kalamazoo, MI); and topotecan (SmithKline Beecham Pharmaceuticals, Philadelphia, PA).

Cytotoxicity studies

AlamarBlue assay. Cells (500/well) were transferred to a 384-well tissue culture plate (Corning, Corning, NY) and allowed to attach for 24 h. Compounds were then applied in an orthogonal pattern as described.[16] Following a 5-day incubation, the redox indicator dye, alamarBlue (AccuMed International, Westlake, OH) was added to each well at a 10% (v/v) ratio, and fluorescence was monitored at 535 nm excitation and 595 nm emission.

Crystal violet assay. Exponentially growing cells were transferred at a density of approximately 5000 cells/well to a 96-well tissue culture plate and allowed to attach for 24 h. Compounds were then applied simultaneously in duplicate serial dilutions. Etoposide, dipyridamole and NBMPR were diluted from DMSO stocks to final concentrations no greater than 0.6% DMSO, which was shown in previous experiments to have no effect on cell proliferation. Each compound was tested separately and mixed together at a single molar ratio approximately equal to the ratio of the individual IC_{50} values. After an additional 72-h incubation, cells were washed once with PBS and stained with 0.5% crystal violet in methanol. Plates were washed gently in water to remove unbound stain and allowed to dry overnight. Crystal violet stain bound to the total protein of attached cells was redissolved in Sorenson's buffer (0.025 M sodium citrate/0.025 M citric acid in 50% ethanol) and absorbence monitored at 535 nM.[17] Each drug combination was tested in duplicate in at least three separate assays.

Calculation of drug interactions

Cytotoxic effect levels and drug interactions were assessed by the combination index method[16,18] using the CalcuSyn software from Biosoft (Ferguson, MO). Briefly, the IC_{50} and the slope parameter (m) for each agent alone were determined from the median-effect plot, an x,y plot of log(D) versus log (f_a/f_u) based on Chou's median-effect equation:

$$f_a/f_u = (D/D_m)^m \quad (1)$$

where D is the dose of the drug, D_m is the IC_{50} as determined from the x-intercept of the median-effect

plot, f_a is the fraction of cells affected, f_u is the fraction of cells unaffected ($f_u = 1 - f_a$) and m is an exponent signifying the steepness of the sigmoid dose–effect curve. Only experiments with linear correlation coefficients (r) > 0.9 were accepted for analysis. A combination index (CI) was then calculated to assess synergism or antagonism according to the following equation which assumes an independent mechanism of drug action (mutual exclusivity):

$$CI = (D)_1/(D_x)_1 + (D)_2/(D_x)_2 + (D)_1(D)_2/(D_x)_1(D_x)_2 \quad (2)$$

where $(D)_1$ and $(D)_2$ are the concentrations of drug 1 and drug 2 which *combined* produce x% inhibition, and $(D_x)_1$ and $(D_x)_2$ are the concentrations of each drug which *alone* produce x% inhibition. CI = 1 indicates an additive interaction, CI < 1 indicates synergy and CI > 1 indicates antagonism. For each experiment CIs from several different effect levels and concentrations of a constant molar ratio were averaged. Statistical tests (Student t-tests) were applied to determine if the average differed significantly from 1.0.

Results

Multiple drug-effect analysis of NB1011 in combination with chemotherapy drugs *in vitro*

Various anti-tumor agents from different mechanistic classes were analyzed in combination with NB1011 to identify those that enhance the anti-proliferative activity against tumor cells. The median-effect/combination index method by Chou and Talalay[16] was used to calculate CI values. CI values > 1 indicate synergy, CI = 1 indicates additivity and CI < 1 indicates antagonism.[16,19] A representative example using the median-effect combination index method is shown for the combination of vinblastine and NB1011 using H630R10 tumor cells (Table 1). CI values were calculated for various dose-effect levels spanning from 10 to 60% inhibition of cell proliferation using parameters derived from dose–response curves constructed for vinblastine alone, NB1011 alone or the combination at a fixed molar ratio (see Materials and methods for details). Table 1 shows that the CI values for the combination of NB1011 and vinblastine are < 1 across a broad range of dose-effect levels, indicating a synergistic interaction. Table 2 shows a summary of the data from the same analysis applied to all 13 anti-tumor agents tested in combination with NB1011 using H630R10 and MCF7TDX tumor cells. The indicated CI value for a given drug/NB1011 combination is the mean CI value calculated from several dose-effect levels.

Of the 13 agents tested, dipyridamole and NBMPR showed synergy (CI < 1.0) with NB1011 against both MCF7TDX and H630R10 tumor cells (Table 2). Vinblastine synergized with NB1011 against the H630R10 cells. Two of the remaining nine agents, irinotecan and taxol, showed an additive or antagonistic interaction (CI = 1–1.4) with NB1011, while all the other agents showed antagonism (CI > 1.5). The most antagonistic interaction was observed with 5-FU which gave CI = 3.19 against MCF7TDX cells (Table 2). This antagonism is expected given the TS-dependent conversion of NB1011 to its cytotoxic product. In light of these results, vinblastine, dipyridamole and NBMPR were chosen for further study.

Potentiation of the cytotoxicity of NB1011 by nucleoside transport inhibitors is selective for tumor cells

We determined whether the synergy with NB1011 was specific for tumor cells by evaluating the same

Table 1. Combination Index values calculated at various levels of growth inhibition of H630R10 cells treated with a combination of NB1011 and vinblastine

Agent	CI values at cell growth inhibitory concentration, $IC_{(x)}$[a]					Parameters		
	IC_{10}	IC_{25}	IC_{35}	IC_{45}	IC_{60}	D_m	m	r
NB1011						170 μM	0.73	0.98
Vinblastine						28 nM	1.27	0.96
NB1011 + vinblastine	0.70	0.52	0.50	0.53	0.45	37 μM	1.07	0.99
Interaction	synergy	synergy	synergy	synergy	synergy			

[a]CI = 1 indicates additivity, CI < 1 indicates synergy and CI > 1 indicates antagonism.[16] $IC_{(x)}$ = concentration at which cell proliferation is inhibited by x%. D_m = median-effect dose (IC_{50}), m = slope parameter and r = correlation coefficient (see Materials and methods for details). Assay done by crystal violet method. Average CI = 0.54 ± 0.04 (Table 3).

Table 2. Mean CI values for various classes of drugs in combination with NB1011

Drug	Class[20]	CI ± SEM	
		H630R10	MCF7TDX
Irinotecan	inhibition of topoisomerase I	1.26 ± 0.20	1.36 ± 0.38
Topotecan		ND	2.45 ± 0.85
Etoposide	inhibition of topoisomerase II	1.96 ± 0.28	3.13 ± 0.58
Vinblastine	inhibition of microtubule assembly	0.78 ± 0.32	1.09 ± 0.16
Taxol	stabilization of microtubules	0.99 ± 0.15	1.41 ± 0.32
Cisplatin	DNA damage	ND	1.51 ± 0.35
Oxaliplatin[a]		1.78 ± 0.06	2.24 ± 0.33
Thiotepa	alkylation	ND	2.23 ± 0.45
Doxorubicin	inhibition of nucleic acid synthesis	1.39 ± 0.13	1.96 ± 0.25
5-FU	inhibition of thymidylate synthase, DNA/RNA incorporation	ND	3.19 ± 0.35
Methotrexate	antifolate, inhibition of DHFR, TS	ND	1.78 ± 0.44
Dipyridamole[a]	inhibition of equilibrative nucleoside transport	0.79 ± 0.17	0.51 ± 0.05
NBMPR[a]		0.25 ± 0.11	0.43 ± 0.10

CI =1 indicates additivity, CI <1 indicates synergy and CI >1 indicates antagonism.[16] CI calculated as the average of at least four consecutive dose/effect levels. ND=not determined.
[a]Assays done by the crystal violet method. All other assays were done by the alamarBlue method.

Table 3. Average CI values for drugs tested in combination with NB1011 in tumor and normal cells

Drug	Cell line	CI ± SEM	*p*-value	Molar ratio[a]	NB1011 dose[b] (μM)	Drug dose[b] (μM)	Interaction
Dipyridamole	H630R10	0.75 ± 0.11	0.052	2	11–150	5.5–75	synergy
	MCF7TDX	0.51 ± 0.06	0.001	0.2	1.1–3.2	5.5–16	synergy
	Det551	1.17 ± 0.23	0.484	5	5.8–375	1.2–75	additivity
	CCD18co	1.30 ± 0.08	0.008	5	81–375	16–75	antagonism
NBMPR	H630R10	0.35 ± 0.07	0.001	1	1.5–500	1.5–500	synergy
	MCF7TDX	0.57 ± 0.17	0.029	3.33	0.15–150	0.045–45	synergy
	Det551	1.43 ± 0.16	0.026	3.33	32–300	9.7–90	antagonism
	CCD18co	3.93 ± 1.00	0.019	3.33	32–300	9.7–90	antagonism
Vinblastine	H630R10	0.54 ± 0.04	0.001	6000	4.1–54	0.0005–0.015	synergy
	MCF7TDX	1.44 ± 0.29	0.186	2000	0.4–1.9	0.0005–0.015	antagonism
	Det551	0.54 ± 0.10	0.003	50000	2.9–47	0.0005–0.015	synergy
	CCD18co	0.65 ± 0.10	0.008	50000	17–135	0.0005–0.015	synergy

[a]Molar ratio NB1011:drug.
[b]Doses at which interaction was assessed.

drug combinations in the two normal cell strains, Det551 and CCD18co. Results of these experiments are shown in Table 3. Consistent with the data shown in Table 2, vinblastine synergized with NB1011 only against the H630R10 tumor cells (CI=0.54) (Table 3). Furthermore, in Det551 and CCD18co normal cells, vinblastine interacted synergistically with NB1011 to a similar extent as in H630R10 cells (CI=0.54 and 0.65, respectively). This lack of selectivity in the potentiation of NB1011 by vinblastine would most likely limit the use of this combination in the clinic possibly by increasing toxicity to normal tissues. The nucleoside transport inhibitor, dipyridamole, synergized with NB1011 against the tumor cells (CI=0.75 and 0.51), but did not show synergy with NB1011 against the normal cells (CI=1.17 and 1.30). An example of an assay analyzing NB1011 in combination with dipyridamole using MCF7TDX cells is shown in Figure 1. Combining NB1011 and dipyridamole (Figure 1, column 5, rows c,d) at drug concentrations that by themselves do not inhibit cell proliferation (Figure 1, column 5, rows a,b and e,f) result in a strong anti-proliferative effect (Figure 1, column 5). Similar results were obtained with another inhibitor of equilibrative nucleoside transport, NBMPR. NBMPR showed synergy with NB1011 in the tumor cells (CI=0.35 and 0.57), but no synergy with NB1011 was observed in the normal cells (CI=1.43 and 3.93). Taken together, these data indicate that two of the 13 agents tested,

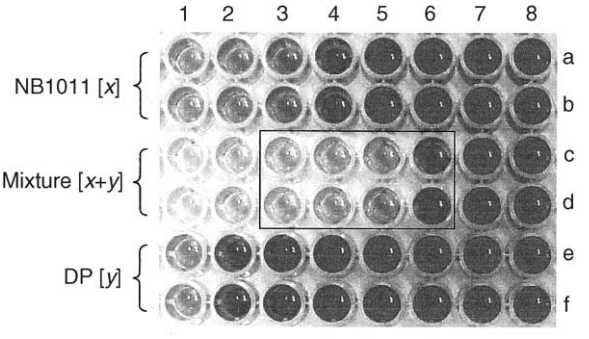

Figure 1. NB1011/dipyridamole combination cytotoxicity assay. Each column contains duplicate wells of the combination of NB1011 and dipyridamole (rows c and d), NB1011 alone (rows a and b), and dipyridamole alone (rows e and f). The concentration of NB1011 and dipyridamole in the mixture is equivalent to the concentration of each single agent in the same column. Compounds were applied in uniform serial dilutions from left to right (NB1011: 18, 10.8, 6.5, 3.9, 2.3, 1.4 and 0.8 μM; dipyridamole: 90, 54, 32.4, 19.4, 11.7, 7.0 and 4.2 μM) and a control (no treatment) was included (column 8). Mixture dilutions in the boxed range (columns 3–6) exhibit a combined cytotoxic effect which is greater than the expected additive effect of each single agent at the same concentration. This result is particularly apparent in columns 4 and 5, where the mixed treatments strongly inhibited cell proliferation (greater than 70%), but the single-agent treatments at comparable concentrations had little effect.

dipyridamole and NBMPR, which are both inhibitors of equilibrative nucleoside transport, potentiate the cytotoxic activity of NB1011. This enhancement of NB1011 cytotoxicity by dipyridamole and NBMPR appears specific for the tumor cells, since no synergy was observed for these combinations against the two normal cell types analyzed.

Synergy between NB1011 and dipyridamole persists in cell culture medium depleted of nucleosides and nucleobases

Dipyridamole potentiates the cytotoxicity of various chemotherapeutic drugs including PALA, MTA, 5-FU and TDX.[14,21–23] The mechanism of potentiation is thought to relate to the ability of dipyridamole to block the uptake and therefore salvage of pyrimidines. However additional non-salvage-dependent mechanisms have been suggested.[23,24] In addition to blocking the influx of nucleosides and nucleobases, dipyridamole has been reported to also inhibit the efflux of deoxyuridine, thereby enhancing the intracellular accumulation of deoxyuridine nucleotides.[14,24] One way to distinguish between dipyridamole's potentiating the cytotoxic activity of NB1011 by inhibition of nucleoside influx versus efflux is to analyze MCF7TDX cells growing in medium supplemented with fetal bovine serum (FBS) or with dialyzed FBS which is free of salvage pathway pyrimidines. If dipyridamole works by affecting pyrimidine uptake, its effects should be specific for the standard serum. Results from these experiments are shown in Table 4. Synergy was observed between NB1011 and dipyridamole at similar levels in experiments using medium supplemented with either FBS or dialyzed FBS. These results suggest that the mechanism by which dipyridamole potentiates the cytotoxicity of NB1011 against MCF7TDX cells does not involve salvage, and may instead involve intracellular accumulation of a toxic product of NB1011 generated by TS.

Discussion

In this study we set out to employ a mechanism-based screen for chemotherapeutic drugs that would enhance the anti-proliferative activity of NB1011 against tumor cells. Our results show that of the 13 compounds tested, the only ones that were effective were the nucleoside transport inhibitors, dipyridamole and NBMPR. Furthermore, potentiation occurred selectively in tumor cell lines expressing high TS levels. Importantly, these agents did not show synergy with NB1011 when tested in the two normal cell types, suggesting the potential

Table 4. Synergy between NB1011 and dipyridamole in MCF7TDX cells using medium supplemented with either FBS or dialyzed FBS

Mixture composition[a]		CI	
[NB1011] (μM)	[dipyridamole] (μM)	FBS	Dialyzed FBS
0.8	4.0	1.07	1.41
1.25	6.25	0.59	0.64
1.65	8.25	0.50	0.46
2.45	12.25	0.68	0.64

[a]Molar ratio of NB1011 and dipyridamole is constant at 1:5.

therapeutic efficacy of this type of drug combination *in vivo*.

Dipyridamole and NBMPR are potent competitive inhibitors of equilibrative nucleoside transport. Equilibrative nucleoside transporters fall into two distinct classes, *es* (equilibrative sensitive) and *ei* (equilibrative insensitive), depending on their characteristic sensitivity to NBMPR. Transporters of the *es* type are fully inhibited by nanomolar concentrations of NBMPR, whereas those of the *ei* type are insensitive to NBMPR concentrations as high as 1 μM. Both equilibrative nucleoside transporter types are inhibited by dipyridamole.[25,26] Dipyridamole (Persantine), used clinically as an antiplatelet agent and a vasodilator,[27] has been shown to enhance the potency of several antitumor agents *in vitro.* These include: 5-FU,[14,28] doxorubicin,[29] etoposide,[28] raltitrexed [23] and methotrexate.[30]

One of the mechanisms by which dipyridamole potentiates the activity of these antitumor agents is by inhibiting the uptake of nucleosides like uridine and thymidine, thereby blocking salvage pathways.[21,27] However, dipyridamole has been shown to still synergize with various drugs in experiments using dialyzed serum, which limits the availability of exogenous nucleosides such as thymidine.[14,23] This suggests the existence of other non-salvage dependent mechanisms. Indeed dipyridamole has, apart from inhibiting nucleoside influx, been reported to block the efflux of nucleosides like deoxyuridine[24] and nucleoside analogs such as fluorodeoxyuridine.[14] The detailed mechanism by which dipyridamole specifically sensitizes tumor cells to NB1011 requires further study. However, the finding that this synergy persists in dialyzed serum (lacking salvage pathway nucleosides) suggests an efflux blockade with a resulting accumulation of toxic metabolites of NB1011.

Although dipyridamole acts to sensitize tumor cells to several antitumor drugs *in vitro,* its clinical application for this purpose has met with limited success.[31,32] Clinically achievable serum concentrations of dipyridamole are limited due to extensive binding to the serum protein, α_1 acid glycoprotein.[33] Depending upon the mode of delivery, a broad range of free dipyridamole concentrations has been reported: by oral dosing (450 mg in six equal doses daily), peak free dipyridamole concentrations in the serum were 24.1 nM on average; by continuous i.v. infusion, steady-state free dipyridamole concentrations were observed at an average of 27.8 nM;[32] and by local infusion, specifically i.p. infusion, higher steady-state free dipyridamole i.p. concentrations above 25 μM have been attained.[34,35] In our *in vitro* combination cytotoxicity experiments potentiation of NB1011 was observed at dipyridamole concentrations ranging from 5.5 to 75 μM (Table 3). Since protein binding in the cell culture medium (supplemented with 10% FBS) is minimal (below 25%),[36] the dipyridamole concentrations used in our experiments reflect free dipyridamole levels. Therefore, a minimum concentration of approximately 5 μM steady-state free dipyridamole would likely be required to bring about potentiation of NB1011 by dipyridamole in patients. This level is achievable by local infusion, which may represent a practical means of delivering dipyridamole in a clinically efficacious form for combination with NB1011. Additionally, recent studies have shown that dipyridamole analogs with reduced serum protein binding can effectively potentiate the cytotoxic activity of various anticancer agents such as CB3717, nolatrexed and 5-FU.[37] These new analogs of dipyridamole may be promising alternatives to overcome the difficulties in achieving effective dose levels which have limited the clinical application of dipyridamole.

Our results show that inhibition of nucleoside transporter function can dramatically increase the sensitivity of high TS-expressing tumor cells to the cytotoxic effects of NB1011. Remarkably, this synergy is observed only with the tested tumor cells and not with the normal cells we have examined. While others have noted enhanced activity of modified nucleosides in the presence of dipyridamole,[14,26] the synergistic activity reported with NB1011 has not been observed previously. This especially applies to the lack of synergistic toxicity on normal cells which have low TS levels. These results support the novel mechanism of action of NB1011 as a nucleotide substrate of TS, as opposed to the classical inhibitors of TS function now in clinical use.

Acknowledgments

We thank Malgorzata Beryt (UCLA School of Medicine) for her expert technical assistance.

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(Received 13 September 2001; accepted 2 October 2001)