Synthesis of 3′- and 5′-Nitrooxy Pyrimidine Nucleoside Nitrate Esters: "Nitric Oxide Donor" Agents for Evaluation as Anticancer and Antiviral Agents

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A group of 3′-*O*-nitro-2′-deoxyuridines, 3′-*O*-nitro-2′-deoxycytidines, and 5′-*O*-nitro-2′-deoxyuridines possessing a variety of substituents (H, Me, F, I) at the C-5 position were synthesized for evaluation as anticancer/antiviral agents that have the ability to concomitantly release cytotoxic nitric oxide (•NO). Although these compounds generally released a greater percent of •NO than the reference drug isosorbide dinitrate upon incubation in the presence of L-cysteine, or serum, their cytotoxicity (CC_{50} = 10^{-3} to 10^{-6} M range) was comparable to 5-iodo-2′-deoxyuridine, but weaker than 5-fluoro-2′-deoxyuridine, against a variety of cancer cell lines. No differences in cytotoxicity against nontransfected (KBALB, 143B), and the corresponding transfected (KBALB−STK, 143B-LTK) cancer cell lines possessing the herpes simplex virus type 1 (HSV-1) thymidine kinase gene (TK^+) were observed, indicating that expression of the viral TK enzyme did not provide a gene therapeutic effect. These nitrate esters were inactive antiviral agents except for 5-iodo-3′-*O*-nitro-2′-deoxyuridine that showed modest activity against HSV-1, HSV-2, and vaccinia virus.

Introduction

Since the discovery that nitric oxide (•NO) is biosynthesized in mammalian cells, •NO has become a molecule of immense interest in medical research. In mammalian systems, •NO is synthesized via an enzymatic oxidation of L-arginine by nitric oxide synthase isozymes [neuronal (nNOS), inducible (iNOS), endothelial (eNOS)] that are differentially expressed and play different physiological roles.[1−4] •NO production in response to immune activation, or inflammatory reaction, has been shown to serve as part of the host defense system against cancerous cells, and intracellular microbes and parasites.[5] In this regard activated macrophages, that produce •NO, have been shown to inhibit DNA synthesis in tumor cells.[6] Pretreatment of various cell lines with a •NO-donor led to inhibition of the replication of poliovirus, picornavirus, and rhinovirus.[3] In addition, IFN-γ treated cells produce •NO that inhibits HSV-1 (herpesvirus).[3] Other studies indicate that the antitumor activity of IL-1α may be mediated by the production of •NO from tumor-derived endothelial cells,[7] and that macrophage •NO synthesis delays progression of ultraviolet light-induced murine skin cancer through to an antitumor immune response.[8]

•NO plays an important role in the action of a number of anticancer drugs due to its ability to influence various aspects of tumor biology including modulation of cell growth,[9] apoptosis,[10] differentiation,[11] metastatic capability,[12] and tumor-induced immunosuppression.[13] Thus, adriamycin stimulates •NO production in EMT-6 cells, and in vivo adriamycin inhibits tumorigenesis partially via a •NO-dependent, nonapoptotic mechanism.[14] Several antiviral acyclic nucleotide analogues activate expression of cytokine genes such as TNF-α and IL-10 in macrophages, and IFN-γ in splenocytes, resulting in an enhanced production of •NO.[15] A group of glyco-*S*-nitrosothiols were found to be cytotoxic to DU-145 human prostate cancer cells in vitro.[16] Induction of •NO production by cytokines indicated that high levels of •NO may contribute to the induction of apoptosis and inhibition of pancreatic tumor growth.[17] A correlation exists between the intracellular levels of •NO with the cytotoxicity of hydroxyguanidine in HL60 cells, where a higher toxicity was observed in hypoxic cells relative to oxic cells.[18] The hypoxic cell radiosensitization effects of the •NO-donor $Et_2N[N(NO)]^- Na^+$, coupled with the vasodilatory effect of •NO on tumor vasculature, suggest that an agent of this type opens a new avenue in radiation oncology treatment.[19] The rate and extent of •NO release from •NO/nucleophile adducts, which release •NO spontaneously in solution, correlated with inhibition of DNA synthesis in A375 human melanoma cells.[9] Tamoxifen has been shown to induce iNOS, producing •NO that is cytotoxic.[20] •NO-induced apoptosis in macrophages activated with cisplatin and IFN-γ requires activation of an endonuclease.[21] •NO-donor agents have been shown to enhance melphalan cytoxicity in breast cancer MCF-7 cells, possibly by delaying entry into the S-phase and a G2/M block.[22] Using a number of aromatic *N*-nitroso compounds, it was shown that the extent of •NO generation was the reciprocal of ID_{50} growth inhibition (cytotoxicity).[23] These data collectively indicate that •NO exhibits a cytotoxic effect and/or enhances the cytotoxic effect of anticancer drugs. However, there are a few instances where •NO is detrimental to anticancer activity. For example, •NO generated from a •NO-donor drug was shown to prevent

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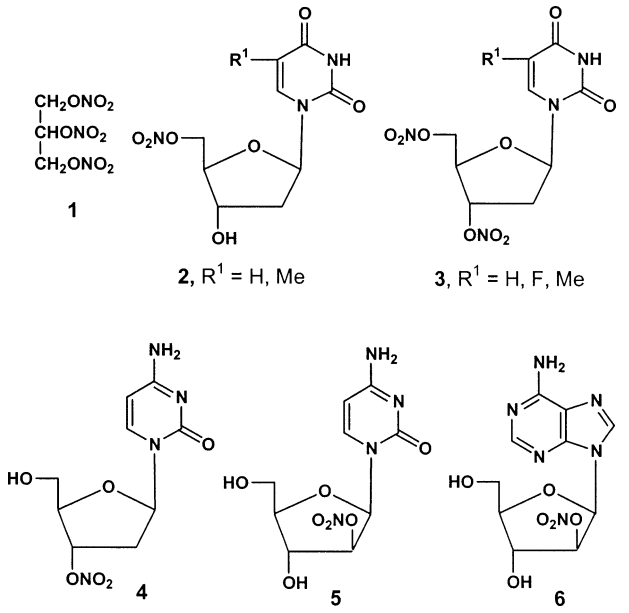
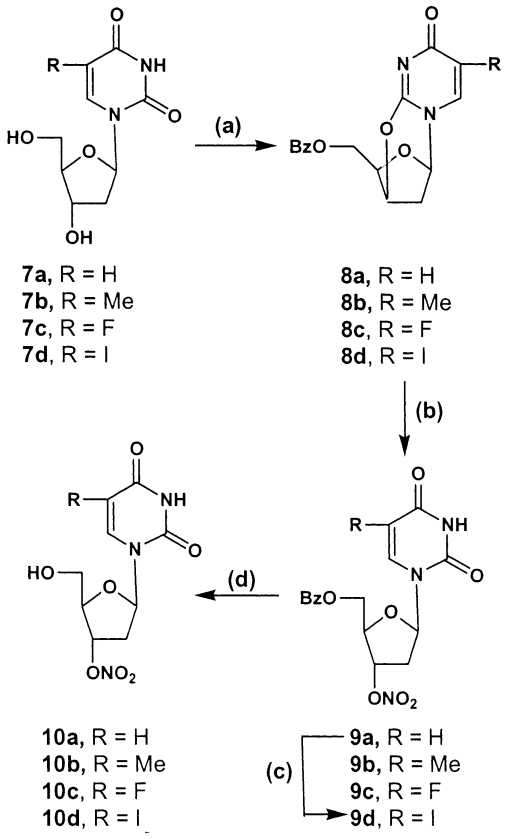


Figure 1. Structures of glycerol trinitrate (**1**), 5′-*O*-nitro-2′-deoxyuridine (**2**), 3′,5′-di-*O*-nitro-2′-deoxyuridine (**3**), 3′-*O*-nitro-2′-deoxycytidine (**4**), nitrara-C (**5**), and nitrara-A (**6**) nitrate esters.

taxol-induced apoptosis, and this was attributed to inhibition of an IL-1 beta converting enzyme-like protease cascade (human neuroblastoma cell line, NB-39-nu).[24] A related study showed that the protective effect of •NO on taxol-induced apoptosis may be partially caused by inhibiting entrance of the cells into the G2/M phase (human leukemia HL-60 cells).[25] These data indicate that the efficacy of an •NO-donor anticancer drug may be dependent upon the specific cell line.

Organic nitrates ($RONO_2$), which are nitric acid esters of mono- or polyhydric alcohols, represent the oldest class of •NO-donor compounds that include the classical drug glycerol trinitrate (**1**) that is used to treat angina pectoris.[26] Although some 5′-*O*-nitro-2′-deoxyuridine (**2**),[27,28] 3′,5′-di-*O*-nitro-2′-deoxyuridine (**3**),[29−31] and 3′-*O*-nitro-2′-deoxycytidine (**4**)[32] compounds have been synthesized, no biological data were reported (see structures in Figure 1). On the other hand, the 2′-*O*-nitro derivatives of arabinocytidine (Nitrara-C, **5**), which resists enzymatic deamination, exhibited antileukemic activity in vitro and in vivo.[33] The related 2′-*O*-nitro derivative of arabinoadenine (Nitrara-A, **6**), that is deaminated about 25-fold slower than Ara-A, inhibited the proliferation of human T-lymphoblasts (CCRF−CEM cell line) in vitro.[34] The design of tumor selective •NO-donor/nucleoside hybrid drugs, that release both cytotoxic •NO and a nucleoside drug in a controlled fashion, constitutes an attractive method to develop a novel class of double-barreled anticancer drugs. The nucleoside component of these hybrid drugs, as the 5′-monophosphate, could act as an irreversible inhibitor of thymidylate synthase (TS), whereas the 5′-triphosphate could serve as an unnatural substrate for, or inhibitor of, DNA polymerase to elicit a cytotoxic anticancer/antiviral effect. Accordingly, we now report the syntheses, in vitro •NO release data, and anticancer and antiviral activities, for a group of 3′-*O*-nitro-2′-deoxyuridines (**10a**−**d**), 3′-*O*-nitro-2′-deoxycytidines (**13a**−**c**), and 5′-*O*-nitro-2′-deoxyuridines (**15a**−**c**).

Scheme 1[a]



[a] Reagents and conditions: (a) C_6H_5COOH, Ph_3P, DIAD, DMF, 25 °C; (b) NH_4NO_3, DMF, 110 °C; (c) ICl, NaN_3, MeCN, 0 °C → 25 °C; (d) NaOMe, MeOH, 25 °C.

Chemistry

A group of 2,3′-anhydro-5′-*O*-benzoyl-2′-deoxyuridines **8a**−**d** possessing a variety of C-5 substituents (R = H, Me, F, I) were prepared from the 2′-deoxyuridine precursors **7a**−**d** by a one-pot tandem Mitsunobu reaction,[35,36] using triphenylphosphine, diisopropyl azodicarboxylate, and benzoic acid in 81%, 74%, 90%, and 82% yield, respectively. Ring opening of the 2,3′-anhydro derivatives **8a**−**d** proceeded readily upon reaction with ammonium nitrate (NH_4NO_3) in dimethyl formamide to form the respective 3′-*O*-nitrate esters **9a**−**d** in 31%, 34%, 50%, and 2.8% yield, respectively. Since conversion of the 5-iodo compound **8d** to **9d** was low (2.8% yield) in this reaction, compound **9d** was prepared by iodination of **9a** using iodine monochloride (ICl) and sodium azide (NaN_3) in acetonitrile[37] in 82.5% yield (see Scheme 1). Deprotection of compounds **9a**−**d** using NaOMe in MeOH afforded the target 5-substituted-3′-*O*-nitro-2′-deoxyuridines **10a**−**d** in 91−95% yield.

The configuration at the C-3′ carbon, and the rotamer orientation of the uracil (**10a**) and thymine (**10b**) ring, was analyzed by nuclear Overhauser enhancement (NOE) ^{1}H NMR difference spectroscopy (Figure 2). Selective irradiation of the H-3′ signal resulted in an enhancement of the arabino (up) H-2′ signal of **10a** (2.2%), and of **10b** (1.9%), indicating that the C-3′ nitrooxy substituent is in the ribo (down) position. No NOE effect was observed between H-3′ and either H-2′ or H-1′ of **10a**,**b**. The observation that selective irradiation of the H-4′ signal resulted in enhancement of the H-1′ signal of **10a** (3.1%), and of **10b** (2.5%), indicates

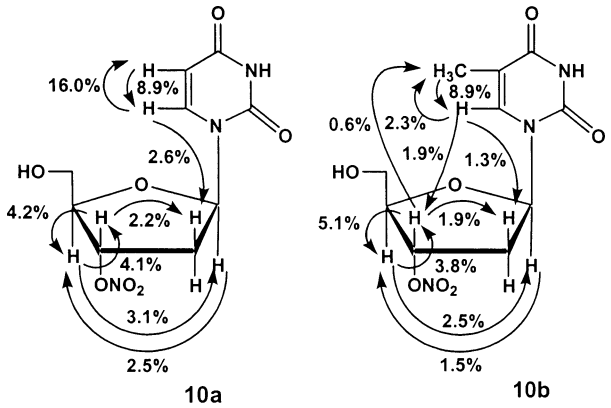


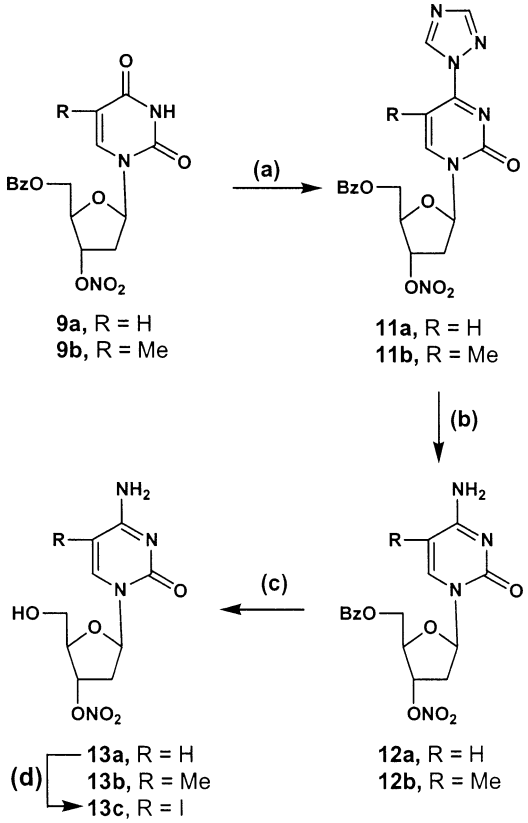
Figure 2. Some NOE measurements to determine the rotameric orientation of the uracil base moiety, and the conformation of the nitrooxy substituent, for the 3′-*O*-nitro-2′-deoxyuridine compounds **10a**,**b** in DMSO-d_6 at 22 °C.

that these two hydrogen atoms are on the same face of the sugar moiety which is indicative of the β-configuration.[38] Selective irradiation of the H-6 signal of **10a** produced an enhancement of the arabino (up) H-2′ signal (2.6%), whereas a similar irradiation for **10b** showed enhancement for the arabino H-2′ (1.3%), and the H-3′ (1.9%), signals. These NOE data indicate the C-6 hydrogen of the uracil moiety of **10a**, or the thymine moiety of **10b**, is oriented in the direction of the sugar ring.

A related group of 3′-*O*-nitro-2′-deoxycytidines **13a**−**c** having a C-5 hydrogen, methyl, or iodo substituent were prepared using the three-step reaction sequence illustrated in Scheme 2. Thus, reaction of the 5′-*O*-benzoyl-3′-*O*-nitro-2′-deoxyuridines **9a**,**b** with 1,2,4-triazole and 4-chlorophenyl dichlorophosphate in pyridine[39−41] afforded the corresponding 4-(1,2,4-triazolo) derivatives **11a** and **11b** in 79 and 71% yield, respectively. Subsequent treatment of **11a**, or **11b**, with aqueous ammonia in dioxane[39−41] yielded the 2′-deoxycytidine compound **12a** (49%), or **12b** (36%), which on deprotection with NaOMe in MeOH to remove the 5′-*O*-benzoyl moiety, afforded the target 3′-*O*-nitro-2′-deoxycytidine **13a** (95%), or 5-methyl-3′-*O*-nitro-2′-deoxycytidine **13b** (94%), respectively. Iodination of **13a** with iodic acid and iodine[42,43] yielded 5-iodo-3′-*O*-nitro-2′-deoxycytidine **13c** (56%). The procedure described above for the synthesis of **13a** is superior to the reported method involving nitration of 2′-deoxycytidine 5′-*O*-monophosphate using nitronium tetrafluoroborate, which gave a mixture of the 5′-*O*-monophosphates of 5-nitro-3′-*O*-nitro-2′-deoxycytidine, 5-nitro-2′-deoxycytidine, and 3′-*O*-nitro-2′-deoxycytidine in a ratio of 12:1:3 that were subsequently dephosphorylated by the action of alkaline phosphatase to the free nucleosides.[32]

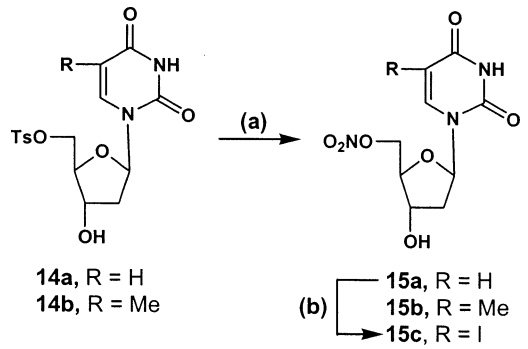
The 5′-*O*-nitro-2′-deoxyuridines **15a**−**c** possessing a C-5 H, Me, or I substituent were also prepared (see Scheme 3). Accordingly, nucleophilic displacement of the 5′-*O*-tosylate moiety in **14a**, or **14b**, using $LiNO_3$ in DMF at 110 °C, afforded the respective 5′-*O*-nitro product **15a** (73%), or **15b** (71%). This synthesis of **15a** was superior to nitration of 2′-deoxyuridine with 90% nitric acid at −70 °C which gave a mixture of **15a** (20%) and 3′,5′-di-*O*-nitro-2′-deoxyuridine (13%).[27] Synthesis of **15b** by displacement of the 5′-*O*-tosylate moiety in **14b**, was equally effective to a reported method involving displacement of either a 5′-*O*-P(=S)OEt_3, or 5′-I, substituent using $AgNO_3$.[28] Electrophilic iodination of **15a** using ICl and NaN_3 in MeCN gave the 5-iodo product **15c** (65%).

Scheme 2[a]



[a] Reagents and conditions: (a) 4-chlorophenyl dichlorophosphate, 1,2,4-triazole, pyridine, 0 °C → 25 °C; (b) NH_4OH, dioxane, 25 °C; (c) NaOMe, MeOH, 25 °C; (d) iodine, iodic acid, acetic acid, H_2O, CCl_4, 25 °C.

Scheme 3[a]



[a] Reagents and conditions: (a) $LiNO_3$, DMF, 100−110 °C, overnight; (b) ICl, NaN_3, MeCN, 0 °C → 25 °C, 48 h.

Results and Discussion

Organic nitrate esters are generally stable in neutral or mild acidic aqueous solution, but nucleophilic hydrolysis to an alcohol and nitrate occur under strongly alkaline conditions. •NO release from organic nitrates may occur as a result of either nonenzymatic or enzymatic biotransformation that proceeds via a three-electron reduction. Accordingly, cellular thiols may play a role in the nonenzymatic production of •NO from glycerol trinitrate (**1**, GTN) whereas, a NADPH-depend-

Table 1. In Vitro Percent Nitric Oxide Release for 3′-*O*-Nitro-2′-deoxyuridines (**10a**−**d**), 3′-*O*-Nitro-2′-deoxycytidines (**13a**−**c**), and 5′-*O*-nitro-2′-deoxyuridines (**15a**−**c**)

	% NO release[a] (18 mM L-cysteine)		% NO release[b] (serum)	
compd	1 hour	16 hours	1 hour	16 hours
10a	4.9 ± 0.0	51.0 ± 0.1	7.7 ± 0.7	9.0 ± 1.5
10b	4.9 ± 0.1	53.6 ± 0.7	7.4 ± 0.9	7.4 ± 0.3
10c	0.0 ± 0.0	3.8 ± 0.1	4.0 ± 0.9	6.6 ± 0.7
10d	3.9 ± 0.1	37.6 ± 0.1	12.3 ± 1.1	13.2 ± 0.3
13a	1.5 ± 0.0	17.1 ± 0.2	18.8 ± 0.9	20.2 ± 0.9
13b	3.8 ± 0.0	50.3 ± 0.7	47.1 ± 3.1	59.5 ± 1.5
13c	4.0 ± 0.1	47.2 ± 0.0	12.5 ± 0.5	17.6 ± 1.7
15a	5.4 ± 0.1	59.9 ± 1.7	2.9 ± 0.8	4.4 ± 1.0
15b	5.0 ± 0.0	59.8 ± 0.2	3.6 ± 0.4	4.8 ± 1.1
15c	3.7 ± 0.1	36.4 ± 0.4	3.9 ± 0.5	4.7 ± 1.0
ISDN[c]	3.5 ± 0.2	24.0 ± 0.2	1.9 ± 0.1	2.6 ± 0.6

[a] The percent nitric oxide released from the test compound was determined as the percent of nitrite (NO_2^-) produced in the presence of L-cysteine (18 mM) as quantitated using the Griess reagent (±SD, $n = 3$). [b] The percent nitric oxide released from the test compound was determined as the percent of nitrite (NO_2^-) produced in the presence of serum as quantitated using the Griess reagent (±SD, $n = 3$). [c] ISDN = isosorbide dinitrate (ISDN possesses two ONO_2 groups which may release •NO, whereas compounds **10**, **13**, and **15** possess only one ONO_2 group that may release •NO).

ent cytochrome P450 pathway and specific isozymes of the glutathione-*S*-transferase group are thought to be operative in the biotransformation/bioactivation of organic nitrates.[26] Although the sole product from reaction of •NO with oxygen in water is nitrite, •NO reacts rapidly with superoxide anion to initially produce a cytotoxic peroxynitrite anion ($ONOO^-$) species that can damage DNA.[5]

The percentages of •NO released, quantitated as nitrite (NO_2^-) using the Griess reaction,[44] upon incubation of the 3′-*O*-nitro derivatives of 2′-deoxyuridine (**10a**−**d**) and 2′-deoxycytidine (**13a**−**c**), and the 5′-*O*-nitro derivatives of 2′-deoxyuridine (**15a**−**c**) in the presence of 18 mM L-cysteine or serum are listed in Table 1. All three groups of compounds (**10**, **13**, **15**) that possess a single ONO_2 moiety, with the exception of 5-fluoro-3′-*O*-nitro-2′-deoxyuridine (**10c**), exhibited comparable or in most instances superior nitric oxide release (1.5−5.4% range at 1 h, and 17.1−59.9% range at 16 h) upon incubation in the presence of L-cysteine compared to the reference drug isosorbide dinitrate (3.5 and 24% release at 1 h and 16 h) that contains two ONO_2 moieties. These results are consistent with the observation that thiols enhance in vitro release of •NO in aqueous buffer solution for •NO-donor compounds although intact cells and tissues show that conversion of organic nitrate esters ($RONO_2$) to •NO is not free-thiol dependent.[45] In contrast, release of •NO from **10**, **13**, and **15** was negligible in the absence of L-cysteine (<0.05% at 1 h and <1.0% at 16 h). A similar study to determine the release of •NO upon incubation in the presence of serum showed that 3′-*O*-nitro-2′-deoxycytidine compounds (**13**) generally provided a greater percent •NO release than the related compounds **10** and **15**. The unusually high release of •NO from 5-methyl-3′-*O*-nitro-2′-deoxycytidine (**13b**, 47.1%) upon incubation with serum at 1 h, relative to that in the presence of 18 mM L-cysteine (3.8% release), indicates that •NO must be released from **13b** by a thiol-independent mechanism in serum. Compounds **10a**−**d**, **13a**−**c**, and **15a**−**c** all exhibited a greater release of •NO in the presence of serum (2.9−47.1% range at 1 h, and 4.4−59.5% range at 16 h) than the reference drug isosorbide dinitrate (1.9% at 1 h and 2.6% at 16 h).

Compounds **10a**−**d**, **13a**−**c**, **15a**−**c**, and the reference compounds 5-iodo-2′-deoxyuridine (IUDR), 5-fluoro-2′-deoxyuridine (FUDR), and thymidine, were evaluated for their tumor cell cytotoxicity using the MTT cytotoxicity assay[46] (see data in Table 2). These 3′- and 5′-*O*-nitro pyrimidine nucleosides exhibited comparable cytotoxicity ($CC_{50} = 10^{-3}$ to 10^{-6} M range) to 5-iodo-2′-deoxyuridine, but weak cytotoxicity in comparison to 5-fluoro-2′-deoxyuridine, against KBALB, KBALB−STK, 143B, 143B-LTK, and EMT-6. 5-Fluoro-3′-*O*-nitro-2′-deoxyuridine (**10c**) was the most cytotoxic compound against this group of cancer cell lines ($CC_{50} = 10^{-6}$ M range). Elaboration of the 3′-OH group of IUDR to a 3′-*O*-NO_2 group (**10d**) decreased cytotoxicity by 2.5- to 26-fold in all cell lines except for EMT-6 cells where cytotoxicity was enhanced by about 6-fold. The position of the *O*-NO_2 substituent was not a determinant of anticancer activity, since 5-iodo-3′-*O*-nitro-2′-deoxyuridine (**10d**) and 5-iodo-5′-*O*-nitro-2′-deoxyuridine (**15c**) were approximately equipotent. The observation that compounds **10c**−**d** and **13c** exhibited similar cytotoxicity against nontransfected (KBALB, 143B), and the corresponding transfected (KBALB−STK, 143B-LTK) cancer cell lines possessing the herpes simplex virus type 1 (HSV-1) thymidine kinase gene (TK^+) indicates that expression of the viral TK enzyme did not provide a gene therapeutic effect. This observation was also made for **10a** and **10b** that did not show increased cytostatic activity in murine mammary (FM3A) and human osteosarcoma cells transfected with the HSV-1 TK gene when compared with their parental counterparts (data not shown). The •NO drug ISDN exhibited comparable cytotoxicity ($CC_{50} = 10^{-4}$ M range) against all cell lines tested (Table 2).

Compounds **10a**−**d** were evaluated for their antiviral activity in a wide variety of assay systems.[47] Antiviral activities against herpes simplex virus type 1 (KOS), herpes simplex virus type 2 (G), thymidine kinase-deficient (TK^-) herpes simplex virus type 1 (KOS, ACV^r), vaccinia virus, and vesicular stomatitis virus in E_6SM cells were determined. These compounds were generally inactive or exhibited negligible activity in these antiviral assay systems (at concentrations up to 400 μg/mL). 5-Iodo-3′-*O*-nitro-2′-deoxyuridine (**10d**) showed some inhibitory activity against HSV-1 (KOS), HSV-2 (G), and vaccinia virus ($IC_{50} = 48$ μg/mL), relative to the reference drug (*E*)-5-(2-iodovinyl)-2′-deoxyuridine ($IC_{50} = 0.0051$, 80.0, and 2.3 μg/mL, respectively). In addition, compounds **10a**−**d** did not reduce cytopathicity induced by parainfluenza-3 virus, reovirus-1, Sindbis virus, Coxsakie B4 virus, or Punta Toro virus in Vero cell cultures ($IC_{50} > 80$ μg/mL), or respiratory syncytial virus in HeLa cell cultures ($IC_{50} > 80$ μg/mL).

The modest anticancer, and near absence of antiviral, efficacy for this novel class of •NO-donor nucleosides could be due to a number of factors. For example, it is possible that the sugar moiety does not undergo phosphorylation by thymidine kinase (TK) to the 5′-mono-

Table 2. In Vitro Cell Cytotoxicity for 3′-*O*-Nitro-2′-deoxyuridines (**10a**−**d**), 3′-*O*-Nitro-2′-deoxycytidines (**13a**−**c**), and 5′-*O*-Nitro-2′-deoxyuridines (**15a**−**c**)

	cellular toxicity (CC_{50}, M) toward various cell lines[a]				
compd	KBALB[b]	KBALB−STK[c]	143-B[d]	143B-LTK[c]	EMT-6[e]
10a	-	-	-	8.5×10^{-4}	-
10b	1.0×10^{-3}	7.0×10^{-3}	-	-	-
10c	8.0×10^{-6}	3.5×10^{-6}	2.4×10^{-6}	7.4×10^{-5}	6.2×10^{-6}
10d	2.4×10^{-4}	2.6×10^{-4}	5.8×10^{-4}	4.1×10^{-4}	2.3×10^{-5}
13a	-	-	-	2.0×10^{-4}	-
13b	-	-	-	1.0×10^{-3}	-
13c	1.0×10^{-3}	2.5×10^{-4}	2.0×10^{-4}	2.0×10^{-4}	3.4×10^{-4}
15a	1.0×10^{-3}	-	-	-	-
15b	-	3.0×10^{-4}	-	-	-
15c	3.4×10^{-4}	2.5×10^{-4}	3.4×10^{-4}	2.1×10^{-5}	-
IUDR[f]	9.7×10^{-5}	1.0×10^{-5}	7.0×10^{-3}	7.4×10^{-3}	3.8×10^{-4}
FUDR[g]	6.0×10^{-11}	8.8×10^{-11}	9.0×10^{-5}	1.0×10^{-4}	9.0×10^{-12}
thymidine	9.5×10^{-5}	1.0×10^{-4}	-	-	1.3×10^{-4}
ISDN[h]	9.7×10^{-4}	6.5×10^{-4}	6.0×10^{-4}	7.0×10^{-4}	-

[a] The molar concentration of the test compound that killed 50% of the cells (or 50% cell survival) upon incubation for 3−5 days at 37 °C in a humidified atmosphere of 95% air and 5% CO_2 (mean value, $n = 6$) was determined using the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) assay. [b]Transformed fibroblast sarcoma cell line. [c] These cells were transfected by, and expressed, the herpes simplex virus type 1 thymidine kinase (HSV-1 TK) gene. [d] Human osteosarcoma cell line. [e] Mouse mammary carcinoma cell line. [f] IUDR = 5-iodo-2′-deoxyuridine. [g] FUDR = 5-fluoro-2′-deoxyuridine. [h] ISDN = isosorbide dinitrate.

phosphate (5′-MP). Support for this explanation is based on the observations that there are generally negligible differences in anticancer/antiviral activities between nontransfected (KBALB, 143B) and viral TK-transfected (KBALB−STK, 143B-LTK) cell lines. Alternatively, this group of nucleoside nitrate esters may be devoid of anticancer/antiviral activity due to the fact that they are not inhibitors of thymidylate synthase (TS). Credence for this possibility is based on the belief that the anticancer activity exhibited by 5-fluoro-2′-deoxyuridine is primarily due to inhibition of DNA biosynthesis by blocking TS, the enzyme which catalyzes the methylation of 2′-deoxyuridine-5′-monophosphate to 2′-deoxythymidine-5′-monophosphate.[48,49] Furthermore, inhibition of TS is the mechanism by which certain nucleosides such as (*E*)-5-(2-iodovinyl)-2′-deoxyuridine and 5-(1-azidovinyl)-2′-deoxyuridine seem to exhibit their cytostatic effect.[50]

Conclusions

A group of nitric oxide donor 3′-*O*-nitro derivatives of 2′-deoxyuridine (**10**) and 2′-deoxycytidine (**13**), and 5′-*O*-nitro-2′-deoxyuridines (**15**), were designed to investigate the concept whether the concomitant release of cytotoxic nitric oxide may enhance the anticancer/antiviral efficacy of pyrimidine nucleosides. This group of compounds generally exhibits comparable and in most instances superior nitric oxide release, upon incubation with phosphate buffer containing 18 mM L-cysteine or in the presence of serum, compared to the reference drug isosorbide dinitrate. This group of 3′- and 5′-*O*-nitrate esters exhibit comparable in vitro cytotoxicity to 5-iodo-2′-deoxyuridine, but weaker cytotoxicity than 5-fluoro-2′-deoxyuridine, in a variety of cancer cell lines. These nitrate esters were devoid of antiviral activity except for 5-iodo-3′-*O*-nitro-2′-deoxyuridine, which showed marginal activity against HSV-1, HSV-2 and vaccinia virus in cell cultures.

Experimental Section

General. Melting points were determined with a Thomas-Hoover capillary apparatus and are uncorrected. Nuclear magnetic resonance (^{1}H NMR, ^{13}C NMR) spectra were recorded on a Bruker AM-300 spectrometer. Proton chemical shifts (δ) are given relative to internal TMS (δ 0), and the assignment of exchangeable protons (N*H*, O*H*) was confirmed by addition of D_2O. The nuclear Overhauser enhancement (NOE) studies were performed under steady-state conditions using the Bruker NOE DIFF.AU software program (signal:noise ratio of 136 for a single pulse). DMSO-d_6 was dried using molecular sieves (type 3A, 1.6-mm pellets) and degassed by passage of dry argon gas at 22 °C just prior to use. Molecular tumbling time was not altered. ^{13}C NMR spectra were acquired using the *J* modulated spin−echo technique where methyl and methine carbon resonances appear as positive peaks, methylene and quaternary carbon resonances appear as negative peaks, and carbon chemical shifts (δ) are given relative to $CDCl_3$ (δ 77). Elemental analyses were performed by the MicroAnalysis Service Laboratory, Department of Chemistry, University of Alberta, and the results were within ±0.4% of theoretical values for all elements listed. Silica gel 60A (Silicycle Co., 230−400 mesh) was used for all flash column chromatography separations. 5′-*O*-Tosyl-2′-deoxyuridine (**14a**)[51] and 5′-*O*-tosyl-2′-deoxythymidine (**14b**)[52] were prepared according to literature procedures. All other reagents were purchased from Aldrich Chemical (Milwaukee, WI).

General Method for the Preparation of 5-Substituted-2,3′-anhydro-5′-*O*-benzoyl-2′-deoxyuridines (8a−d). A solution containing either **7a**, **7b**, **7c**, or **7d** (65.7 mmol) and triphenylphosphine (Ph_3P, 25.9 g, 98.6 mmol) in DMF (90 mL) was prepared, a solution comprised of diisopropyl azodicarboxylate (DIAD, 19.5 mL, 98.6 mmol) and benzoic acid (12.1 g, 98.6 mmol) in DMF (40 mL) was added dropwise with stirring at 25 °C, and the reaction was allowed to proceed for 30 min at 25 °C. An additional aliquot of Ph_3P (98.6 mmol) and DIAD (98.6 mmol) was added, the reaction was allowed to proceed for 1 h, Et_2O (400 mL) was added, and the mixture was stirred for 10 min at 25 °C. The resulting suspension was cooled to ice-bath temperature, and the white crystalline precipitate was collected by filtration and washed with cold Et_2O to give the respective product **8a**, **8b**, **8c**, or **8d.** Physical and spectral data for **8a**−**d** are listed below.

2,3′-Anhydro-5′-*O*-benzoyl-2′-deoxyuridine (8a). White solid; yield, 81%; mp 225−226 °C; ^{1}H NMR (DMSO-d_6): δ 7.93 (d, $J = 7.8$ Hz, 2H, *o*-benzoyl hydrogens), 7.66 (d, $J = 7.5$ Hz, 1H, H-6), 7.63−7.68 (m, 1H, *p*-benzoyl hydrogen), 7.47−7.52 (m, 2H, *m*-benzoyl hydrogens), 5.97 (d, $J = 3.3$ Hz, 1H, H-1′), 5.78 (d, $J = 7.5$ Hz, 1H, H-5), 5.45 (br s, 1H, H-3′), 4.50−4.60 (m, 2H, H-5′), 4.37 (dd, $J = 11.7$, 6.3 Hz, 1H, H-4′), 2.52−2.88 (m, 2H, H-2′); ^{13}C NMR (DMSO-d_6): δ 170.05, 165.14, 153.50, 140.57, 133.34, 129.06, 128.92, 128.55, 107.88, 86.94, 81.86, 77.28, 62.48, 32.68. Anal. ($C_{16}H_{14}N_2O_5$) C, H, N.

2,3′-Anhydro-5′-*O*-benzoyl-2′-deoxythymidine (8b). White solid; yield, 74%; mp 245−246 °C; ^{1}H NMR (DMSO-d_6): δ 7.90 (dd, J = 6.9, 1.2 Hz, 2H, *o*-benzoyl hydrogens), 7.63−7.68 (m, 1H, *p*-benzoyl hydrogen), 7.57 (s, 1H, H-6) 7.46−7.57 (m, 2H, *m*-benzoyl hydrogens), 5.90 (d, J = 3.6 Hz, 1H, H-1′), 5.42 (br s, 1H, H-3′), 4.50−4.61 (m, 2H, H-5′), 4.36 (dd, J = 11.7, 5.4 Hz, 1H, H-4′), 2.53−2.88 (m, 2H, H-2′), 1.72 (s, 3H, CH_3); ^{13}C NMR (DMSO-d_6): δ 170.63, 165.14, 153.13, 136.32, 133.30, 129.04, 128.70, 128.37, 116.00, 86.81, 81.80, 77.07, 62.30, 32.71, 12.85. Anal. ($C_{17}H_{16}N_2O_5$) C, H, N.

5-Fluoro-2,3′-anhydro-5′-*O*-benzoyl-2′-deoxyuridine (8c). White solid; yield, 90%; mp 235−236 °C; ^{1}H NMR (DMSO-d_6): δ 8.10 (d, J = 5.4 Hz, 1H, H-6), 7.89−7.91 (m, 2H, *o*-benzoyl hydrogens), 7.62−7.68 (m, 1H, *p*-benzoyl hydrogen), 7.46−7.51 (m, 2H, *m*-benzoyl hydrogens), 5.92 (d, J = 3.9 Hz, 1H, H-1′), 5.48 (br s, 1H, H-3′), 4.54−4.65 (m, 2H, H-5′), 4.43 (dd, J = 11.7, 6.3 Hz, 1H, H-4′), 2.67−2.71 (m, 1H, H-2′α), 2.56 (dt, J = 13.5, 2.7 Hz, 1H, H-2′β); ^{13}C NMR (DMSO-d_6): δ 165.11 (*C*O$_2$), 162.16 (d, J_{CCF} = 16.5 Hz, C-4 *C*O), 151.22 (*C*-2), 144.31 (d, J_{CF} = 248.3 Hz, *C*-5), 133.32, 128.99 and 128.51 (phenyl *C*H), 128.90 (phenyl *C*-1), 125.34 (d, J_{CCF} = 33.0 Hz, *C*-6), 87.45 (*C*-1′), 82.11 (*C*-3′), 77.70 (*C*-4′), 62.21 (*C*-5′), 32.46 (*C*-2′). Anal. ($C_{16}H_{13}FN_2O_5$) C, H, N.

5-Iodo-2,3′-anhydro-5′-*O*-benzoyl-2′-deoxyuridine (8d). White solid; yield, 82%; mp 224−225 °C; ^{1}H NMR (DMSO-d_6): δ 8.30 (s, 1H, H-6), 7.86−7.94 (m, 2H, *o*-benzoyl hydrogens), 7.60−7.67 (m, 1H, *p*-benzoyl hydrogen), 7.46−7.51 (m, 2H, *m*-benzoyl hydrogens), 5.98 (d, J = 3.6 Hz, 1H, H-1′), 5.46 (br s, 1H, H-3′), 4.54−4.61 (m, 2H, H-5′), 4.42 (ddd, J = 11.7, 6.3, 5.4 Hz, 1H, H-4′), 2.55−2.88 (m, 2H, H-2′); ^{13}C NMR (DMSO-d_6): δ 166.57, 165.14, 153.80, 145.05, 133.34, 128.99, 128.88, 128.55, 87.15, 81.99, 80.68, 77.64, 62.16, 32.48. Anal. ($C_{16}H_{13}IN_2O_5$) C, H, N.

General Method for the Preparation of 5-Substituted-3′-*O*-nitro-5′-O-benzoyl-2′-deoxyuridines (9a−d). A mixture containing either **8a**, **8b**, **8c**, or **8d** (26.1 mmol) and NH_4NO_3 (31.3 g, 391.5 mmol) in dry DMF (120 mL) was stirred at 110−120 °C for 12 h under argon. The solvent was removed in vacuo, and the residue was purified via silica gel flash column chromatography using hexanes−EtOAc (1:3, 1:2, 1:7 or 1:1, v/v, respectively) as eluent to give the corresponding product **9a**, **9b**, **9c**, or **9d**. Physical and spectral data for **9a**−**d** are listed below.

3′-*O*-Nitro-5′-*O*-benzoyl-2′-deoxyuridine (9a). White crystals (CH_2Cl_2−hexane); yield, 31%; mp 168−170 °C; ^{1}H NMR (DMSO-d_6): δ 11.43 (s, 1H, *NH*), 7.99 (d, J = 7.8 Hz, 2H, *o*-benzoyl hydrogens), 7.67 (d, J = 7.8 Hz, 1H, H-6), 7.65−7.70 (m, 1H, *p*-benzoyl hydrogen), 7.52−7.57 (m, 2H, *m*-benzoyl hydrogens), 6.13 (dd, J = 7.8, 6.3 Hz, 1H, H-1′), 5.76 (dd, J = 5.4, 1.2 Hz, 1H, H-3′), 5.60 (d, J = 7.8 Hz, 1H, H-5), 4.48−4.61 (m, 3H, H-4′, H-5′), 2.56−2.74 (m, 2H, H-2′); ^{13}C NMR (DMSO-d_6): δ 165.32, 162.77, 150.15, 140.44, 133.48, 129.15, 129.06, 128.72, 102.06, 84.78, 83.29, 79.17, 64.04, 34.24. Anal. ($C_{16}H_{15}N_3O_8$) C, H, N.

3′-*O*-Nitro-5′-*O*-benzoyl-2′-deoxythymidine (9b). White crystals (CH_2Cl_2−hexane); yield, 34%; mp 109−110 °C; ^{1}H NMR (DMSO-d_6): δ 11.40 (s, 1H, *NH*), 8.02 (dd, J = 8.4, 1.5 Hz, 2H, *o*-benzoyl hydrogens), 7.66−7.71 (m, 1H, *p*-benzoyl hydrogen), 7.52−7.57 (m, 2H, *m*-benzoyl hydrogens), 7.44 (s, 1H, H-6), 6.17 (dd, J = 8.4, 6.9 Hz, 1H, H-1′), 5.80 (br d, J = 6.6 Hz, 1H, H-3′), 4.62 (dt, J = 7.8, 2.1 Hz, 1H, H-4′), 4.49−4.57 (m, 2H, H-5′), 2.57−2.72 (m, 2H, H-2′), 1.59 (s, 3H, CH_3); ^{13}C NMR (DMSO-d_6): δ 165.30, 163.35, 150.16, 135.43, 133.49, 129.14, 129.06, 128.74, 109.89, 83.99, 83.32, 78.97, 64.03, 34.21, 11.79. Anal. ($C_{17}H_{17}N_3O_8$) C, H, N.

5-Fluoro-3′-*O*-nitro-5′-*O*-benzoyl-2′-deoxyuridine (9c). White crystals (EtOAc−hexane); yield, 50%; mp 168−170 °C; ^{1}H NMR (DMSO-d_6): δ 11.15 (br s, 1H, *NH*), 7.86 (dd, J = 8.4, 1.5 Hz, 2H, *o*-benzoyl hydrogens), 7.76 (d, J = 6.0 Hz, 1H, H-6), 7.63−7.68 (m, 1H, *p*-benzoyl hydrogen), 7.47−7.52 (m, 2H, *m*-benzoyl hydrogen), 6.55 (d, J = 3.3 Hz, 1H, H-1′), 5.46 (br d, J = 1.5 Hz, 1H, H-3′), 4.52−4.62 (m, 2H, H-5′), 4.43 (dd, J = 11.7, 6.0 Hz, 1H, H-4′), 2.56−2.63 (m, 2H, H-2′); ^{13}C NMR (DMSO-d_6): δ 165.11 (*C*O$_2$), 153.34 (d, J_{CCF} = 26.4 Hz, C-4 *C*═O), 149.82 (d, J = 12.8 Hz, C-2 *C*═O), 145.28 (d, J_{CF} = 242.8 Hz, C-5), 136.47 (d, J_{CCF} = 25.3 Hz, C-6), 133.36, 129.02, 128.53 and 128.38 (phenyl carbons), 82.61 (C-1′), 78.92 (C-3′), 77.57 (C-4′), 62.16 (C-5′), 31.75 (C-2′). Anal. ($C_{16}H_{14}FN_3O_8$) C, H, N.

5-Iodo-3′-*O*-nitro-5′-*O*-benzoyl-2′-deoxyuridine (9d). Method A: White foam; yield, 2.8%; mp 85−86 °C; ^{1}H NMR ($CDCl_3$): δ 9.40 (s, 1H, *NH*), 8.02−8.11 (m, 2H, *o*-benzoyl hydrogens), 7.89 (s, 1H, H-6), 7.60−7.70 (m, 1H, *p*-benzoyl hydrogens), 7.43−7.52 (m, 2H, *m*-benzoyl hydrogens), 6.21 (dd, J = 6.6, 5.7 Hz, 1H, H-1′), 5.64 (d, J = 6.6 Hz, 1H, H-3′), 4.68−4.76 (m, 2H, H-5′), 4.58 (br s, 1H, H-4′), 2.81 (dd, J = 14.4, 5.1 Hz, 1H, H-2′α), 2.37 (ddd, J = 14.7, 7.5, 7.2 Hz, 1H, H-2′β); ^{13}C NMR ($CDCl_3$): δ 165.87, 159.53, 149.47, 143.43, 133.84, 129.60, 128.87, 128.81, 85.92, 82.60, 81.52, 69.03, 63.91, 36.97. Anal. ($C_{16}H_{14}IN_3O_8$) C, H, N.

Method B: A mixture of **9a** (1.0 g, 2.65 mmol) and NaN_3 (0.69 g, 10.6 mmol) in dry MeCN (50 mL) was cooled in an ice-bath, a solution of ICl (1.07 g, 6.6 mmol) in MeCN (5 mL) was added dropwise during 5 min, and the reaction was allowed to proceed at 25 °C for 48 h with stirring under an argon atmosphere. Removal of the solvent in vacuo gave a residue that was purified via flash silica gel column chromatography using hexanes−EtOAc (1:1, v/v) as eluent to afford **9d** (1.1 g, 82.5%) as a white foam, which was identical (mp, ^{1}H NMR) to **9d** described above under Method A.

General Method for the Preparation of 5-Substituted-3′-*O*-nitro-2′-deoxyuridines (10a−d). A solution of NaOMe in MeOH (12.75 mL of 0.5 M) was added to either **9a**, **9b**, **9c**, or **9d** (3.18 mmol) and the reaction was allowed to proceed at 25 °C for 1 h with stirring. The reaction was quenched by addition of NH_4Cl (0.5 g), and the solvent was removed in vacuo. Purification of the residue by flash silica gel column chromatography using MeOH−CH_2Cl_2 (1:9, v/v) as eluent yielded the respective product **10a**, **10b**, **10c**, or **10d**. Physical and spectral data for **10a**−**d** are listed below.

3′-*O*-Nitro-2′-deoxyuridine (10a) was obtained directly as white crystals; yield, 91%; mp 188−190 °C; ^{1}H NMR (DMSO-d_6): δ 11.35 (br s, 1H, N*H*), 7.88 (d, J = 8.4 Hz, 1H, H-6), 6.13 (dd, J = 8.7, 5.7 Hz, 1H, H-1′), 5.67 (d, J = 8.4 Hz, 1H, H-5), 5.58 (d, J = 6.0 Hz, 1H, H-3′), 5.25 (br s, 1H, 5′-*OH*), 4.21 (d, J = 1.8 Hz, 1H, H-4′), 3.60−3.74 (m, 2H, H-5′), 2.45−2.56 (m, 1H, H2′α), 2.40 (ddd, J = 14.7, 8.7, 6.3 Hz, 1H, H-2′β); ^{13}C NMR (DMSO-d_6): δ 162.86, 150.25, 140.12, 102.13, 84.59, 83.84, 82.46, 61.26, 35.23. Anal. ($C_9H_{11}N_3O_7$) C, H, N.

3′-*O*-Nitro-2′-deoxythymidine (10b). White crystals (CH_2Cl_2−hexane); yield, 92%; mp 137−139 °C; ^{1}H NMR (DMSO-d_6): δ 11.36 (s, 1H, N*H*), 7.72 (s, 1H, H-6), 6.14 (dd, J = 8.4, 6.0 Hz, 1H, H-1′), 5.59 (d, J = 5.7 Hz, 1H, H-3′), 5.31 (t, J = 5.4 Hz, 1H, 5′-O*H*), 4.18 (d, J = 1.8 Hz, 1H, H-4′), 3.67 (dd, J = 5.1, 3.3 Hz, 2H, H-5′), 2.37−2.52 (m, 2H, H-2′), 1.78 (s, 3H, CH_3); ^{13}C NMR (DMSO-d_6): δ 163.50 (C-4 *C*═O), 150.31 (C-2 *C*═O), 135.66 (C-6), 109.76 (C-5), 84.54 (C-1′), 83.45 (C-3′), 82.23 (C-4′), 61.28 (C-5′), 34.91 (C-2′), 12.24 (*C*H$_3$). Anal. ($C_{10}H_{13}N_3O_7$) C, H, N.

5-Fluoro-3′-*O*-nitro-2′-deoxyuridine (10c) was obtained directly as white crystals; yield, 95%; mp 195−197 °C; ^{1}H NMR (DMSO-d_6): δ 11.20 (br s, 1H, N*H*), 7.76 (d, J = 6.0 Hz, 1H, H-6), 6.49 (d, J = 2.7 Hz, 1H, H-1′), 5.28 (d, J = 1.2 Hz, 1H, H-3′), 5.02 (br s, 1H, 5′-O*H*), 4.20 (dt, J = 6.3, 2.4 Hz, 1H, H-4′), 3.42−3.58 (m, 2H, H-5′), 2.45−2.56 (m, 2H, H-2′); ^{13}C NMR (DMSO-d_6): δ 153.31 (d, J_{CCF} = 26.4 Hz, C-4 *C*═O), 150.12 (C-2 *C*═O), 145.17 (d, J_{CF} = 241.7 Hz, C-5), 136.04 (d, J_{CCF} = 29.7 Hz, C-6), 85.85 (C-1′), 78.62 (C-3′), 77.26 (C-4′), 59.28 (C-5′), 31.68 (C-2′). Anal. ($C_9H_{10}FN_3O_7$) C, H, N.

5-Iodo-3′-*O*-nitro-2′-deoxyuridine (10d) was obtained directly as white crystals; yield, 92%; mp 170−172 °C; ^{1}H NMR (DMSO-d_6): δ 11.74 (s, 1H, N*H*), 8.36 (s, 1H, H-6), 6.09 (dd, J = 8.4, 6.0 Hz, 1H, H-1′), 5.59 (d, J = 5.7 Hz, 1H, H-3′), 5.40 (t, J = 5.1 Hz, 1H, 5′-O*H*), 4.23 (br d, J = 1.8 Hz, 1H, H-4′), 3.62−3.80 (m, 2H, H-5′), 2.40−2.56 (m, 2H, H-2′); ^{13}C NMR (DMSO-d_6): δ 160.13, 149.87, 144.51, 84.34, 84.18, 82.69, 69.76, 61.10, 35.54. Anal. ($C_9H_{10}IN_3O_7$) C, H, N.

General Method for the Preparation of 5-Substituted-4-(1,2,4-triazolo)-4-deoxy-3′-*O*-nitro-5′-*O*-

benzoyl-2′-deoxyuridine (11a,b). 4-Chlorophenyl dichlorophosphate (0.88 mL, 5.32 mmol), and then 1,2,4-triazole (0.74 g, 10.64 mmol), was added to a solution of either **9a** or **9b** (5.32 mmol) in pyridine (20 mL) at 0 °C with stirring. The reaction was allowed to proceed at 25 °C for 4 days with stirring, and the solvent was removed in vacuo. The residue was dissolved in CH_2Cl_2 (200 mL), this solution was washed with water (2 × 50 mL) and aqueous $NaHCO_3$ (50 mL), the organic fraction was dried (Na_2SO_4), and the solvent was removed in vacuo. Purification of the residue by flash silica gel column chromatography using hexanes−EtOAc (1:3 and 1:5, v/v, respectively) gave the respective product **11a** or **11b**. Physical and spectral data for **11a**,**b** are listed below.

4-(1,2,4-Triazolo)-4-deoxy-3′-*O*-nitro-5′-*O*-benzoyl-2′-deoxyuridine (11a) was obtained directly as white crystals that were washed with cold ether; yield, 79%; mp 85−87 °C; ^{1}H NMR ($CDCl_3$): δ 9.20 (s, 1H, triazole hydrogen), 8.23 (d, J = 7.2 Hz, 1H, H-6), 8.09 (s, 1H, triazole hydrogen), 7.89 (dd, J = 8.7, 1.2 Hz, 2H, *o*-benzoyl hydrogens), 7.49−7.58 (m, 1H, *p*-benzoyl hydrogen), 7.32−7.42 (m, 2H, *m*-benzoyl hydrogens), 6.94 (d, J = 7.2 Hz, 1H, H-5), 6.20 (dd, J = 7.5, 5.7 Hz, 1H, H-1′), 5.65 (d, J = 6.9 Hz, 1H, H-3′), 4.81 (dd, J = 12.0, 3.3 Hz, 1H, H-5′a), 4.72 (dd, J = 5.1, 3.0 Hz, 1H, H-4′), 4.66 (dd, J = 12.0, 3.6 Hz, 1H, H-5′b), 3.18 (ddd, J = 15.3, 7.2, 1.5 Hz, 1H, H-2′α), 2.33−2.43 (m, 1H, H-2′β); ^{13}C NMR ($CDCl_3$): δ 165.72 (*C*O$_2$), 159.42 (C-2 *C*═O), 153.88 (C-4), 145.19 (C-6), 143.18 (phenyl C-1), 133.78, 129.34, 128.77 and 128.68 (phenyl and triazole *C*H), 94.74 (C-5), 88.47 (C-1′), 82.78 (C-3′), 82.46 (C-4′), 63.85 (C-5′), 38.01 (C-2′). Anal. ($C_{18}H_{16}N_6O_7$) C, H, N.

4-(1,2,4-Triazolo)-4-deoxy-3′-*O*-nitro-5′-*O*-benzoyl-2′-deoxythymidine (11b). White foam; yield, 71%; mp 59−60 °C; ^{1}H NMR (DMSO-d_6): δ 9.29 (s, 1H, triazole hydrogen), 8.35 (s, 1H, triazole hydrogen), 8.24 (s, 1H, H-6), 7.89 (dd, J = 8.4, 1.5 Hz, 2H, *o*-benzoyl hydrogens), 7.57−7.62 (m, 1H, *p*-benzoyl hydrogen), 7.43−7.48 (m, 2H, *m*-benzoyl hydrogens), 6.15 (dd, J = 7.2, 6.6 Hz, 1H, H-1′), 5.85 (dd, J = 4.8, 2.1 Hz, 1H, H-3′), 4.83 (br dd, J = 6.3, 4.2 Hz, 1H, H-4′), 4.73 (dd, J = 12.3, 3.9 Hz, 1H, H-5′a), 4.60 (dd, J = 12.3, 4.5 Hz, 1H, H-5′b), 2.91 (ddd, J = 15.3, 6.6, 1.5 Hz, 1H, H-2′α), 2.68−2.80 (m, 1H, H-2′β), 2.10 (s, 3H, C*H*$_3$); ^{13}C NMR (DMSO-d_6): δ 165.24, 157.85, 152.71, 147.35, 147.29, 133.39, 129.08, 128.96, 128.92, 128.58, 104.66, 87.76, 83.64, 80.95, 64.14, 36.32, 15.76. Anal. ($C_{19}H_{18}N_6O_7$) C, H, N.

General Method for the Preparation of 5-Substituted-3′-*O*-nitro-5′-*O*-benzoyl-2′-deoxycytidines (12a,b). A solution of either **11a** or **11b** (3.74 mmol) in NH_4OH/dioxane (1:3, v/v, 80 mL) was stirred at 25 °C for 5 h prior to removal of the solvent in vacuo. The residue was purified via flash silica gel column chromatography using MeOH:CH_2Cl_2 (1:9, v/v) as eluent to give the respective products **12a** and **12b**. Physical and spectral data for **12a**,**b** are listed below.

3′-*O*-Nitro-5′-*O*-benzoyl-2′-deoxycytidine (12a) was obtained directly as white crystals, which were washed with cold ether; yield, 49%; mp 139−141 °C; ^{1}H NMR (DMSO-d_6): δ 7.98 (dd, J = 8.4, 1.2 Hz, 2H, *o*-benzoyl hydrogens), 7.68 (ddt, J = 14.7, 7.5, 1.2 Hz, 1H, *p*-benzoyl hydrogen), 7.62 (d, J = 7.8 Hz, 1H, H-6), 7.51−7.56 (m, 2H, *m*-benzoyl hydrogens), 7.23 (s, 2H, N*H*$_2$), 6.13 (dd, J = 7.8, 6.0 Hz, 1H, H-1′), 5.77 (d, J = 4.8 Hz, 1H, H-3′), 5.68 (d, J = 7.8 Hz, 1H, H-5), 4.51−4.60 (m, 3H, H-4′, H-5′), 2.52−2.61 (m, 2H, H-2′); ^{13}C NMR (DMSO-d_6): δ 165.80, 165.43, 165.24 and 154.57 (quaternary carbons), 140.68 (C-6), 133.34, 129.05, and 128.60 (phenyl *C*H), 94.30 (C-5), 85.60 (C-1′), 83.77 (C-3′), 79.21 (C-4′), 64.19 (C-5′), 35.04 (C-2′). Anal. ($C_{16}H_{16}N_4O_7$) C, H, N.

5-Methyl-3′-*O*-nitro-5′-*O*-benzoyl-2′-deoxycytidine (12b) was obtained directly as white crystals, which were washed with cold ether; yield, 36%; mp 158−160 °C; ^{1}H NMR (DMSO-d_6): δ 8.15 (d, J = 8.1 Hz, 2H, *o*-benzoyl hydrogens), 7.76−7.85 (m, 1H, *p*-benzoyl hydrogen), 7.59−7.71 (m, 2H, *m*-benzoyl hydrogens), 7.55 (br s, 1H, N*H*a), 7.49 (s, 1H, H-6), 7.00 (br s, 1H, N*H*b), 6.32 (dd, J = 8.1, 6.0 Hz, 1H, H-1′), 5.90−5.96 (m, 1H, H-3′), 4.63−4.79 (m, 3H, H-4′, H-5′), 2.60−2.70 (m, 2H, H-2′), 1.77 (s, 3H, C*H*$_3$); ^{13}C NMR (DMSO-d_6): δ 165.30, 165.22, 165.16, 154.66, 137.54, 133.48, 129.06, 128.73, 101.81, 84.98, 83.87, 79.08, 64.22, 35.04, 12.90. Anal. ($C_{17}H_{18}N_4O_7$) C, H, N.

General Method for the Preparation of 5-Substituted-3′-*O*-nitro-2′-deoxycytidines (13a,b). A solution of NaOMe in MeOH (3.7 mL of 0.5 M) was added to either **12a** or **12b** (0.93 mmol), the mixture was stirred at 25 °C for 15 min, and the solvent was removed in vacuo. Purification of the residue by flash silica gel column chromatography using MeOH:CH_2Cl_2 (1:4, v/v) as eluent afforded the respective products **13a** and **13b**. Physical and spectral data for **13a**,**b** are listed below.

3′-*O*-Nitro-2′-deoxycytidine (13a). White foam; yield, 95%; mp 65−66 °C (lit.[32] mp 177−178 °C); ^{1}H NMR (DMSO-d_6): δ 7.80 (d, J = 7.8 Hz, 1H, H-6), 7.20 and 7.26 (two s, 1H each, N*H*$_2$), 6.14 (dd, J = 8.7, 5.4 Hz, 1H, H-1′), 5.76 (d, J = 7.8 Hz, 1H, H-5), 5.59 (d, J = 6.0 Hz, 1H, H-3′), 5.25 (t, J = 5.4 Hz, 1H, 5′-O*H*), 4.19 (br d, J = 1.8 Hz, 1H, H-4′), 3.62−3.66 (m, 2H, H-5′), 2.45−2.52 (m, 1H, H-2′α), 2.29 (ddd, J = 14.7, 6.0, 2.7 Hz, 1H, H-2′β); ^{13}C NMR (DMSO-d_6): δ 163.67, 152.34, 141.70, 94.37, 85.02, 84.80, 82.78, 61.09, 35.88. Anal. ($C_9H_{12}N_4O_6$) C, H, N.

5-Methyl-3′-*O*-nitro-2′-deoxycytidine (13b). Colorless oil, which was crystallized from EtOAc−hexane to give white crystals; yield, 94%; mp 141−143 °C; ^{1}H NMR (DMSO-d_6): δ 7.62 (s, 1H, H-6), 7.35 and 6.90 (two s, 1H each, N*H*$_2$), 6.14 (dd, J = 9.0, 5.7 Hz, 1H, H-1′), 5.57 (d, J = 5.1 Hz, 1H, H-3′), 5.35 (br s, 1H, 5′-O*H*), 4.16 (d, J = 2.1 Hz, 1H, H-4′), 3.60−3.70 (m, 2H, H-5′), 2.41−2.49 (m, 1H, H-2′α), 2.25−2.36 (m, 1H, H-2′β), 1.84 (s, 3H, C*H*$_3$); ^{13}C NMR (DMSO-d_6): δ 165.40 (C-2 *C*═O), 155.10 (C-4), 138.12 (C-6), 101.96 (C-5), 84.88 and 84.79 (C-1′ and C-3′), 82.36 (C-4′), 61.45 (C-5′), 35.70 (C-2′), 13.30 (*C*H$_3$). Anal. ($C_{10}H_{14}N_4O_6$) C, H, N.

5-Iodo-3′-*O*-nitro-2′-deoxycytidine (13c). A mixture of **13a** (0.144 g, 0.53 mmol), iodine (0.135 g, 0.53 mmol), and iodic acid (0.093 g, 0.53 mmol) in acetic acid, H_2O, and CCl_4 (8:3:2, v/v/v, 26 mL) was stirred at 25 °C for 14 h. Removal of the solvents in vacuo, coevaporation of toluene (2 × 20 mL) from the residue, dissolution of the residue in CH_2Cl_2 (100 mL), filtration, washing with aqueous $NaHCO_3$ (50 mL), and drying the organic fraction (Na_2SO_4) were carried out consecutively. The solvent was removed in vacuo to give a residue that was purified via flash silica gel column chromatography using MeOH:CH_2Cl_2 (1:9, v/v) as eluent to afford **13c** (0.118 g, 56%) as a white foam: mp 81−82 °C; ^{1}H NMR (DMSO-d_6): δ 8.23 (s, 1H, H-6), 7.95 and 6.75 (two br s, 1H each, N*H*$_2$), 6.09 (dd, J = 8.7, 5.7 Hz, 1H, H-1′), 5.59 (d, J = 6.0 Hz, 1H, H-3′), 5.36 (t, J = 5.4 Hz, 1H, 5′-O*H*), 4.20−4.28 (m, 1H, H-4′), 3.62−3.76 (m, 2H, H-5′), 2.49−2.56 (m, 1H, H-2′α), 2.36 (ddd, J = 15.0, 8.4, 6.6 Hz, 1H, H-2′β); ^{13}C NMR (DMSO-d_6): δ 163.58, 153.61, 147.07, 85.11, 84.83, 82.63, 61.13, 57.04 (C-5), 36.10. Anal. ($C_9H_{11}IN_4O_6$) C, H, N.

General Method for the Preparation of 5-Substituted-5′-*O*-nitro-2′-deoxyuridines (15a,b). A mixture of either **14a** or **14b** (6.54 mmol) and $LiNO_3$ (4.48 g, 65 mmol) in dry DMF (30 mL) was stirred at 100 °C for 14 h under argon. Removal of the solvent in vacuo gave a residue, toluene (20 mL) was added, and the solvent was removed in vacuo. Purification of the residue via flash silica gel column chromatography using a gradient of 5% →10% MeOH in CH_2Cl_2 as eluent gave a pale yellow oil which was recrystallized from MeOH to yield the respective product **15a** or **15b**. Physical and spectral data for **15a**,**b** are listed below.

5′-*O*-Nitro-2′-deoxyuridine (15a). Yield, 73%; mp 170−172 °C (lit.[27] mp 175 °C); ^{1}H NMR (DMSO-d_6): δ 11.34 (s, 1H, N*H*), 7.65 (d, J = 8.1 Hz, 1H, H-6), 6.16 (dd, J = 7.2, 6.6 Hz, 1H, H-1′), 5.65 (d, J = 8.1 Hz, 1H, H-5), 5.53 (d, J = 3.9 Hz, 1H, 3′-O*H*), 4.79 (dd, J = 11.4, 3.6 Hz, 1H, H-5′a), 4.66 (dd, J = 11.4, 6.9 Hz, 1H, H-5′b), 4.20−4.27 (m, 1H, H-3′), 3.95−4.00 (m, 1H, H-4′), 2.09−2.28 (m, 2H, H-2′); ^{13}C NMR (DMSO-d_6) δ 162.81, 150.19, 140.65, 101.98, 84.59, 81.76, 73.05 (C-5′), 70.10, 38.19. Anal. ($C_9H_{11}N_3O_7$) C, H, N.

5′-*O*-Nitro-2′-deoxythymidine (15b). Yield, 71%; mp 183−185 °C (lit.,[28] mp 189 °C); ^{1}H NMR (DMSO-d_6): δ 11.32 (s, 1H, N*H*), 7.46 (s, 1H, H-6), 6.19 (t, J = 6.9 Hz, 1H, H-1′), 5.51 (d, J = 3.3 Hz, 1H, 3′-O*H*), 4.81 (dd, J = 11.4, 3.9 Hz, 1H,

H-5′a), 4.69 (dd, J = 11.4, 6.6 Hz, 1H, H-5′b), 4.28−4.30 (m, 1H, H-3′), 3.96 (ddd, J = 6.6, 3.9, 3.6 Hz, 1H, H-4′), 2.25 (ddd, J = 14.1, 7.2, 6.9 Hz, 1H, H-2′α), 2.11 (ddd, J = 14.1, 6.3, 3.9 Hz, 1H, H-2′β), 1.78 (s, 3H, CH_3); ^{13}C NMR (DMSO-d_6): δ 163.50, 150.28, 135.99, 109.79, 84.08, 81.69, 73.07 (C-5′), 70.26, 38.08, 12.07. Anal. ($C_{10}H_{13}N_3O_7$) C, H, N.

5-Iodo-5′-*O*-nitro-2′-deoxyuridine (15c). A mixture of **15a** (0.2 g, 0.74 mmol) and NaN_3 (0.19 g, 2.94 mmol) in dry MeCN (20 mL) was cooled in an ice-bath. A solution of ICl (0.36 g, 2.2 mmol) in MeCN (3 mL) was added dropwise during 5 min, and the reaction was allowed to proceed at 25 °C for 48 h with stirring under argon. The solvent was removed in vacuo and the residue was purified via silica gel flash column chromatography using a gradient of 5% → 10% MeOH in CH_2Cl_2 as eluent to give **15c** (0.19 g, 65%) as white crystals: mp 181−183 °C; ^{1}H NMR (DMSO-d_6): δ 11.80 (s, 1H, NH), 8.10 (s, 1H, H-6), 6.18 (dd, J = 6.9, 3.9 Hz, 1H, H-1′), 5.59 (d, J = 4.2 Hz, 1H, 3′-OH), 4.75−4.94 (m, 2H, H-5′), 4.33−4.37 (m, 1H, H-3′), 4.06 (ddd, J = 6.9, 6.6, 3.6 Hz, 1H, H-4′), 2.40 (ddd, J = 14.1, 7.2, 6.9 Hz, 1H, H-2′α), 2.19 (ddd, J = 14.1, 6.6, 3.9 Hz, 1H, H-2′β); ^{13}C NMR (DMSO-d_6): δ 160.21(C-4 C=O), 149.86 (C-2 C=O), 144.80 (C-6), 85.18 (C-1′), 82.08 (C-3′), 72.85 (C-5′), 70.13 (C-4′), 69.78 (C-5), 38.32. Anal. ($C_9H_{10}IN_3O_7$) C, H, N.

In Vitro Nitric Oxide Release Assays. 1. Incubation With 18 mM L-cysteine in Phosphate Buffer (pH 7.4). In vitro nitric oxide release was assayed using a modification of the previously reported procedure.[44] Briefly, a solution of the test compound (1 mL of a 2 mM solution in 0.1 M phosphate buffer (pH 7.4) was mixed thoroughly with a freshly prepared solution of L-cysteine (1 mL of a 36 mM solution in 0.1 M phosphate buffer, pH 7.4), and the mixture was incubated at 37 °C, for 1 and 16 h in the absence of air. After exposure to air for 10 min at 25 °C, an aliquot of the Griess reagent (1 mL) [freshly prepared by mixing equal volumes of 1.0% sulfanilamide (prepared and stored in aqueous 5% phosphoric acid) and 0.1% *N*-naphthylethylenediamine dihydrochloride in water] was added to an equal volume (1 mL) of each test compound's incubation solution with mixing. After 10 min had elapsed, absorbance was measured at 540 nm using a Philips PU 8740 UV/VIS scanning spectrophotometer. Solutions of 0−60 μM sodium nitrite were used to prepare a nitrite absorbance versus concentration curve under the same experimental conditions. The percent nitric oxide released (quantitated as nitrite ion) was calculated (±SEM, n = 3) from the standard nitrite versus concentration curve.

2. Incubation with Phosphate Buffer (pH 7.4). This assay was performed as described under procedure 1 above, except that a solution of the test compound (2 mL of a 1 mM solution in 0.1 M phosphate buffer pH 7.4) was used, and no L-cysteine was added.

3. Incubation with Rat Serum. Nitric oxide release was measured by modification of the reported procedure for in vivo serum samples.[53] Briefly, rat serum was prepared by filtration with a Millipore UL trafree-15 centrifugal filter. Stock solutions of test compounds (1×10^{-2} M) were prepared, and 10 μL of each test compound solution was mixed with filtered serum (90 μL), which corresponds to the reaction sample. Each reaction sample was incubated for either 1 h or 16h at 37 °C in the presence of 0.2 U/mL Aspergillus nitrate reductase, 50 mM HEPES buffer, 5 μM FAD, and 0.1 mM NADPH to provide a total volume of 500 μL that was comprised of the following composition for the blank and the reaction sample (in brackets), respectively: water 300 μL (290 μL), 1 M HEPES 25 μL (25 μL), serum 90 μL (100 μL), 0.1 mM FAD 25 μL (25 μL), 1 mM NADPH 50 μL (50 μL), 10U/mL Nase 10 μL (10 μL). Following the incubation, 5 μL of lactate dehydrogenase (1500 U/mL) and 50 μL of 100 mM pyruvic acid were added to each tube to oxidize any unreacted NADPH, and samples were incubated for 10 min. Greiss reagent (550 μL) was then added to each tube, the mixture was allowed to stand at 25 °C for 10 min, absorbance for each sample was determined at 540 nm, and % nitric oxide release was calculated as indicated under Procedure 1 described above.

In Vitro Cell Cytotoxicity (MTT assay). KBALB, KBALB−STK, human 143B, and human 143B-LTK cells were cultured in complete DMEM medium supplemented with 10% fetal bovine serum (FBS), and EMT-6 cells were cultured in complete WAYMOUTH medium in 12.5% FBS. Exponentially growing cells were trypsinized, centrifuged, and resuspended in growth medium, and the cell number was readjusted to 8×10^3 cells/mL. Cells were seeded into 96-well plates at 8×10^2 cells/well and incubated at 37 °C in a humidified 5% CO_2 atmosphere for 24 h.

The test compound was dissolved in DMEM medium, and 100 μL of this solution was added to cells in 96-well plates to produce the preselected test compound concentration. DMEM medium (100 μL) was added to control wells. The plates were incubated for 3 days at 37 °C in a humidified atmosphere consisting of 95% air and 5% CO_2. At the end of the incubation, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT, Sigma) was dissolved in phosphate-buffered saline (PBS) to produce a concentration of 5 mg/mL, filtered through a 0.45 μm membrane filter, and diluted (1:5) with prewarmed DMEM medium. An aliquot of this solution (50 μL) was added to each well, and the plates were incubated at 37 °C for 4 h. The medium was removed from the wells, dimethyl sulfoxide (150 μL) was added to each well, and the plates were placed on a shaker for 15 min to dissolve the formazan crystals. The absorbance at 540 nm (A_{540}) was measured immediately in each well using a scanning multiwell spectrophotometer (ELISA reader). A_{540} values, corrected for the absorbance in medium blanks, reflected the concentration of viable cells. The CC_{50} values reported refer to the test drug concentrations that reduced the A_{540} to 50% of the control value (mean value, n = 6). This assay,[46] which depends on the metabolic reduction of MTT to colored formazan, measures cytostatic and cytotoxic effects of the test drug.

Antiviral Activity Assays. The antiviral assays were based on an inhibition of virus-induced cytopathicity in either E_6SM, HeLa or Vero cell cultures, following previously established procedures.[47] Herpes simplex virus type 1 (HSV-1) (KOS), HSV-2 (G), vaccinia virus, vesicular stomatitis virus (VSV), and the thymidine kinase-deficient HSV-1 TK$^-$ KOS (ACV^r) strains were propagated in E_6SM cell cultures, parainfluenza-3 virus, reovirus-1, Sindbis virus, Coxsackie B4 virus and Punta Toro virus in Vero cell cultures, and respiratory syncytial virus in HeLa cell cultures. Briefly, confluent cell cultures in microtiter plates were exposed to 100 $CCID_{50}$ of virus, 1 $CCID_{50}$ being the virus dose required to infect 50% of the cell cultures. After a 1 h virus adsorption period, residual virus was removed, and the cell cultures were incubated in the presence of varying concentrations (400, 100, etc., μg/mL) of the test compounds. Viral cytopathicity was recorded as soon as it reached completion in the control virus-infected cell cultures.

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