Drug Design

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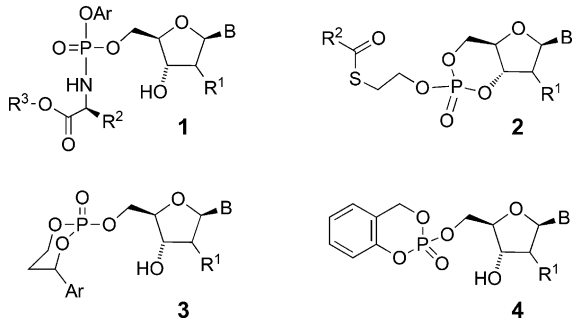
Cyclic-Disulfide-Based Prodrugs for Cytosol-Specific Drug Delivery**

Gabor Butora, Ning Qi, Wenlang Fu, Truyen Nguyen, Hsueh-Cheng Huang, and Ian W. Davies*

Abstract: *The cytosolic conversion of therapeutically relevant nucleosides into bioactive triphosphates is often hampered by the inefficiency of the first kinase-mediated step. Nucleoside monophosphate prodrugs can be used to bypass this limitation. Herein we describe a novel cyclic-disulfide class of nucleoside monophosphate prodrugs with a cytosol-specific, reductive release trigger. The key event, a charge-dissipating reduction-triggered cyclodeesterification leads to robust cytosolic production of the cyclic 3′,5′-monophosphate for downstream enzymatic processing. The antiviral competence of the platform was demonstrated with an O-benzyl-1,2-dithiane-4,5-diol ester of 2′-C-methyluridine-3′,5′-phosphate. Both in vitro and in vivo comparison with the clinically efficacious ProTide prodrug of 2′-deoxy-2′-α-fluoro-β-C-methyluridine is provided. The cytosolic specificity of the release allows for a wide range of potential applications, from tissue-targeted drug delivery to intracellular imaging.*

Unnatural nucleosides are an indispensable part of modern antiviral treatment and chemotherapy. In the vast majority of cases, the nucleosides themselves are inactive, and a kinase-mediated transformation is necessary to produce the bioactive nucleotides. Incorporation of the suitably modified nucleoside triphosphate into the nascent oligonucleotide is then followed by a chain-termination event, which in turn leads to the inhibition of viral replication (antiviral agents) or arrest of uncontrolled cell proliferation (chemotherapeutic antimetabolites).[1]

The chemical modification that induces chain termination often causes the nucleoside to become a poor substrate of the kinase responsible for production of its monophosphate. In this instance, prodrugs of nucleoside monophosphates can be utilized to "bypass" this first, usually rate-limiting phosphorylation step.[2] The most notable examples of this approach are aryloxy phosphoramidates **1** (ProTides, esterase, McGuigan),[3] *S*-acyl thioethyl phosphates **2** (SATE, esterase, Imbach),[4] cyclic arylpropanyl phosphates **3** (HepDirect, CYP450, Erion),[5] and salicyl alcohol phosphates **4** (*cyclo*Sal, esterase, Meier; Scheme 1).[6] In spite of the inherent elegance



Scheme 1. Nucleoside monophosphate prodrugs. B = nucleobase.

of these solutions, none is able to ensure exclusive cytosol-specific and tissue-independent delivery of antiviral agents or chemotherapeutic antimetabolites.

The bioactivation of SATE prodrugs **2** is initiated by enzyme-mediated ester hydrolysis (Scheme 2). The thioethyl phosphate **5** undergoes spontaneous cyclodeesterification to yield **6** with the concomitant release of thiirane. The cyclic phosphodiester **6** is hydrolyzed to produce the nucleoside monophosphate **7**, and then further phosphorylated to the respective di- and triphosphates. In theory, the transient intermediate **5** can also be generated by a cytosol-specific reduction of a linear disulfide **8**; however, the insufficient chemical stability of such diisulfides renders this approach intractable. The release of reactive thiirane (SATE)[7] or a Michael acceptor (HepDirect)[8] has raised safety and toxicological concerns.

The reducing environment of the cytosol is controlled by the action of a ubiquitous tripeptide glutathione **13** (Scheme 3). The intracellular concentration is maintained at a millimolar level through energy-dependent anabolic processes, whereas the extracellular concentration is merely micromolar. In healthy cells, the equilibrium between glutathione (GSH) and glutathione disulfide is heavily shifted in favor of the reduced form by the action of NADPH-dependent glutathione reductase (NADPH: reduced form of nicotinamide adenine dinucleotide phosphate). In this sense, GSH can be viewed as a hydride shuttle between NADPH and the substrate being reduced.[9–11]

[*] G. Butora, N. Qi, W. Fu, I. W. Davies
Department of Process Chemistry, Merck & Co., Inc.
Rahway, New Jersey 07065 (USA)
E-mail: gbutora@gmail.com
T. Nguyen
Department of Safety Assessment, Merck & Co., Inc.
Kenilworth, New Jersey 07033 (USA)
H.-C. Huang
Department of Infectious Diseases, Merck & Co., Inc.
Kenilworth, New Jersey 07033 (USA)

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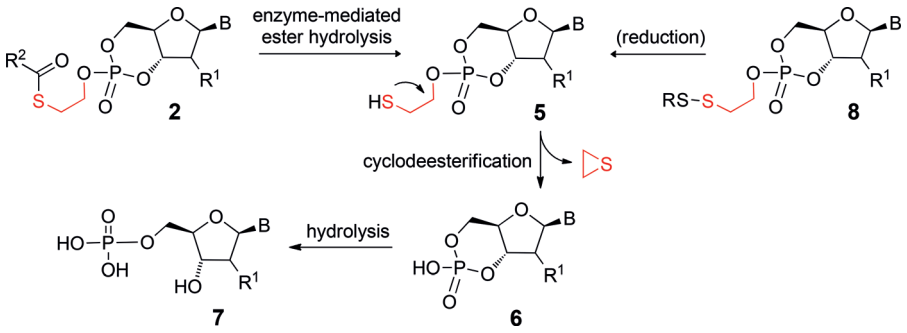
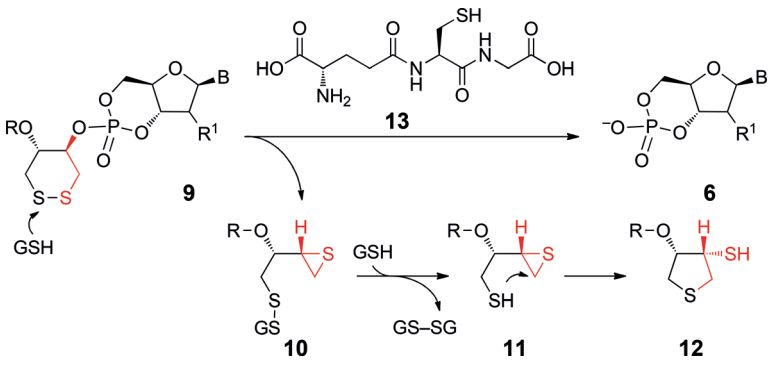


Scheme 2. Mechanism of activation and release of SATE prodrugs.



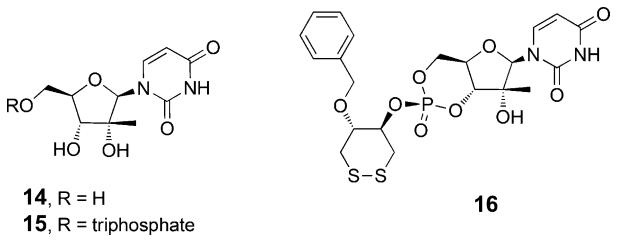
Scheme 3. Cyclic-disulfide-based prodrugs: Mechanism of release.

The sharp transmembrane gradient of glutathione can be utilized to trigger cytosol-specific payload release.[12] As opposed to either esterase-activated SATE thioesters or ProTides, a GSH-activated prodrug would remain stable in human serum. Only upon cell uptake would it undergo reductive bioactivation, and this intracellular specificity would make this class of prodrugs ideally suited for the delivery of antiviral or antimetabolic therapeutics.

We envisioned that the inclusion of the disulfide bond in an appropriately sized ring would largely alleviate the stability concerns associated with linear disulfides. Cyclic disulfides, such as those found in the oxidized form of dithiothreitol and dithioerythritol, are chemically quite stable,[13] and prodrugs such as **9** retain the two-carbon-atom tether necessary for the cyclodeesterification process. Furthermore, the transiently produced thiirane derivative **11** is rapidly disarmed by a facile ring closure to produce a stable tetrahydrothiophene derivative **12** (Scheme 3). The modification of groups attached to the 1,2-dithiane core can then be used to tune the pharmacological and physicochemical properties of the prodrug.[14]

For the synthesis of a representative example of this novel prodrug class, 2′-*C*-methyluridine (**14**, Scheme 4) was treated in the presence of diisopropylethylamine with bis(diisopropylamino)chlorophosphine to produce intermediate **17** (Scheme 5). Cyclization to phosphoramidite **18** was induced by diisopropylethylamine hydrochloride generated in situ and could be further accelerated by the addition of dimethylaminopyridine. Surprisingly, the cyclic phosphoramidite **18** was produced in the form of a single P^{III} diastereoisomer with the *R* configuration, probably as a result of the diastereoselective displacement of one of the two protonated diisopropylamino groups by the 3′-hydroxy group of intermediate **17**. If optimization is desired, the cyclic phosphoramidite **18** can serve as an ideal point of differentiation, as it is chemically stable and readily accessible on a multigram scale. *O*-Benzyl-*trans*-4,5-dihydroxy-1,2-dithiane (**20**) was prepared by a phase-transfer-mediated monoalkylation of racemic **19** and separated into the single enantiomers (+)-**20** (*R*,*R*) and (−)-**20** (*S*,*S*). The absolute configuration was assigned by vibrational circular dichroism (VCD; see the Supporting Information).

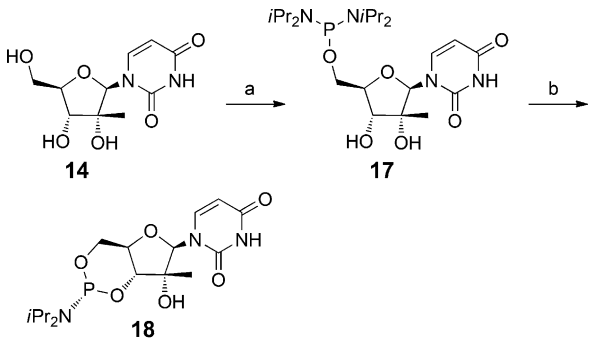
Herein we demonstrate the utility of the proposed technology with 2′-*C*-methyluridine (**14**). This nucleoside is poorly phosphorylated, hence it is only weakly active against hepatocyte virus C (HCV) in cell-based antiviral assays, such as the subgenomic replicon ($EC_{50} = 15.2$ μM, genotype 1b). On the other hand, its triphosphate **15** is a good inhibitor of the HCV NS5B polymerase ($IC_{50} = 1.32$ μM, genotype 1b).[15] A benzyl-modified *trans*-4,5-dihydroxy-1,2-dithiane was utilized as the prodrug moiety (compounds **16**).

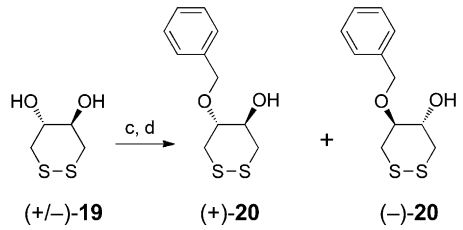


Scheme 4. Structures of 2′-*C*-methyluridine (**14**) and the corresponding nucleoside triphosphate **15** and cyclic-disulfide prodrug **16**.

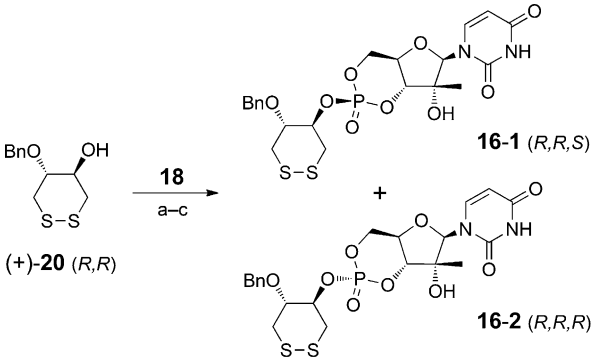
The 1,2-dithianes (+)-**20** and (−)-**20** were converted into prodrugs **16-1**, **16-2** and **16-3**, **16-4** in two synthetic steps performed in a one-pot operation (Scheme 6). Thus, the exposure of alcohol **20** and amidite **18** to mildly acidic ethylthiotetrazole produced the respective P^{III} triester, which was oxidized in situ to the final P^{V} triester with *tert*-butyl hydroperoxide. By detailed NMR spectroscopic analysis of isomers **16-1** and **16-3**, we unambiguously established the absolute configuration at the P^{V} center to be *S*.

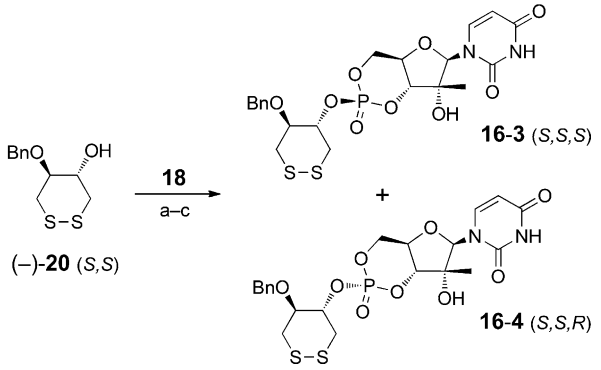
The antiviral activities of prodrugs **16** were evaluated in a cell-based subgenomic replicon assay (Table 1). The 1,2-dithiane prodrugs exhibited a high level of pan-genotypic antiviral activity, thus indicating robust generation of the active triphosphate metabolite **15** in the replicon-containing Huh-7 cells. The most active isomers **16-1** and **16-3** exhibited the *S* absolute configuration at the P^{V} center. The steric





Scheme 5. Synthesis of 1,2-dithiane-based prodrugs. Reaction conditions: a) bis(diisopropylamino)chlorophosphine, DIEA, CH_2Cl_2; b) DIEA·HCl, 4-dimethylaminopyridine, CH_2Cl_2; c) benzyl bromide, MeTHF, 5 N aqueous KOH; d) supercritical fluid chromatography–HPLC, Diacel, Chiralpak AS. MeTHF = 2-methyltetrahydrofuran.





Scheme 6. Synthesis of cyclic-disulfide-based prodrugs **16**. Reaction conditions: a) ethylthiotetrazole, MeCN; b) *t*BuOOH, MeCN; c) preparative HPLC separation. Bn = benzyl.

Table 1: Cell-based antiviral potency of 1,2-dithiane-based prodrugs against HCV genotypes 1a, 1b, and 2a (subgenomic replicon in Huh-7 cells).

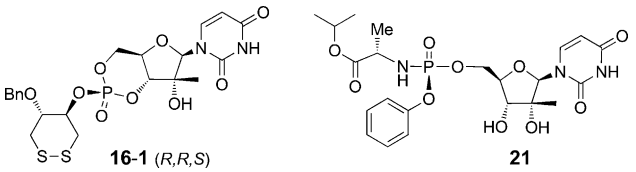
		16-1	**16-2**	**16-3**	**16-4**
Replicon EC_{50} [nM][a]	genotype 1a	14 ± 2	81 ± 7	19 ± 7	332 ± 19
	genotype 1b	27 ± 20	135 ± 50	43 ± 25	471 ± 203
	genotype 2a	14 ± 3	106 ± 45	19 ± 1	420 ± 84

[a] The respective EC_{90} values are listed in the Supporting Information. Values shown are the mean ± standard deviation from $N = 2$ experiments.

arrangement of the diol moiety was less significant: The *R*,*R* isomers were found to be slightly more potent.

To provide a direct comparison of the present technology with the well-established ProTide platform, phosphoramidate **21** was synthesized according to reported procedures.[16] The high antiviral competence of prodrug **16-1** correlated well with both the total level and maximum concentration of the triphosphate **15** in human hepatocytes; which were found to be almost five times those delivered by the ProTide platform **21** (Table 2).

Table 2: Comparison of the ProTide and 1,2-dithiane prodrug platforms.

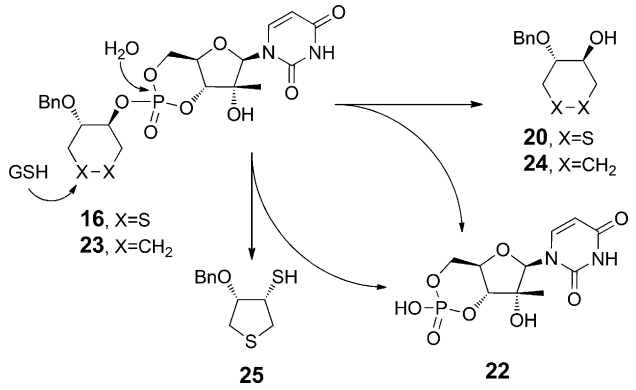


		16-1	**21**
Replicon[a] EC_{50} [nM]	genotype 1a	14 ± 2	75 ± 80
	genotype 1b	27 ± 20	47 ± 23
	genotype 2a	14 ± 3	118 ± 69
Cytotoxicity CC_{50} [μM]		3	26
NTP in vitro[b]	$AUC_{0-48\,h}$ [μM h]	46155	10055
	C_{max} [μM]	1324	251

[a] Subgenomic replicon in Huh-7 cells ($N \geq 2$). [b] Primary human hepatocytes (10 μM dose).

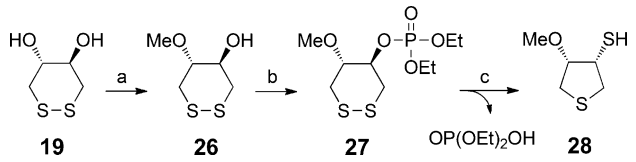
Besides the release mechanism described in Scheme 3, prodrugs such as **16** can in theory also release the cyclic monophosphate **22** by enzymatic hydrolysis of the P^V ester (Scheme 7). To assess the contribution of this pathway, we synthesized the cyclohexyl isostere of **16**, compound **23** (Scheme 7). All four isomers **23-1**, **23-2** and **23-3**, **23-4** were found to be inactive in the replicon assay ($EC_{50} > 100$ μM), thus suggesting that hydrolysis of the P^V phosphotriester does not significantly contribute to the anticipated reductive release pathway in Huh-7 replicon cells.

The therapeutic window of any preclinical candidate is dictated by the toxicological properties of nucleotides generated downstream and prodrug-related metabolites and is maximized during the lead-optimization process. To assess the target-independent, mechanism-related safety profile and de-



Scheme 7. Hydrolytic versus reductive release mechanism of dithiane prodrugs. For a full description of **25**, see the Supporting Information.

risk from the novel platform, we focused on the effect of the release mechanism and metabolites on cytosolic glutathione levels. To eliminate the contribution of downstream nucleotides and more complex, application-specific prodrug breakaway fragments, we synthesized simplified versions of prodrug **16** and its rearrangement product **28** (Scheme 8).

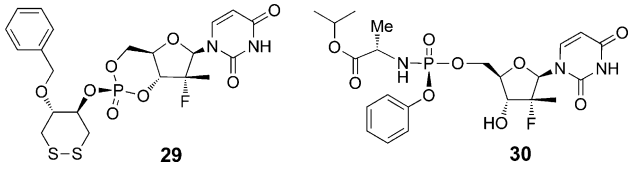


Scheme 8. Simplified 1,2-dithiane prodrugs. Reaction conditions: a) dimethyl sulfate, MeTHF, 5 N aqueous KOH; b) diethyl chlorophosphate, lithium 2-methylbutan-2-olate, THF; c) TCEP, aqueous K_2CO_3. TCEP = tris(2-carboxyethyl)phosphine.

Time-course experiments were conducted in a hepatoma cell line (HepG2) with the "empty" prodrug **27** or its rearrangement product **28** at concentrations of 10 and 100 μM. Neither **27** nor **28** caused measurable reduction of the GSH levels (data not shown) or adversely affected cell viability. In contrast, treatment of the cells with ethacrynic acid[17] caused a decrease in viability of cells predepleted of GSH with buthionine sulfoximine (BSO; see the Supporting Information).

To assess the therapeutic potential of the present platform, we synthesized and evaluated **29**, the 1,2-dithiane prodrug of 2′-deoxy-2′-α-fluoro-β-*C*-methyluridine, and compared it to the respective ProTide prodrug **30** (sofosbuvir), currently marketed for the treatment of HCV infections (Table 3). The 1,2-dithiane-based prodrug **29** exhibited slightly higher antiviral potency against all three HCV genotypes when compared to the ProTide prodrug in a subgenomic replicon assay. Similarly, in an in vitro experiment conducted in human hepatocytes, the 1,2-dithiane was capable of delivering a twofold higher maximum concentration (C_{max}) and level (AUC_{0-48h}) of the respective triphosphate. Remarkably, this trend was also observed in vivo. After a 25 mpk dose (p.o., rats), the concentration of the respective

Table 3: Comparison of ProTide and 1,2-dithiane prodrugs of 2′-deoxy-2′-α-fluoro-β-*C*-methyluridine.



		29	**30**
Replicon[a] EC_{50} [nM]	genotype 1a	48 ± 38	168 ± 96
	genotype 1b	64 ± 38	183 ± 84
	genotype 2a	56 ± 39	205 ± 52
Cytotoxicity CC_{50} [μM]		30	> 100
NTP in vitro[b]	AUC_{0-48h} [μM h]	14587	7166
	C_{max} [μM]	395	202
NTP in vivo[c]	1 h/4 h [μM]	20/49	2/7

[a] Subgenomic replicon in Huh-7 cells ($N \geq 3$). [b] Primary human hepatocytes (10 μM dose). [c] Rat-liver 2′-deoxy-2′-α-fluoro-β-*C*-methyluridine-5′-triphosphate (25 mpk p.o.). mpk = milligram per kilogram, p.o. = oral administration.

triphosphate in liver cells reached 49 μM after 4 h, as compared to 7 μM in the case of the ProTide prodrug **30**: a sevenfold improvement in the level of the active metabolite.

In summary, we have described a promising new class of cyclic-disulfide-based prodrugs that are activated by a non-enzymatic, cytosol-specific reductive release trigger. The fidelity of the release mechanism was demonstrated in vitro with 1,2-dithiane-based prodrugs of 2′-*C*-methyluridine, and a direct comparison to the state-of-the art ProTide platform was provided. The preclinical relevance was further highlighted by the comparison of cyclic-disulfide and ProTide prodrugs of clinically efficacious 2′-deoxy-2′-α-fluoro-β-*C*-methyluridine. We believe that this technology provides a valuable addition to the existing approaches and has the potential to become a preferred therapeutic modality in cases in which cytosol-specific delivery is of essence.

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[1] L. P. Jordheim, D. Durantel, F. Zoulim, C. Dumontet, *Nat. Rev. Drug Discovery* **2013**, *12*, 447 – 464.
[2] M. J. Sofia, *Antiviral Chem. Chemother.* **2011**, *22*, 23 – 49.
[3] D. Cahard, C. McGuigan, J. Balzarini, *Mini-Rev. Med. Chem.* **2004**, *4*, 371 – 381.
[4] C. Périgaud, G. Gosselin, I. Lefebvre, J. L. Girardet, S. Benzaria, I. Barber, J. L. Imbach, *Bioorg. Med. Chem. Lett.* **1993**, *3*, 2521 – 2526.
[5] M. D. Erion, K. R. Reddy, S. H. Boyer, M. C. Matelich, J. Gomez-Galeno, R. H. Lemus, B. G. Ugarkar, T. J. Colby, J.

Schanzer, P. D. Van Poelje, *J. Am. Chem. Soc.* **2004**, *126*, 5154 – 5163.
[6] C. Meier, *Mini-Rev. Med. Chem.* **2002**, *3*, 219 – 234.
[7] D. R. Bobeck, R. F. Schinazi, S. Coats, *Antivir. Ther.* **2010**, *15*, 935 – 950.
[8] A. T. Dinkova-Kostova, M. A. Massiah, R. E. Bozak, R. J. Hicks, P. J. Talalay, *Proc. Natl. Acad. Sci. USA* **2001**, *98*, 3404 – 3409.
[9] V. Lushchak, *J. Amino Acids* **2012**, 2012.
[10] H. Ostergaard, C. Tachibana, J. R. Winther, *J. Cell Biol.* **2004**, *166*, 337 – 345.
[11] P. Maher, *Ageing Res. Rev.* **2005**, *4*, 288 – 314.
[12] G. Saito, J. A. Swanson, K. D. Lee, *Adv. Drug Delivery Rev.* **2003**, *55*, 199 – 215.
[13] J. A. Burns, G. M. Whitesides, *J. Am. Chem. Soc.* **1990**, *112*, 6296 – 6303.
[14] For examples of nucleoside payloads, modifying groups, and potential therapeutic applications, see: G. Butora (Merck & Co.), WO2014/088923, **2013**.
[15] A. Cho, L. Zhang, J. Xu, D. Babusis, T. Butler, R. Lee, O. L. Saunders, T. Wang, J. Parrish, J. Perry, J. Y. Feng, A. S. Ray, C. U. Kim, *Bioorg. Med. Chem. Lett.* **2012**, *22*, 4127 – 4132.
[16] Y. Mehellou, R. Valente, H. Mottram, E. Walsby, K. I. Mills, J. Balzarini, C. McGuigan, *Bioorg. Med. Chem.* **2010**, *18*, 2439 – 2446.
[17] B. Ji, K. Ito, S. Sekine, A. Tajima, T. Horie, *Free Radical Biol. Med.* **2004**, *37*, 1718 – 1729.