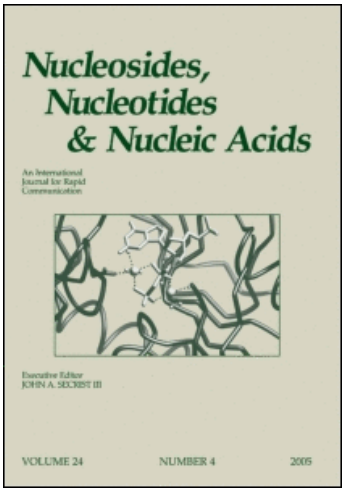


Nucleosides, Nucleotides and Nucleic Acids
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597286

Ribonucleotide Reductase Targets for Chemotherapy; Mechanistic Aspects and Biologically Active Agents

Morris J. Robins[a]; Mirna C. Samano[ab]; Vicente Samano[ab]
[a] Department of Chemistry and Biochemistry, Brigham Young University, Provo, UT, U.S.A. [b] Burroughs Wellcome Co., Research Triangle Park, NC

To cite this Article Robins, Morris J. , Samano, Mirna C. and Samano, Vicente(1995) 'Ribonucleotide Reductase Targets for Chemotherapy; Mechanistic Aspects and Biologically Active Agents', Nucleosides, Nucleotides and Nucleic Acids, 14: 3, 485 — 493
To link to this Article: DOI: 10.1080/15257779508012411
URL: http://dx.doi.org/10.1080/15257779508012411

NUCLEOSIDES & NUCLEOTIDES, 14(3-5), 485-493 (1995)

RIBONUCLEOTIDE REDUCTASE TARGETS FOR CHEMOTHERAPY; MECHANISTIC ASPECTS AND BIOLOGICALLY ACTIVE AGENTS

Morris J. Robins,* Mirna C. Samano,¶ and Vicente Samano ¶

Department of Chemistry and Biochemistry, Brigham Young University, Provo, UT 84602 U.S.A.

Abstract: Ribonucleotide reductases are essential for the *de novo* biosynthesis of the 2'-deoxynucleotide components of DNA. These enzymes have complex cofactors and execute novel chemistry involving C2' via radical abstraction of H3'. Mechanistic aspects of these transformations and selected nucleotide analogues that cause mechanism-based inactivation of ribonucleotide reductases are discussed.

Introduction

Ribonucleotide reductases are crucial enzymes present in organisms from viruses to humans.[1] They convert ribonucleotides to 2'-deoxy derivatives in the unique *de novo* pathways to these essential monomers for DNA biosynthesis. Of the known classes, ribonucleoside diphosphate reductases (RDPRs, EC 1.17.4.1) occur most widely (*e.g.* in *Escherichia coli*, mammalian cells, herpes simplex virus, yeast). The enzyme from *E. coli* has been studied extensively and the RDPR from mammalian cells has functional and structural similarities. These RDPRs are composed of two major nonidentical subunits R1 and R2 (formerly designated B1 and B2, M1 and M2, etc.). In turn, R1 and R2 each are composed of two polypeptide chains to provide an overall $\alpha_2\beta_2$ structure.

The R1 subunit contains binding sites for the four natural substrates ADP, CDP, GDP, and UDP; binding sites for nucleoside triphosphates which act as allosteric effectors to control velocities of formation of the product 2'-deoxyNDPs; and appropriately located

*Author to whom correspondence should be addressed at Brigham Young University.

¶Present address Burroughs Wellcome Co., Research Triangle Park, NC.

cysteine units for dithiol–disulfide redox functionality. This proximate redox system is connected by a complex electron transfer pathway to the ultimate reductant NADPH.

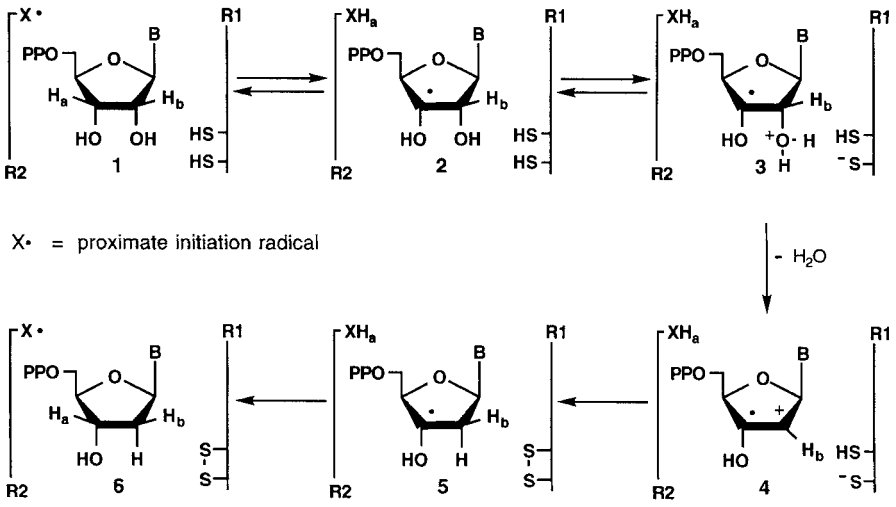
The R2 subunit contains a binuclear iron(III) center and a "stable" free radical associated with the oxyphenyl moiety of a tyrosine residue in the polypeptide backbone. This intriguing phenoxy free radical serves as the initiator of the rather complex mechanism associated with overall removal of the hydroxyl group from C2' and its replacement by hydrogen with complete stereoretention at the α-face of the furanosyl ring.

Ribonucleoside 5'-triphosphate reductase (RTPR) from *Lactobacillus leichmannii* is the most widely studied RTPR. Although it is composed of a single polypeptide chain and utilizes adenosylcobalamin (coenzyme B_{12}) as a radical initiation cofactor, it appears to execute 2'-deoxygenation analogously. Parallel mechanistic working hypotheses have been invoked for this RTPR and the noted RDPRs in terms of overall substrate reactions and inactivation by 2'-modified nucleotide analogues.

Mechanistic Aspects

Stubbe and coworkers[1c,2] have proposed a generic mechanism for RDPR (Scheme 1): The hydrogen atom at C3' of the nucleoside diphosphate substrate **1** is abstracted by a radical (possibly generated by electron transfer between the phenoxy free radical and an atom/group in closer proximity to **1**) to give the C3' radical **2**. The O2' is lost heterolytically from **3** by departure of a water molecule, generated by hydrogen-bonding of **2** to a thiol of a dithiol pair, to produce the resonance stabilized cation radical **4**. Transfer of a hydride equivalent (possibly by electron transfer followed by abstraction of thiol hydrogen) to C2' at the α-face results in formation of 2'-deoxy C3' radical **5**. Return of the hydrogen atom, originally abstracted from C3' by the transient proximate radical, completes the catalytic cycle and regenerates the biological radical initiator. The disulfide created on the R1 subunit is reduced to a dithiol pair by the complex electron transfer pathway from NADPH, and the enzyme is recharged for the next turnover to synthesize another 2'-deoxyNDP **6**. Analogous chemistry with coenzyme B_{12} as the ultimate radical initiator was proposed for the RTPR system.[1c,3]

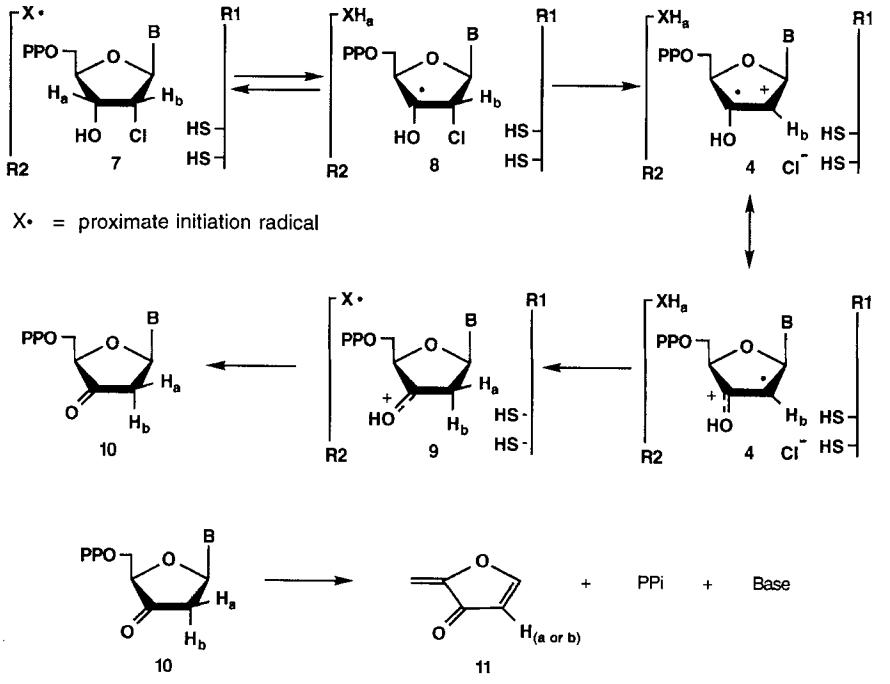
Thelander *et al.* reported irreversible inactivation of *E. coli* RDPR by 2'-chloro-2'-deoxynucleoside 5'-diphosphates.[4] Stubbe and coworkers studied these inactivations in detail and proposed mechanisms which begin in the same manner as the normal substrate turnover.[1c,5] Thus, the radical initiator abstracts H3' from 2'-chloro-2'-deoxy analogue **7**



Scheme 1. Proposed mechanism for ribonucleoside diphosphate reductase.

(Scheme 2) to give the 3'-radical **8**. Spontaneous loss of chloride was invoked to generate cation-radical **4** without protonation by the proximate thiols [*i.e.* H-bonding to OH and loss of H_2O (pKa ~ 15.7) in Scheme 1 *versus* spontaneous loss of Cl^- (HCl, pKa ~ -7) in Scheme 2]. Delivery of a hydrogen atom to C2' of the resonance-stabilized **4** followed by loss of the proton from O3' of **9** would give the 2'-deoxy-3'-keto intermediate **10**. Analogous 2'-deoxy-3'-ketonucleosides are known to undergo spontaneous β-elimination of H2' and the heterocyclic base at ambient temperature in solutions of dimethylsulfoxide.[6] Accompanying β-elimination of H4' and inorganic pyrophosphate would produce the 2-methylene-3(2*H*)-furanone (**11**) proposed by Stubbe and coworkers to be the Michael acceptor which effects covalent alkylation causing time-dependent irreversible inactivation of the enzyme.[3b]

Irreversible inactivation of RDPR by 2'-azido-2'-deoxynucleoside 5'-diphosphates (**12**) also occurred,[4] and Sjöberg *et al.* found a new EPR signal consistent with a nitrogen-centered radical.[7] This was the first direct experimental evidence for free radical chemistry in the proposed reductase mechanisms. Sjöberg *et al.* reconstituted a RDPR containing the R2 subunit from *E. coli* grown on [^{15}N]-ammonium sulfate. However, EPR spectra of both the [^{15}N]-R2 and natural [^{14}N]-R2 RDPRs inactivated with [^{14}N]-azido nucleotide



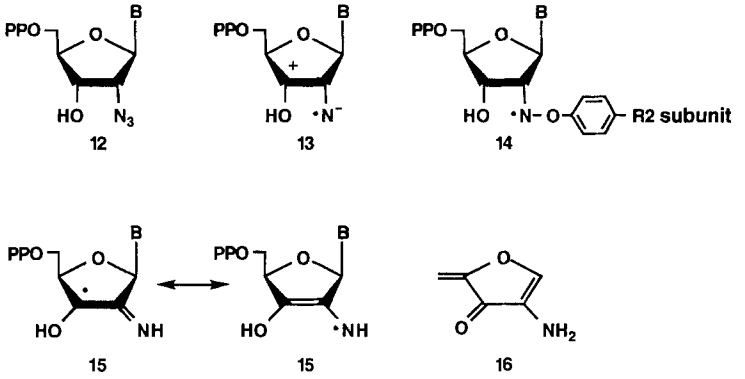
Scheme 2. Proposed mechanism for inactivation of RDPR by 2'-chloro-2'-deoxyNDPs.

were identical.[7] Stubbe and coworkers reinvestigated this[8,9] and analogous inactivation of RTPR by 2'-azido-2'-deoxynucleoside triphosphates including the *arabino* epimer of 2'-azido-dATP.[1c] Sjöberg *et al.* found that inactivation by the azido analogues resulted in disappearance of the tyrosyl radical EPR signal,[7] and Stubbe noted that the azido compounds were much more efficient inactivators than their chloro counterparts.[9]

Identification of the nitrogen radical species has proven to be elusive. Stubbe and coworkers[8] found altered splitting of the EPR signal in harmony with the unpaired electron on a [^{15}N]-radical upon inactivation of RDPR with a [^{15}N]-azido nucleotide. However, no EPR changes were observed upon substitution of sugar hydrogens H1', H2', H3', or H4' by deuterium.[1c,8,10] Inactivation with a [$^{15}N_3$]-azido nucleotide resulted in evolution of [$^{30}N_2$]-dinitrogen gas[9] and gave the same EPR signal as the [^{15}N]-azido analogue.[10] A sensitive biological assay failed to detect azide in the RDPR incubation mixture.[9]

Based on these observations Sjöberg *et al.*[7] suggested structures **13** and **14** as possibilities for the new nitrogen-centered radical, and Stubbe and coworkers proposed the

resonance stabilized radical **15**.[9] These proposed structures have the α-nitrogen of the azido group bound to the sugar residue. Stubbe's working hypothesis for generation of **15** involved abstraction of H3' from the azido nucleotide to give the 2'-azido-3'-radical (see the initiation steps of Schemes 1 and 2) followed by expulsion of dinitrogen and migration of H2' to give **15**. Abstraction of hydrogen and elimination of inorganic pyrophosphate and the base was proposed[9] to give 4-amino-2-methylene-3(2*H*)-furanone (**16**), which would inactivate the enzyme by analogous Michael alkylation.



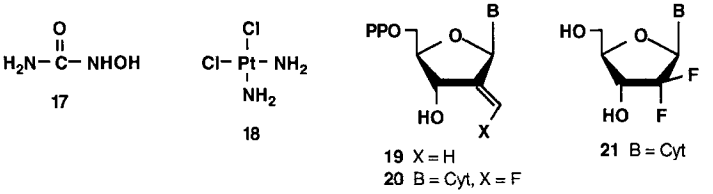
We had concerns with certain aspects of this mechanism. (1) Sjöberg *et al.* had reconstituted RDPR from their [^{15}N]-R2 subunit and [^{14}N]-R1 (B2 and B1).[7] Thus, their inactivation experiment with a [^{14}N]-azido nucleotide and observation of an identical EPR spectrum to that with the native enzyme might have detected effects involving only the R2 (B2) subunit. Information relative to effects on R1 (B1) might have been overlooked. (2) The absence of effects on EPR spectra by substitution of sugar hydrogens H1'-H4' with deuterium is not consistent with the α-nitrogen of the azido group remaining bound to the sugar. (3) Unprecedented chemistry for fragmentation of a β-azido radical intermediate had been invoked. (4) Failure to detect azide in the "bulk solution" of the inactivation mixtures, observation of [$^{30}N_2$]-nitrogen gas evolution, and observation of a [^{15}N]-nitrogen-centered free radical did not preclude departure of an intact azide species within the active site of the enzyme complex followed by rapid destruction of azide to give the observed products.

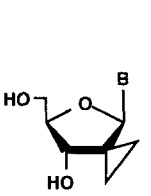
In order to probe the crucial question of whether the α-nitrogen of the azido group remained bonded to C2' in the radical, a known procedure[11] was employed to prepare the doubly labeled 2'[$^{15}N_3$]-azido-2'[^{13}C]-deoxyuridine.[10] The 5'-diphosphate of this compound caused inactivation of RDPR and produced a [^{15}N]-centered radical whose EPR

spectrum was identical to that from a parallel inactivation with 2'[^{15}N]-azido-dUDP.[10] This powerful evidence for cleavage of the azido group from C2' (*i.e.* no observed [^{15}N]–[^{13}C] hyperfine interaction) was corroborated by studies with site-directed mutants with serine for cysteine substitutions, and with RDPR pre-oxidized by turnover with CDP in the absence of a biological reducing system. The cysteine-225 mutant gave a modified RDPR which degraded 2'-azido-dUDP with concomitant loss of the tyrosine-122 radical, but without formation of the nitrogen-centered radical, and released azide into the bulk medium. The pre-oxidized RDPR also suffered loss of the tyrosine-122 radical, released azide into the medium, and failed to generate the nitrogen-centered radical. In addition, ESEEM spectroscopy with RDPR inactivated by selectively deuterated ([^{2}H]1' - [^{2}H]4') 2'-azido-dUDPs provided spatial information consistent with the nitrogen-centered radical being located "below" the α-face of the sugar residue, and closer to H4'. New mechanistic interpretations have been proposed which involve azide loss from the initial C3' radical intermediate and subsequent reaction of an azide species with the sulfhydryl group to generate nitrogen gas and a nitrogen-centered radical.[10] Further biological and chemical modelling studies are in progress to probe this fascinating enzyme chemistry more deeply. Preliminary chemical modelling results are consistent with radical eliminations for the 2'-azido- and 2'-chloro-2'-deoxy analogues. However, involvement of specific enzyme functionalities undoubtedly are required to execute the biologically crucial 2'-deoxygenation process, and likely also the removal of fluoride from 2'-deoxy-2'-fluoro analogues.

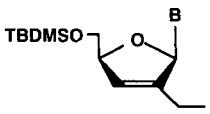
Selected Biologically Active Agents

The simple molecule hydroxyurea (**17**) is the only drug in general clinical use for which the primary target is thought to be RDPR.[12] The widely employed cisplatin (*cis*-diamminedichloroplatinum, **18**) has been found to cause strong, stereoselective inhibition of RDPR.[13] Allosteric repression of RDPR activity is effected by a number of nucleoside triphosphates,[1] but examples of nucleosides whose diphosphates function as mechanism-based inhibitors of RDPR have been reported recently.

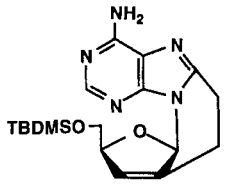




22



23 B = Ade



24

The 2'-deoxy-2'-methylenenucleoside 5'-diphosphates (**19**) were designed to be mechanism-based inhibitors that could be activated by abstraction of H3' to give an allylic radical.[14] Potent time-dependent inactivation of RDPR by the cytosine (MdCDP)[15] as well as uracil[15] and purine analogues was observed, and antitumor activity was demonstrated with MdCyd.[14b,16] McCarthy and coworkers prepared the (fluoro)methylene analogue (FMdCDP, **20**) of MdCDP, which has enhanced potency as an inactivator of RDPR,[17] and FMdCyd is an investigational agent with activity against a number of tumor systems. Gemcitabine (2'-deoxy-2',2'-difluorocytidine, **21**) is a recent agent with good activity against solid tumors. The diphosphate of **21** is a potent inactivator of RDPR,[15] although its primary mode of biological activity likely is at the triphosphate level as an inhibitor of DNA polymerase[18] and this also appears to be the case with the 2'-methylene analogues.[19] Higher levels of triphosphate, relative to diphosphate, were found with **21** in biological systems.[18] The new 2'-deoxynucleoside-2'-spirocyclopropanes[20] (**22**) were designed as mechanism-based inactivators of RDPR in which abstraction of H3' would generate a cyclopropylcarbinyl radical. Chemical generation of a 3'-deoxy C3' radical in the adenine series resulted in rearrangement to the ethyl-vinyl product **23** plus the cyclized **24** formed by radical attack at C8.[20] The 5'-diphosphates of **22** are not accepted well by RDPR, which was known to have restrictive steric requirements in the 2'-region. However, preliminary results with a triphosphate and the more promiscuous RTPR appear promising.

In summary, ribonucleotide reductases are enzymes that have intriguing cofactors and execute novel chemistry involving C2' via radical abstraction of H3'. They provide the only *de novo* source of 2'-deoxynucleotides for DNA biosynthesis, and thus are appealing targets for chemotherapeutic intervention in diseases which involve cellular proliferative or other replicative hyperactivity. Mechanistic studies have illuminated fascinating chemical transformations.[1c] We now propose that modification of Stubbe's hypothesis (uniform anionic heterolysis at C2' to produce a stabilized sugar radical cation)[1c] to include radical

homolysis at C2' with certain 2'-substituents is more plausible. This homolytic mode also is consistent with alternative interpretations of the data. However, involvement of specific functionalities within the enzyme complex undoubtedly is required for the normal substrate turnover (*i.e.* 2'-deoxygenation) and removal of fluoride at C2'. Small molecules such as hydroxyurea and cisplatin inactivate RDPR and are used in clinical medicine against cancer. New nucleosides have been shown to cause mechanism-based inactivation of RDPR and development of investigational new agents is in progress.

Acknowledgments. We thank the American Cancer Society (Grant No. DHP-34) and Brigham Young University Development Funds for generous support and Mrs. Hazel Dunsmore for assistance with the manuscript.

REFERENCES

1. For extensive reviews see: (a) Thelander, L.; Reichard, P. *Annu. Rev. Biochem.* **1979**, *48*, 133-158. (b) Lammers, M.; Follmann, H. *Struct. Bonding (Berlin)* **1983**, *54*, 27-91. (c) Ashley, G. W.; Stubbe, J. In *Inhibitors of Ribonucleoside Diphosphate Reductase Activity.* International Encyclopedia of Pharmacology and Therapeutics, Section 128; Cory, J. G., Cory, A. H., Eds.; Pergamon Press: New York, 1989; pp. 55-87.
2. Stubbe, J; Ator, M.; Krenitsky, T. *J. Biol. Chem.* **1983**, *258*, 1625-1631.
3. (a) Harris, G.; Ashley, G. W.; Robins, M. J.; Tolman, R. L.; Stubbe, J. *Biochemistry* **1987**, *26*, 1895-1902. (b) Ashley, G. W.; Harris, G.; Stubbe, J. *Biochemistry* **1988**, *27*, 4305-4310; 7841-7845.
4. Thelander, L.; Larsson, B.; Hobbs, J.; Eckstein, F. *J. Biol. Chem.* **1976**, *251*, 1398-1405.
5. Ator, M. A.; Stubbe, J. *Biochemistry* **1985**, *24*, 7214-7221.
6. Hansske, F.; Madej, D.; Robins, M. J. *Tetrahedron* **1984**, *40*, 125-135.
7. Sjöberg, B.-M.; Gräslund, A.; Eckstein, F. *J. Biol. Chem.* **1983**, *258*, 8060-8067.
8. Ator, M.; Salowe, S. P.; Stubbe, J.; Emptage, M. H.; Robins, M. J. *J. Am. Chem. Soc.* **1984**, *106*, 1886-1887.
9. Salowe, S. P.; Ator, M. A.; Stubbe, J. *Biochemistry* **1987**, *26*, 3408-3416.
10. Salowe, S.; Bollinger, J. M., Jr.; Ator, M.; Stubbe, J.; McCracken, J.; Peisach, J.; Samano, M. C.; Robins, M. J. *Biochemistry* **1993**, *32*, 12749-12760.

11. Verheyden, J. P. H.; Wagner D.; Moffatt, J. G. *J. Org. Chem.* **1971**, *36*, 250-254.

12. Moore, E. C.; Hurlbert, R. B. In *Inhibitors of Ribonucleoside Diphosphate Reductase Activity.* International Encyclopedia of Pharmacology and Therapeutics, Section 128; Cory, J. G., Cory, A. H., Eds.; Pergamon Press: New York, **1989**; pp. 165-201.

13. Smith, S. L.; Douglas, K. T. *Biochem. Biophys. Res. Commun.* **1989**, *162*, 715-723.

14. (a) Samano, V.; Robins, M. J. *J. Org. Chem.* **1991**, *56*, 7108-7113. (b) Robins, M. J.; Samano, V.; Zhang, W.; Balzarini, J.; De Clercq, E.; Borchardt, R. T.; Lee, Y.; Yuan, C.-S. *J. Med. Chem.* **1992**, *35*, 2283-2293.

15. Baker, C. H.; Banzon, J.; Bollinger, J. M.; Stubbe, J.; Samano, V.; Robins, M. J.; Lippert, B.; Jarvi, E.; Resvick, R. *J. Med. Chem.* **1991**, *34*, 1879-1884.

16. (a) Takenuki, K.; Matsuda, A.; Ueda, T.; Sasaki, T.; Fujii, A.; Yamagami, K. *J. Med. Chem.* **1988**, *31*, 1063-1064. (b) Yamagami, K.; Fujii, A.; Arita, M.; Okumoto, T.; Sakata, S.; Matsuda, A.; Ueda, T.; Sasaki, T. *Cancer Res.* **1991**, *51*, 2319-2323.

17. McCarthy, J. R.; Matthews, D. P.; Stemerick, D. M.; Huber, E. W.; Bey, P.; Lippert, B. J.; Snyder, R. D.; Sunkara, P. S. *J. Am. Chem. Soc.* **1991**, *113*, 7439-7440.

18. (a) Heinemann, V.; Xu, Y.-Z.; Chubb, S.; Sen, A.; Hertel, L. W.; Grindey, G. B.; Plunkett, W. *Mol. Pharmacol.* **1990**, *38*, 567-572. (b) Huang, P.; Chubb, S.; Hertel, L. W.; Grindey, G. B.; Plunkett, W. *Cancer Res.* **1991**, *51*, 6110-6117.

19. Cory, A. H.; Samano, V.; Robins, M. J.; Cory, J. G. *Biochem. Pharmacol.* **1994**, *47*, 365-371.

20. Samano, V.; Robins, M. J. *J. Am. Chem. Soc.* **1992**, *114*, 4007-4008.