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Camptothecin analogs with bulky, hydrophobic substituents at the 7-position via a Grignard reaction

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Abstract—By developing a new synthetic procedure for introduction of side chains onto the camptothecin ring system, we were able to achieve the preparation of a number of analogs bearing bulky, hydrophobic groups directly attached to the 7-position. These include 7-*tert*-butylcamptothecin, 7-benzylcamptothecin and the corresponding 10,11-methylenedioxycamptothecins. This method involves the reaction of an appropriate orthoaminobenzonitrile with various Grignard reagents to give the corresponding orthoaminoketones. Friedlander condensation of the latter with the key tricyclic ketone leads to 7-substituted camptothecin analogs. We report the activity of these compounds as topoisomerase I poisons and their ability to inhibit growth of selected tumor cell lines.

In 1966, Wall and colleagues discovered that camptothecin (CPT) was the component in the extract from the stem of the Chinese tree *Camptotheca acuminata* active against L1210 murine leukemia cells.[1] CPT is a specific topoisomerase I inhibitor (or poison).[2,3] Topoisomerase I (topo I) relaxes DNA supercoiling by making transient single-strand breaks.[4,5] These breaks are coupled with the formation of a covalent DNA–enzyme intermediate termed the cleavable complex.[2,3] CPT and analogs specifically and reversibly stabilize cleavable complexes by inhibiting DNA religation (reviewed in Ref. 6). The mechanism of CPT cytotoxicity is thought to be the consequence of a collision between moving replication forks and CPT-stabilized cleavable DNA–topo I complexes.[3,7,8] Due to the activity of CPT, a new series of compounds based on CPT that trapped topo I–DNA complexes and led to cell death were developed.[9–16] More recently, an emphasis has been placed on the utility of extremely hydrophobic CPT analogs that may bind to serum albumin or be formulated for slow release.[17–19] A review of the clinical use of very lipophilic analogs has recently appeared.[20]

In this report, we describe a new scheme for synthesizing CPT analogs with bulky, hydrophobic moieties at the 7-position, and describe the in vitro activity of these new compounds. A number of synthetic routes have been used to synthesize CPT analogs (recently reviewed in Refs. 21,22). The basic procedure for the total synthesis of CPT and 10,11-methylenedioxy-CPT (MD-CPT) that we use is the Friedlander reaction of orthoaminobenzaldehyde with a (*S*) tricyclic synthon (Scheme 1).[16,23–26] We previously used this scheme to synthesize a large number of CPT analogs.[15]

Other groups have attached bulky, hydrophobic groups, such as *tert*-butyl to the 7-position by the use of a dimethylsilane linker (silatecan[27]) or through an oxyimino linker (ST1481[28]). Our initial attempts to directly attach the *tert*-butyl group to the 7-position of CPT and MD-CPT by the Sawada method[13] were unsuccessful due to the poor reactivity of the *tert*-butanal. However, by developing a new scheme, we were able to achieve the preparation of 7-*tert*-butylCPT. The general synthetic approach to the CPT analogs with bulky hydrophobic groups at the 7-position is shown in Scheme 1. Thus, reaction of a commercially available 2-cyanoaniline (**1a** or **1b**) with an appropriate Grignard reagent yields the corresponding aminoketone (**2a** or **2b**). Friedlander condensation of an appropriate aminoketone with the key tricyclic intermediate in the (*S*) configuration

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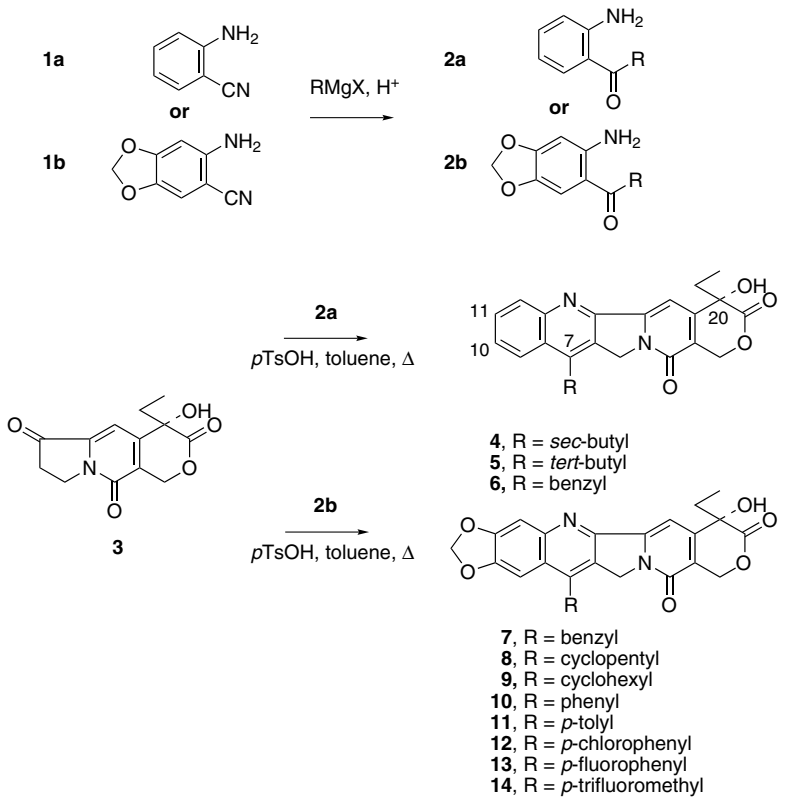

Scheme 1.

(**3**)[16,24] leads to the formation of the corresponding 20-(*S*)-7-substituted-CPT analog (**4**–**14**). For example, compound **7** was synthesized by the reaction of 0.025 mol of 2-cyano-4,5-methylenedioxyaniline, CuI, and 0.1 mol of benzylmagnesiumbromide in THF. The reaction mixture was stirred overnight at room temperature after the addition of 15% H_2SO_4 (5 mL). The resulting aminoketone **2b** was isolated by silica gel column chromatography in 58% yield. Equimolar amounts of **2b** and the tricyclic intermediate **3** were refluxed in toluene containing *p*-toluenesulfonic acid (30 mg) for 8 h. The resulting camptothecin analog **7** was isolated by column chromatography and obtained in 78% yield.

Using this synthetic scheme, a number of bulky, lipophilic CPT analogs (**4**–**14**) were synthesized (Scheme 1). The newly synthesized analogs were characterized for their ability to inhibit topo I (EC_{50}), stabilize topo I–DNA cleavable complexes (k_{app} by competitive DNA assay), and inhibit growth of HeLa (cervical) and PC-3 (prostate) tumor cells (three-day continuous exposure MTT assay). The experimental protocols for these assays are those published previously by us.[29,30] Briefly, CPT analog-induced cleavable complex formation was performed using pBR322 plasmid DNA (Gibco BRL) and human topo I enzyme (Topogen, Inc.). The samples were analyzed by electrophoresis for 16 h at 30 V on a 1% agarose gel in TAE buffer (containing 0.5 μg/mL ethidium bromide). After electrophoresis, the gel was stained with SYBR Green (Molecular Probes, Eugene, OR) and photographed using a Kodak Image Station 440CF. The resulting photograph was analyzed digitally to determine EC_{50} (the concentration of drug required to produce 50% nicked DNA). Due to the poor solubility of the hydrophobic analogs in high salt solutions, reversal of the topo I cleavage activity of the pBR322 plasmid DNA was accomplished by the method of Hecht and colleagues.[31] Cleavable complexes were formed in the presence of sufficient analog to induce ~90% nicked DNA (as determined from EC_{50} curves) for more water soluble analogs (Fig. 1A), or to the maximum of water solubility for less soluble analogs (Fig. 1B). A 100-fold excess of sonicated salmon sperm linear DNA (Gibco BRL, 10 mg/mL) was added to the reaction mixture to initiate reversal of complex formation. All reactions were performed at 37 °C. Aliquots were removed at selected time intervals for analysis and k_{app} determined by fits to a single exponential decay. Under the conditions used here, this is approximately the dissociation constant for the ternary complex (discussed at length in Ref. 29). For inhibition of cell growth, the human prostate carcinoma cell line PC-3 and the cervical carcinoma HeLa line were maintained in DMEM supplemented with 10% fetal bovine serum. Exponentially growing cells ($1–2 \times 10^3$ cells, unless otherwise specified) in 0.1 mL medium were seeded on day 0 in a 96-well microtiter plate. On day 1, 0.1 mL aliquots of medium containing graded concentrations of test analogs were added in triplicate to the cell plates. After incubation for three days, cell growth was monitored by the standard MTT assay. The IC_{50} is the concentration of drug

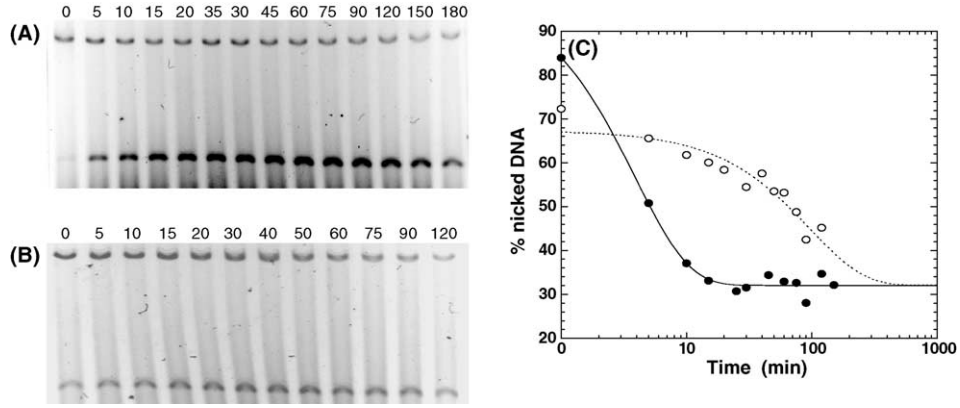

Figure 1. Comparison of cleavable complex stability for (A) 7-*t*-butyl-CPT and (B) 7-*p*-trifluoromethylphenyl-MD-CPT. Times following addition of excess salmon sperm DNA are shown above each lane. Data are plotted in (C) as % nicked DNA present in solution (upper band in panels (A) and (B)) for 7-*t*-butyl-CPT (∫) and 7-*p*-trifluoromethylphenyl-MD-CPT (), respectively.

inhibiting cell growth by 50%. The results are recorded in Table 1. We also include previously synthesized related analogs in Table 1 for comparison.

All of the new analogs were active as inhibitors of topo I. The ability of the 7-*sec*-butyl- and 7-*tert*-butyl- analogs to inhibit topo I was comparable to CPT alone and the 7-*n*-butyl- analog, indicating a general tolerance for branched chain alkyl groups at this position. However, those analogs bearing a 7-benzyl- and certain 7-aryl- substituents are considerably less potent at inhibiting topo I than the analog without those substituents. Against cultured cell lines, the growth inhibition activity of the new compounds was comparable to related analogs, and was generally better than CPT alone. This is most likely due to efficient cellular accumulation of the more hydrophobic compounds. However, the EC_{50} values are not correlated with IC_{50}. This phenomenon has been observed before by us and by other laboratories.[30,32–34] The exceptions are the 7-*sec*-butyl- and 7-*tert*-butyl-CPT analogs, which were less active than CPT alone. The reason for this appears to be the poor stability of the cleavable complex formed with some of these analogs (see below).

The stability of the cleavable complexes formed with the hydrophobic analogs is highly correlated with the ability of these analogs in inhibiting tumor cell growth (Fig. 2). Some interesting features become apparent from the data in Table 1. The 7-benzyl- and 7-*tert*-butyl-CPT, both of which would have bulky groups above or below the plane of the CPT ring system, form less stable complexes than CPT itself. Curiously, this effect can be overcome by the MD-CPT analogs, where complexes formed with 7-benzyl-MD-, 7-cyclopentyl-MD-, and 7-cyclohexyl-MD-CPT are similar to those obtained for MD-CPT alone. Even more surprising is that the 7-aryl-MD analogs form remarkably stable cleavable

Table 1. Activity of CPT analogs with bulky 7-substituents

CPT analog	EC_{50} for topo I (μM)	k_{app} for complex stability (min^{-1})	Half-life for complex stability (min)	IC_{50} versus HeLa cells (nM)	IC_{50} versus PC-3 cells (nM)
CPT[a]	0.35 ± 0.01	0.149 ± 0.010	4.7 ± 0.3	117.2 ± 30.5	141.4 ± 5.9
7-Ethyl-10-hydroxy-CPT (SN-38)[a]	0.32 ± 0.04	0.061 ± 0.010	11.4 ± 1.9	25.7 ± 2.0	35.6 ± 4.5
7-Ethyl[a]	0.07 ± 0.03	0.128 ± 0.070	5.4 ± 3.0	38.8 ± 3.1	35.3 ± 4.0
7-*n*-Butyl[a]	0.20 ± 0.11	0.051 ± 0.014	13.6 ± 3.7	37.3 ± 0.8	48.7 ± 7.2
7-*sec*-Butyl	0.45 ± 0.01	0.116 ± 0.015	6.0 ± 0.8	131.0 ± 30.9	170.5 ± 1.9
7-*tert*-Butyl	0.24 ± 0.03	0.265 ± 0.095	2.6 ± 0.9	204.1 ± 13.2	307.6 ± 108.3
7-Benzyl	4.56 ± 3.01	0.240 ± 0.021	2.9 ± 0.3	80.9 ± 13.4	51.2 ± 4.7
MD-CPT[a]	0.05 ± 0.02	0.058 ± 0.003	12.0 ± 0.6	9.6 ± 2.8	8.1 ± 1.2
7-Ethyl-MD[a]	0.27 ± 0.05	0.037 ± 0.020	18.7 ± 10.1	13.9 ± 2.4	10.0 ± 1.1
7-Benzyl-MD	1.95 ± 1.10	0.057 ± 0.005	12.2 ± 1.1	20.5 ± 7.7	18.9 ± 3.1
7-Cyclopentyl-MD	0.19 ± 0.15	0.029 ± 0.008	23.9 ± 6.6	6.2 ± 0.4	9.0 ± 1.0
7-Cyclohexyl-MD	1.0[b]	0.031 ± 0.003	22.4 ± 2.2	9.0 ± 0.8	11.5 ± 1.8
7-Phenyl-MD	0.15 ± 0.08	0.005 ± 0.001	138.6 ± 27.7	8.1 ± 0.9	8.1 ± 0.2
7-*p*-Tolyl-MD	1.04 ± 0.20	0.005 ± 0.001	138.6 ± 27.7	2.0 ± 2.0	8.3 ± 1.0
7-*p*-Chlorophenyl-MD	1.16 ± 0.10	0.008 ± 0.001	86.6 ± 10.8	26.0 ± 16.0	8.2 ± 0.7
7-*p*-Fluorophenyl-MD	0.39 ± 0.04	0.004 ± 0.001	173.3 ± 43.3	16.2 ± 5.6	6.3 ± 1.1
7-*p*-Trifluoromethylphenyl-MD	0.16 ± 0.13	0.007 ± 0.003	99.0 ± 42.4	6.9 ± 2.0	13.4 ± 2.1

[a] The synthesis and characterization of these compounds has been described previously.[29]

[b] Inhibition (poisoning) of topo I was approximately 50% complete at 1 μM 7-cyclohexyl-MD-CPT. However, poor aqueous solubility above this concentration prevented establishment of a complete concentration-dependent inhibition curve.

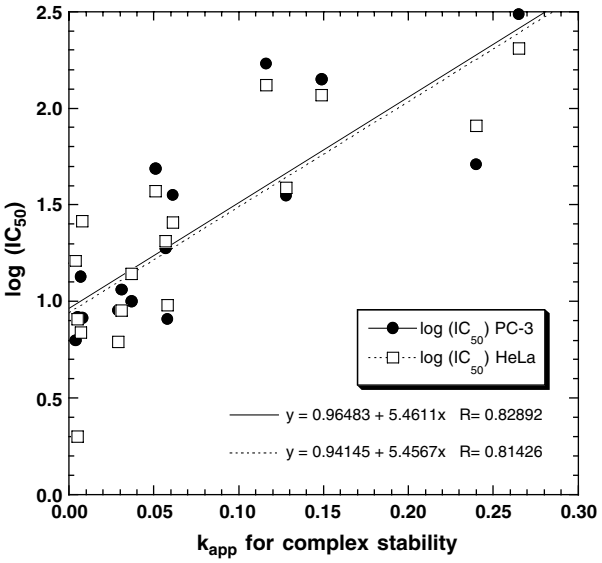

Figure 2. Correlation of cleavable complex stability with growth inhibition.

complexes. Our previous studies had suggested that hydrophilic substituents at the 7-position resulted in more stable cleavable complexes.[30] Clearly, our data on the 7-aryl-MD- analogs suggests that hydrophilicity is not a requirement for complex stability. Instead, the substituent at the 7-position may be required to interact with specific residues of topo I within the cleavable complex.[35] It should be noted, however, that the aqueous solubility of the 7-aryl-MD- analogs is poor. For example, in Fig. 1B (at 0 min), achieving 100% entrapment of topo I on DNA was not feasible due to the limiting solubility of the 7-*p*-trifluoromethylphenyl-MD-CPT analog.

The use of very hydrophobic, 7-substituted CPT as antitumor drugs would require an appropriate balance among hydrophobicity for good cellular accumulation, substituents that do not destabilize cleavable complexes, and water solubility for formulation. The analogs described in this report are the first of a class of analogs with bulky, hydrocarbon groups directly attached to the 7-position of camptothecin (e.g., 7-cyclohexyl-CPT), and provide a methodological template for additional modifications to these hydrocarbons that could be used to achieve the desired balance of properties for novel antitumor agents.

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References and notes

1. Wall, M. E.; Wani, M. C.; Cook, C. E.; Palmar, K. H.; MacPhail, A. T.; Sim, G. A. *J. Am. Chem. Soc.* **1966**, *88*, 3888.
2. Hsiang, Y.-H.; Hertzberg, R.; Hecht, S.; Liu, L. F. *J. Biol. Chem.* **1985**, *260*, 14873.
3. Hsiang, Y.-H.; Lihou, M. G.; Liu, L. F. *Cancer Res.* **1989**, *49*, 5077.
4. Champoux, J. Mechanistic Aspects of Type-I Topoisomerases. In *DNA Topology and Its Biological Effects*; Wang, J. C., Cozarelli, N. R., Eds.; Cold Spring Harbor Laboratory; Cold Spring Harbor, 1990; p 217.
5. Wang, J. C. *J. Biol. Chem.* **1991**, *266*, 6659.
6. Chen, A. Y.; Liu, L. F. *Annu. Rev. Pharmacol. Toxicol.* **1994**, *94*, 194.
7. Holm, C.; Covey, J. M.; Kerrigan, D.; Pommier, Y. *Cancer Res.* **1989**, *49*, 6365.
8. Pommier, Y.; Leteurtre, F.; Fesen, M.; Fujimori, A.; Betrand, R.; Solary, E.; Kohlhagen, G.; Kohn, K. W. *Cancer Invest.* **1994**, *12*, 530.
9. Peel, M. R.; Milstead, M. W.; Sternbach, D. D.; Besterman, J. M.; Leitner, P.; Morton, B.; Wall, M. E.; Wani, M. C. *Bioorg. Med. Chem. Lett.* **1995**, *5*, 2129.
10. Luzzio, M. J.; Besterman, J. M.; Emerson, D. L.; Evans, M. G.; Lackey, K.; Leitner, P. L.; McIntyre, G.; Morton, B.; Myers, P. L.; Peel, M.; Sisco, J. M.; Sternbach, D. D.; Tong, W. Q.; Truesdale, A.; Uehling, D. E.; Vuong, A.; Yates, J. *J. Med. Chem.* **1995**, *38*, 395.
11. Jaxel, C.; Kohn, K. W.; Wani, M. C.; Wall, M. E.; Pommier, Y. *Cancer Res.* **1989**, *49*, 1465.
12. Yaegashi, T.; Sawada, S.; Nagata, H.; Furuta, T.; Yokokura, T.; Miyasaka, T. *Chem. Pharm. Bull.* **1994**, *42*, 2518.
13. Sawada, S.; Nokata, K.; Furuta, T.; Yokokura, T.; Miyasaka, T. *Chem. Pharm. Bull.* **1991**, *39*, 2574.
14. Wall, M. E.; Wani, M. C. Camptothecin and Analogs: Synthesis, Biological In vitro and In vivo activities and Clinical Possibilities. In *Human Medicinal Agents from Plants*; Kinghorn, A. D., Balandrin, M. F., Eds.; ACS Symposium Series; 1993; Vol. 534, p 149.
15. Wall, M. E.; Wani, M. C.; Nicholas, A. W.; Manikumar, G.; Tele, C.; Moore, L.; Truesdale, A.; Leitner, P.; Besterman, J. M. *J. Med. Chem.* **1993**, *36*, 2689.
16. Wall, M. E.; Wani, M. C.; Natschke, S. M.; Nicholas, A. W. *J. Med. Chem.* **1986**, *29*, 1553.
17. Van Hattum, A. H.; Pinedo, H. M.; Schluper, H. M.; Hausheer, F. H.; Boven, E. *Int. J. Cancer* **2000**, *88*, 260.
18. Bom, D.; Curran, D. P.; Zhang, J.; Zimmer, S. G.; Bevins, R.; Kruszewski, S.; Howe, J. N.; Bingcang, A.; Latus, L. J.; Burke, T. G. *J. Controlled Release* **2001**, *74*, 325.
19. De Cesare, M.; Pratesi, G.; Perego, P.; Carenini, N.; Tinelli, S.; Merlini, L.; Penco, S.; Pisano, C.; Bucci, F.; Vesci, L.; Pace, S.; Capocasa, F.; Carminati, P.; Zunino, F. *Cancer Res.* **2001**, *61*, 7189.
20. Zunino, F.; Pratesi, G. *Expert Opin. Invest. Drugs* **2004**, *13*, 269.
21. Du, W. *Tetrahedron* **2003**, *59*, 8649.
22. Thomas, C. J.; Rahier, N. J.; Hecht, S. M. *Bioorg. Med. Chem.* **2004**, *12*, 1585.
23. Wani, M. C.; Wall, M. E. *J. Org. Chem.* **1969**, *34*, 1364.
24. Wani, M. C.; Ronman, P. E.; Lindley, J. T.; Wall, M. E. *J. Med. Chem.* **1980**, *23*, 554.
25. Wani, M. C.; Nicholas, A. W.; Manijumar, G.; Wall, M. E. *J. Med. Chem.* **1987**, *30*, 1774.
26. Wani, M. C.; Nicholas, A. W.; Wall, M. E. *J. Med. Chem.* **1987**, *30*, 2317.
27. Bom, D.; Curran, D. P.; Kruszewski, S.; Zimmer, S. G.; Thompson Strode, J.; Kohlhagen, G.; Du, W.; Chavan, A. J.; Fraley, K. A.; Bingcang, A. L.; Latus, L. J.; Pommier, Y.; Burke, T. G. *J. Med. Chem.* **2000**, *43*, 3970.
28. Dallavalle, S.; Ferrari, A.; Biasotti, B.; Merlini, L.; Penco, S.; Gallo, G.; Marzi, M.; Tinti, M. O.; Martinelli, R.; Pisano, C.; Carminati, P.; Carenini, N.; Beretta, G.;

Perego, Y.; De Cesare, M.; Pratesi, G.; Zunino, F. *J. Med. Chem.* **2001**, *44*, 3264.
29. Vladu, B.; Woynarowski, J. M.; Manikumar, G.; Wani, M.; Wall, M. E.; Von Hoff, D. D.; Wadkins, R. M. *Mol. Pharmacol.* **2000**, *57*, 243.
30. Wadkins, R. M.; Bearss, D.; Manikumar, G.; Wani, M. C.; Wall, M. E.; Von Hoff, D. D. *Curr. Med. Chem. Anticancer Agents* **2004**, *4*, 327.
31. Hertzberg, R. P.; Caranfa, M. J.; Hecht, S. M. *Biochemistry* **1989**, *28*, 4629.
32. Tanizawa, A.; Fujimori, A.; Fujimori, Y.; Pommier, Y. *J. Natl. Cancer Inst.* **1994**, *86*, 836.
33. Tanizawa, A.; Kohn, K. W.; Kohlhagen, G.; Leteurtre, F.; Pommier, Y. *Biochemistry* **1995**, *34*, 7200.
34. Wadkins, R. M.; Bearss, D.; Manikumar, G.; Wani, M. C.; Wall, M. E.; Von Hoff, D. D. *Cancer Res.*, in press.
35. Staker, B. L.; Hjerrild, K.; Feese, M. D.; Behnke, C. A.; Burgin, A. B., Jr.; Stewart, L. *Proc. Natl. Acad. Sci. U.S.A.* **2002**, *99*, 15387.