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Dumbbell-shaped circular oligonucleotides as inhibitors of human topoisomerase I

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Abstract—A dumbbell-shaped circular oligonucleotide containing topoisomerase I-binding sites and two mismatched base pairs in its sequence has been designed and synthesized. Our further studies demonstrate that this particularly designed oligonucleotide displays an IC_{50} value of 9 nM in its inhibition on the activity of human topoisomerase I, a magnitude smaller than that of camptothecin, an anticancer drug currently in clinical use.

Topoisomerase I is an enzyme that releases the topological stress of DNA generated by replication and transcription and several other cellular processes, and is critical for cell growth and proliferation.[1] In the course of its action, this enzyme introduces a nick into one strand of DNA, allows free rotation about sigma bonds in the strand opposite the nick, and then reseals the original break.[2] It has been demonstrated in the past years that human topoisomerase I is a molecular target of various anticancer agents such as camptothecin, a plant secondary metabolite that has been clinically used for the treatment of colon and ovarian carcinomas.[3] With the aim of searching for a new type of agents beyond the chemical class of organic compounds for interfering the action of human topoisomerase I, we have recently examined the possibility of using oligonucleotides as irreversible inhibitors of this enzyme. Here, we report our construction of a mismatched dumbbell-shaped circular oligonucleotide and examination of its inhibitory effect on the activity of human topoisomerase I in its relaxation of negatively supercoiled DNA.

The endowed substrates of topoisomerase I in organisms are positive and negative supercoiled structures of DNA. Besides these supercoiled entities, it has been discovered in the past decades that some nonsupercoiled forms of duplex oligonucleotides can serve as substrates of eukaryotic topoisomerase I in very limited cases.[4] A double-stranded structure containing such a topoisomerase I-recognition sequence (Duplex 1) is illustrated in Figure 1. Further investigation revealed that when gap, nick, bulge, mismatch,[5] and certain other structural variations[6] were introduced into the nonsupercoiled substrates, the resultant duplex structures could form covalent linkages with topoisomerase I in irreversible fashions. The previous discoveries[5,6] have inspired us to speculate that this nonsupercoiled oligonucleotide substrate, if modified properly in its structure, could serve as an inhibitor of topoisomerase I in the relaxation reaction of supercoiled DNA.

A dumbbell-shaped circular oligonucleotide[7] was accordingly designed during our investigations (Oligonucleotide 1 in Fig. 1). Two 'extremely stable hairpins'[8] were introduced into the circular oligonucleotide, which in theory could lead to an increase of thermal stability of this duplex structure. In addition, the circular backbone of Oligonucleotide 1 should resist exonuclease hydrolysis, a property desirable for the development of pharmaceutical agents for in vivo use. Most importantly, Oligonucleotide 1 was designed to contain a topoisomerase I-recognition site[4,5] and two mismatched base pairs near the cutting site of this enzyme. Topoisomerase I could in theory bind to Oligonucleotide 1 and further cause a scission of one of the two duplex strands[5] when these two types of biomolecules are allowed to interact with each. After a nick is generated in Oligonucleotide 1 by topoisomerase I, religation reaction between the cut fragments might not be able to take place properly because the presence of two

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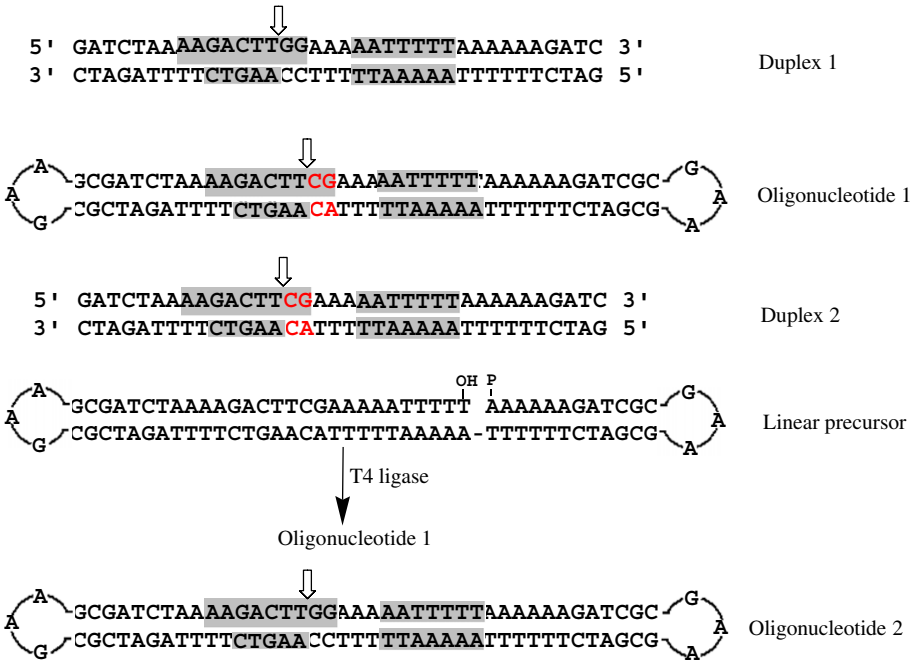

Figure 1. Sequences of oligonucleotides used in the current studies. The shadowed tracts in Duplex 1 denote the topoisomerase I-binding sequences. The cutting site by topoisomerase I is indicated by ⇩ while the bases marked in red are mismatched pairs.

mismatched base pairs near the cut site could cause improper conformational alteration, thus leading to chemically irreversible damages to the enzyme.[5]

Synthesis of Oligonucleotide 1 was subsequently carried out in our laboratories through ligation reaction of a linear 86-mer (lanes 2, 3, and 4 in Fig. 2) catalyzed by

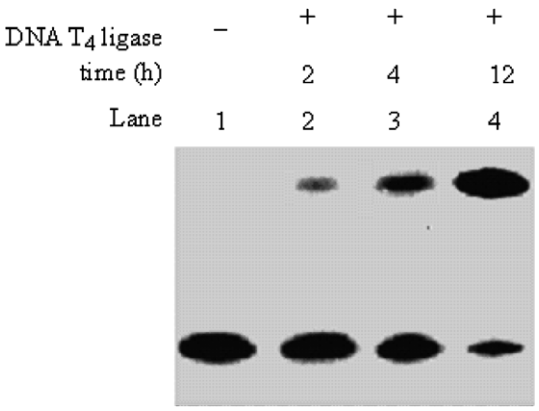

Figure 2. Polyacrylamide gel electrophoretic analysis of formation of Oligonucleotide 1. 86-mer linear precursor (Fig. 1) with free 5′-hydroxyl terminus was labeled with [γ-^{32}P]ATP (Amersham) at its 5′-end catalyzed by T_4 polynucleotide kinase (New England Biolabs). A mixture containing 50 mM Tris (pH 7.5), 10 mM $MgCl_2$, 1 mM ATP, 10 mM dithiothreitol, 25 μg/ml BSA, and 10 μM ^{32}P-labeled 86-mer linear precursor (Fig. 1) was then kept at 95 °C for 3 min and further allowed to cool down to room temperature over 30 min. 10 U of T_4 DNA ligase was next added to the above mixture, which was further incubated at 15 °C for different time periods. The final products were then analyzed using polyacrylamide gel electrophoresis (15%) followed by autoradiography for visualization using a PhosphorImager (Typhoon 8600, Amersham). Lane 1: linear precursor alone, lanes 2–4: linear precursor + T_4 DNA ligase for 2, 4, and 12 h, respectively.

DNA T_4 ligase. Circularity of the backbone of Oligonucleotide 1 was further confirmed via hydrolysis test using T_7 exonuclease (Fig. S1). Expression and activity of topoisomerase I have, on the other hand, been found to be higher in colorectal, prostate, and ovarian cancers than in their adjacent normal tissues[9] where this enzyme is not associated with its supercoiled DNA substrates at all times. Under these circumstances, topoisomerase I alone could in theory be taken as the target of therapeutical agents. As a model study to target topoisomerase I in the tissues where this enzyme is overexpressed, Oligonucleotide 1 was allowed to react with topoisomerase I for 3 min without the disturbance of its regular substrate of pBR322 in our studies. This preincubation would give a chance to let topoisomerase I bind to and further form irreversible linkage with Oligonucleotide 1. After 3 min of preincubation, pBR322 as the regular substrate was added to the pre-mixed solution of topoisomerase I and Oligonucleotide 1. As shown in Figure 3, the relaxation efficiency of human topoisomerase I decreased with the increase of concentration of Oligonucleotide 1 (lanes 1–6). The measured IC_{50} value of Oligonucleotide 1 under this circumstance was 9 nM (Fig. S2A) and the inhibitory effect of this dumbbell-shaped sequence was still observable when its concentration was set as low as 0.5 nM (lane 5 in Fig. 3). The above observations are the indications that Oligonucleotide 1 could indeed reduce the activity of topoisomerase I and act as an inhibitor of this enzyme in the relaxation reaction of negatively supercoiled DNA.

Duplex 2 is a structural entity of oligonucleotides containing the same duplex sequence as Oligonucleotide 1 does (Fig. 1). Different from Oligonucleotide 1, on the other hand, Duplex 2 is a bimolecular complex rather than a unimolecular unit. In addition, there is absence

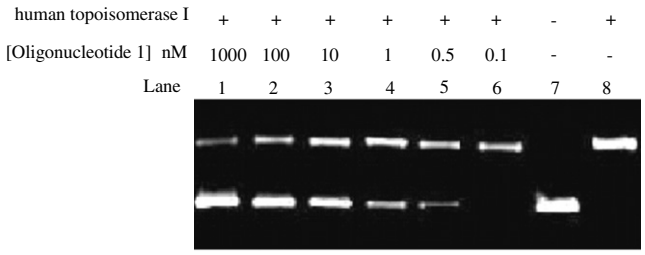

Figure 3. Agarose gel electrophoretic analysis of inhibitory effect of Oligonucleotide 1 on human topoisomerase I. Oligonucleotide 1 and 1 U human topoisomerase I (TopoGEN, Cat No.: 2005H-2) were incubated in a buffer of 10 mM Tris–HCl (pH 7.9), 150 mM NaCl, 0.1% bovine serum albumin (BSA), 0.1 mM spermidine, and 5% glycerol in a total volume of 20 μL at 25 °C for 3 min. When this preincubation was over, pBR322 was added and the new mixtures were adjusted to contain 10 mM Tris–HCl (pH 7.9), 150 mM NaCl, 0.1% bovine serum albumin (BSA), 0.1 mM spermidine, 5% glycerol, 400 ng pBR322, 1 U of human topoisomerase I, and different concentrations of Oligonucleotide 1, which was further allowed to react at 37 °C for 20 min. The samples were next analyzed using agarose gel (1.0%, w/v) electrophoresis (100 V for 60 min) followed by ethidium bromide staining. The DNA bands were visualized using Gel Documentation System (G:Box HR, Syngnene, Cambridge, UK) equipped with Gene Tools Software. Concentrations of Oligonucleotide 1 in the samples loaded in lanes 1–6 and lane 8 were 1000 nM, 100 nM, 10 nM, 1 nM, 0.5 nM, 0.1 nM, and 0 nM, respectively, while the sample in the lane 7 contained pBR322 alone.

of two 'extremely stable hairpins' in the structure of Duplex 2, which should in theory possess a lower melting point than Oligonucleotide 1 does. The inhibitory effects of Duplex 2 on human topoisomerase I were examined as well during our investigations. As shown in Figures 4 and S2B, this biomolecular complex exhibited inhibitory effect on topoisomerase I as well and gave an IC_{50} value of 93 nM when it was preincubated with topoisomerase I for 3 min. The relatively lower inhibitory efficiency of Duplex 2 (IC_{50} = 93 nM) as compared to the one obtained from Oligonucleotide 1 (IC_{50} = 9 nM) was presumably resulted in by the fact that Oligonucleotide 1 as a unimolecular entity could form double-stranded structures more effectively than the bimolecular complex of Duplex 2 does. In addition, the obtained melting points of Duplex 2 and Oligonucleotide 1 under our experimental conditions were 55 °C and 75 °C, respectively, which confirms that a regular duplex structure of oligonucleotides possesses indeed a lower thermal stability than those that contain 'extremely stable hairpins' in its structure.[8]

As control experiments, inhibitory effect of Duplex 1 on topoisomerase I was also examined during our investigation. Duplex 1 is a 36-mer linear duplex structure containing topoisomerase I-binding and cutting sites (Fig. 1). Different from Oligonucleotide 1 and Duplex 2, on the other hand, this duplex entity contains no mismatched base pair in its double-stranded structure. As shown in Figures 5 and S2C, the inhibitory effect of Duplex 1 on topoisomerase I could only be observed at a concentration as high as 100 nM and displayed an IC_{50} value greater than ~1.4 μM. Moreover, a perfectly matched dumbbell-shaped oligonucleotide containing topoisomerase I-binding site (Oligonucleotide 2 in Fig. 1) was synthesized and its inhibitory effect on the activity of topoisomerase I was examined in our laboratory. Similar to Duplex 1, Oligonucleotide 2 displayed a relatively low inhibitory efficiency and showed an IC_{50} value as high as ~1.2 μM (Fig. S2E). Since neither Duplex 1 nor Oligonucleotide 2 contains mismatched base pairs in its structure, the processes of strand scission and rejoining driven by topoisomerase I on these sequences could take place reversibly and uninterruptedly,[5,6] which could consequently be the cause of inefficient inhibition by the perfectly matched duplex structures.

Unlike the preincubation experiments discussed above, new tests were further carried out in our studies in which Oligonucleotide 1, topoisomerase I, and pBR322 were mixed together simultaneously (Fig. 6). Under this circumstance, Oligonucleotide 1 displayed an IC_{50} value of 83 nM (Fig. S2D). In addition, the inhibitory effect of Duplex 2 in the absence of preincubation period with topoisomerase I was examined, from which an IC_{50} value of ~400 nM (Fig. S2F) was obtained. These relatively higher IC_{50} values of Oligonucleotide 1 and Duplex 2 in the absence of preincubation period as compared to the ones obtained when preincubation was given could be caused by the competitions between

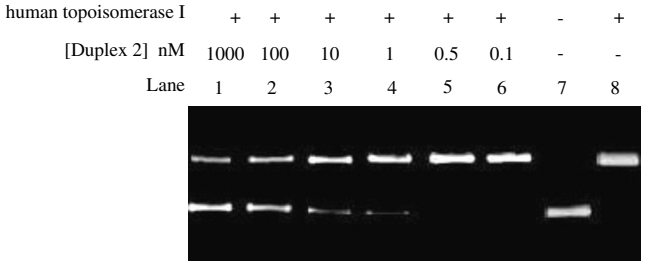

Figure 4. Agarose gel electrophoretic analysis of inhibitory effect of Duplex 2 on human topoisomerase I. The same procedures as those for the samples in Figure 3 were used except for replacing Oligonucleotide 1 with Duplex 2. Concentrations of Duplex 2 in the samples loaded in lanes 1–6 and lane 8 were 1000 nM, 100 nM, 10 nM, 1 nM, 0.5 nM, 0.1 nM, and 0 nM, respectively, while the sample in the lane 7 contained pBR322 alone.

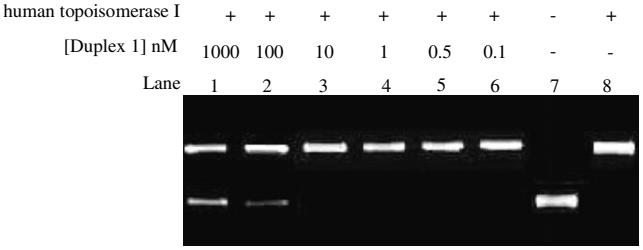

Figure 5. Agarose gel electrophoretic analysis of inhibitory effect of Duplex 1 on human topoisomerase I. The same procedures as those for the samples in Figure 3 were used except for replacing Oligonucleotide 1 with Duplex 1. Concentrations of Duplex 1 in the samples loaded in lanes 1–6 and lane 8 were 1000 nM, 100 nM, 10 nM, 1 nM, 0.5 nM, 0.1 nM, and 0 nM, respectively, while the sample in the lane 7 contained pBR322 alone.

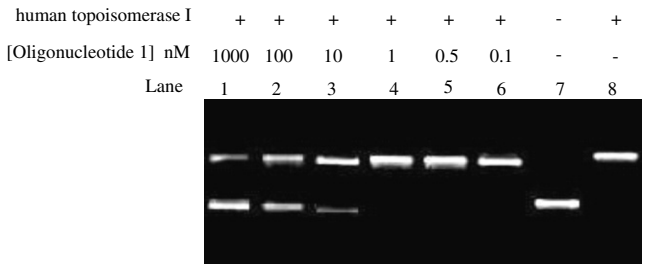

Figure 6. Agarose gel electrophoretic analysis of inhibitory effect of Oligonucleotide 1 on human topoisomerase I without preincubation. A mixture containing 10 mM Tris–HCl (pH 7.9), 150 mM NaCl, 0.1% bovine serum albumin (BSA), 0.1 mM spermidine, 5% glycerol, 400 ng pBR322, 1 U of human topoisomerase I, and different concentrations of Oligonucleotide 1 was prepared and further incubated at 37 °C for 20 min. Concentrations of Oligonucleotide 1 in the samples loaded in lanes 1–6 and lane 8 were 1000 nM, 100 nM, 10 nM, 1 nM, 0.5 nM, 0.1 nM, and 0 nM, respectively, while the sample in the lane 7 contained pBR322 alone.

the inhibitory oligonucleotides and pBR322 in their binding to topoisomerase I. Given the fact that the IC_{50} value of camptothecin, a clinically used drug, in its inhibition of topoisomerase I is about 700 nM,[10] on the other hand, Oligonucleotide 1 could be taken as an effective human topoisomerase I inhibitor since it has an IC_{50} value of 9 nM with preincubation and 83 nM in the absence of preincubation.

DNA-based biopharmaceuticals have been recognized to be promising drug candidates for a wide range of diseases such as cancer, AIDS, and some other neurological disorders.[11] The examples of these types of therapeutical agents include aptamers, DNAzymes, oligonucleotides for antisense, and small interfering RNA.[12] Since oligonucleotides and DNA are not capable of passing freely across membrane of eukaryotic cells due to the polyanionic characteristics in their backbones, transfecting agents and other physical measures are constantly needed to facilitate the cellular uptake of DNA-based therapeutical agent.[13] In consideration of the recent development in the introduction of oligonucleotides and DNA into eukaryotic cells and the reduced potentials for toxicity of DNA-based biopharmaceuticals,[13] it is our expectation that the results shown in the current studies could serve as useful information to benefit the future design of some new DNA-based therapeutical agents that target topoisomerase I.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2007.06.035.

References and notes

1. (a) Wang, J. C. *Nat. Rev. Mol. Cell Biol.* **2002**, *3*, 430; (b) Leppard, J. B.; Champoux, J. J. *Chromosoma* **2005**, *114*, 75; (c) Champoux, J. J.; Dulbecco, C. R. *Proc. Natl. Acad. Sci. U.S.A.* **1972**, *69*, 143.
2. (a) Corbett, K. D.; Berger, J. M. *Annu. Rev. Biophys. Biomol. Struct.* **2004**, *33*, 95; (b) Champoux, J. J. *Annu. Rev. Biochem.* **2001**, *70*, 369.
3. (a) Pommier, Y. *Nat. Rev. Cancer* **2006**, *6*, 789; (b) Topcu, Z. *J. Clin. Pharm. Ther.* **2001**, *26*, 405; (c) Rothenberg, M. L. *Ann. Oncol.* **1997**, *8*, 837; (d) Li, T. K.; Liu, L. F. *Annu. Rev. Pharm. Toxicol.* **2001**, *41*, 53.
4. (a) Christiansen, K.; Svejstrup, A. B.; Andersen, A. H.; Westergaard, O. *J. Biol. Chem.* **1993**, *268*, 9690; (b) Christiansen, K.; Westergaard, O. *J. Biol. Chem.* **1994**, *269*, 721; (c) Sikder, D.; Nagaraja, V. *J. Mol. Biol.* **2001**, *312*, 347; (d) Bugreev, D. V.; Buneva, V. N.; Sinitsyna, O. I.; Nevinsky, G. A. *Russ. J. Bioorg. Chem. (Translation of Bioorganicheskaya Khimiya)* **2003**, *29*, 143; (e) Svejstrup, J. Q.; Christiansen, K.; Andersen, A. H.; Lund, K.; Westergaard, O. *J. Biol. Chem.* **1990**, *265*, 12529.
5. (a) Pourquier, P.; Ueng, L. M.; Kohlhagen, G.; Mazumder, A.; Gupta, M.; Kohn, K. W.; Pommier, Y. *J. Biol. Chem.* **1997**, *272*, 7792; (b) Pourquier, P.; Pilon, A. A.; Kohlhagen, G.; Mazumder, A.; Sharma, A.; Pommier, Y. *J. Biol. Chem.* **1997**, *272*, 26441; (c) Yeh, Y. C.; Chang, D. Y.; Masin, J.; Lu, A. L. *J. Biol. Chem.* **1991**, *266*, 6480; (d) Yeh, Y. C.; Liu, H. F.; Ellis, C. A.; Lu, A. L. *J. Biol. Chem.* **1994**, *269*, 15498.
6. (a) Antony, S.; Arimondo, P. B.; Sun, J. S.; Pommer, Y. *Nucleic Acids Res.* **2004**, *32*, 5163; (b) Straub, T.; Knudsen, B. R.; Boege, F. *Biochemistry* **2000**, *39*, 7552; (c) Wang, X.; Henningfeld, K. A.; Hecht, S. M. *Biochemistry* **1998**, *37*, 2691; (d) Colley, W. C.; van der Merwe, M.; Vance, J. R.; Burgin, A. B., Jr.; Bjornsti, M. A. *J. Biol. Chem.* **2004**, *279*, 54069; (e) Svejstrup, J. Q.; Christiansen, K.; Gromova, I. I.; Andersen, A. H.; Westergaard, O. *J. Mol. Biol.* **1991**, *222*, 669; (f) Cheng, C.; Shuman, S. *J. Biol. Chem.* **1998**, *273*, 11589; (g) Bugreev, D. V.; Vasyutina, E. L.; Kolocheva, T. I.; Buneva, V. N.; Andoh, T.; Nevinsky, G. A. *Biochimie* **1998**, *80*, 303.
7. (a) Yamakawa, H.; Abe, T.; Saito, T.; Takai, K.; Yamamoto, N.; Takaku, H. *Bioorg. Med. Chem.* **1998**, *6*, 1025; (b) Hosoya, T.; Takeuchi, H.; Kanesaka, Y.; Yamakawa, H.; Naoko, M. K.; Takai, K.; Yamamoto, N.; Takaku, H. *FEBS Lett.* **1999**, *461*, 136; (c) Owczarzy, R.; Vallone, P. M.; Goldstein, R. F.; Benight, A. S. *Biopolymers* **1999**, *52*, 29; (d) Abe, T.; Takai, K.; Nakada, S.; Yokota, T.; Takaku, H. *FEBS Lett.* **1998**, *425*, 91; (e) Clusel, C.; Ugarte, E.; Enjolras, N.; Vasseur, M.; Blumenfeld, M. *Nucleic Acids Res.* **1993**, *21*, 3405; (f) Gao, H.; Chidambaram, N.; Chen, B. C.; Pelham, D. E.; Patel, R.; Yang, M.; Zhou, L.; Cook, A.; Cohen, J. S. *Bioconjug. Chem.* **1994**, *5*, 445; (g) Kuhn, H.; Frank-Kamenetskii, M. D.; Demidov, V. V. *Antisense Nucleic Acid Drug Dev.* **2001**, *11*, 149.
8. Yoshizawa, S.; Ueda, T.; Ishido, Y.; Miura, K.; Watanabe, K.; Hirao, I. *Nucleic Acids Res.* **1994**, *22*, 2217.
9. (a) Giovanella, B. C.; Stehlin, J. S.; Wall, M. E.; Wani, M. C.; Nicholas, A. W.; Liu, L. F.; Silber, R.; Potmesil, M. *Science* **1989**, *246*, 1046; (b) Husain, I.; Mohler, J. L.; Seigler, H. F.; Besterman, J. M. *Cancer Res.* **1994**, *54*, 539; (c) Van der Zee, A. G. J.; Hollema, H.; De Jong, S.; Boonstra, H.; Gouw, A.; Willemse, P. H. B.; Zijlstra, J. G.; De Vries, E. G. E. *Cancer Res.* **1991**, *51*, 5915; (d) Hirabayashi, N.; Kim, R.; Nishiyama, M. *Proc. Am. Soc. Cancer Res.* **1992**, *33*, 436.
10. (a) Mejia, E. G. D.; Chandra, S.; Ramirez-Mares, M. V.; Wang, W. Y. *Food Chem. Toxicol.* **2006**, *44*, 1191; (b) Jo, J. Y.; Mejia, E. G. D.; Lila, M. A. *J. Agric. Food Chem.* **2006**, *54*, 2083; (c) Jo, J. Y.; Mejia, E. G. D.; Lila, M. A. *J. Agric. Food Chem.* **2005**, *53*, 2489; (d) Peel, M. R.;

Milstead, M. W.; Sternbach, D. D.; Besterman, J. M.; Leitner, P.; Morton, B. *Bioorg. Med. Chem. Lett.* **1995**, *5*, 2129; (e) Li, C. J.; Averboukh, L.; Pardee, A. B. *J. Biol. Chem.* **1993**, *268*, 22463.
11. Fattal, E.; Bochot, A. *Adv. Drug Deliver. Rev.* **2006**, *58*, 1203.
12. (a) Crooke, S. T. *Antisense Nucleic Acid Drug Dev.* **1998**, *8*, 115; (b) Stull, R. A.; Szoka, F. C., Jr. *Pharm. Res.* **1995**, *12*, 465.
13. Patil, S. D.; Rhodes, D. G.; Burgess, D. J. *AAPS J.* **2005**, *7*, E61.
14. Laine, J. P.; Opresko, P. L.; Indig, F. E.; Harrigan, J. A.; Kobbe, C. V.; Bohr, V. A. *Cancer Res.* **2003**, *63*, 7136.