Published in final edited form as:

Invest New Drugs. 2010 February ; 28(1): 20–25. doi:10.1007/s10637-008-9209-1.

Anti-proliferative effects of Simocyclinone D8 (SD8), a novel catalytic inhibitor of Topoisomerase II

Ahad A. Sadiq, M.D.^{1,*}, Manish R. Patel, D.O.^{1,*}, Blake A. Jacobson, Ph.D.¹, Marco Escobedo, B.S.¹, Keith Ellis, Ph.D.², Lisa M. Oppegard, Ph.D.³, Hiroshi Hiasa, Ph.D.³, and Robert A. Kratzke, M.D.¹

¹ Division of Hematology-Oncology-Transplant, Department of Medicine, University of Minnesota Medical School, Minneapolis, MN 55455

² Department of Medicinal Chemistry and the Institute for Therapeutic Discovery and Development, College of Pharmacy, University of Minnesota, Minneapolis 55414

³ Department of Pharmacology, University of Minnesota Medical School, Minneapolis, Minnesota 55455

INTRODUCTION

Type-II topoisomerase targeted agents are proven therapies and the drugs of first choice for the treatment of many different cancers including testicular and small cell lung cancer. DNA topoisomerases are enzymes that change the topology of DNA molecules while maintaining their chemical integrity and structure. Two of the most prescribed anti-cancer therapies are the human topoisomerase II (hTopoII) poisons; etoposide (VP-16) [3] and doxorubicin [9]. While these drugs have proven efficacy, their use is limited by toxicity as well as the risk of developing secondary leukemia [2].

Non-small cell lung cancer (NSCLC) and malignant mesothelioma (MM) are devastating diseases that are incurable when metastatic. Despite recent advances for both diseases, the median survival remains only one year [16, 19]. Better chemotherapeutic agents are desperately needed to improve the care of patients with these diseases. Topoisomerase II (TopoII) poisons have been used clinically for both NSCLC and MM [3, 18]. The currently available TopoII targeted drugs, such as etoposide (VP-16) and anthracyclines are poisons that stabilize the covalent topoisomerase-DNA complex after enzymatic cleavage of the double-stranded DNA. Thus, both their antitumor effect and toxicity are a result of induction of DNA damage. It is this DNA damage that is associated with chromosomal aberrations that lead to chemotherapy-related leukemia [2].

There has been recent interest in the proliferative function of TopoII in cancer. Specifically, there are two isoforms of TopoII in humans, α and β . While TopoII β appears to be constitutively expressed, TopoII α is more prevalent during times of cell growth and proliferation leading to the conclusion that the function of TopoII α may be critical to the

* A.A.S and M.R.P. contributed equally to this work

growth of cancer cells. In NSCLC, it has been shown that TopoII expression is increased compared to normal lung tissue, though it is unclear if this predicts response to TopoII inhibitors [7]. In the case of breast cancer, TopoII gene amplification has been associated with an increased response to anthracyclines [15]. This has led to the search for catalytic inhibitors of TopoII which inhibit enzymatic activity by binding to the enzyme in the DNA binding site and preventing the enzyme from binding to DNA. These enzyme-binding TopoII inhibitors should result in inhibition of DNA replication and chromosome segregation. These compounds should also inhibit enzymatic cleavage of DNA by TopoII which is a potentially safer mechanism to target TopoII than that of etoposide and anthracyclines.

Simocyclinone D8 (SD8) is a novel compound derived from *Streptomyces antibioticus* Tu6040 that is a potent catalytic inhibitor of bacterial DNA gyrase, a prokaryotic homologue to human TopoII. In addition, SD8 has been demonstrated to have antiproliferative effects on MCF-7 breast cancer cells in culture [17]. In the current work, we show that SD8 inhibits the catalytic activity of TopoII. Furthermore, we have tested the antiproliferative effects of SD8 on NSCLC and MM cell lines as well as their ability to induce apoptosis.

MATERIALS AND METHODS

Simocyclinone D8 Fermentation, Isolation, Purification, and Analytical Characterization

Full details of the fermentation, isolation, purification, and characterization will be published elsewhere¹. Briefly, SD8 was produced by fermentation on a 20L scale using glycerol and L-lysine as carbon and nitrogen sources, respectively, according to the procedure of Fiedler and coworkers [17]. Isolation with methanol was followed by purification on diol-bonded silica gel on a CombiFlashXL automated purification system. A final purification with reverse phase HPLC (C18, 1:1 acetonitrile/0.5% formic acid) afforded SD8 that was identical to an authentic sample provided by Dr. Fiedler by NMR (¹H and ¹³C), mass spec, and HPLC.

CELL LINES—NSCLC cell lines (H2009 and H2030) and MM cell lines (H2596 and H2461) were obtained from the American Tissue Culture Collection (ATCC) and were grown in RPMI + L-glutamine (Gibco, Invitrogen) media containing 10% calf serum and 1% antibiotic (penG, streptomycin and amphotericin B). Non-transformed mesothelial cell line, LP9, was obtained from the National Institute of Aging and were grown in 1:1 mixture of 199E media:MCDB and supplemented with 15% calf serum, 15 ng/mL epidermal growth factor, and hydrocortisone. Detailed karyotype and origins of this cell line have been described previously [14].

CELL PROLIFERATION ASSAY—Cells were seeded in triplicate onto 96-well plates with 2000 cells/well along with appropriate controls containing only media and drug. Cells were treated with SD8 24 hours after plating. All samples and controls were treated with equal volume of the drug's vehicle, dimethyl sulfoxide (DMSO). Cell growth was measured

¹L.M.O., Kathryn R. Streck, Bree L. Hamann, K.C.E., Hans-Peter Fiedler, Arkady B. Khodursky, and H.H., manuscript in preparation)

using the Cell Counting Kit-8 (CCK-8) following the manufacturer's (Dojindo Molecular Technologies, Inc., Gaithersburg, MD) protocol. 10 μ L of CCK-8 reagent was added to each well and allowed to incubate for 3 hours. The amount of CCK-8 reagent reduced to formazan by cellular dehydrogenase indicating cell viability was assayed by reading the absorbance at 405 nm on a 96-well plate reader. The absorbance reading was subtracted from background control. An average of 3 readings was taken and statistical analysis was performed by a simple t-test. A p value of <0.05 was taken as significant.

APOPTOSIS ASSAY—Apoptosis was assessed by the Poly(ADP) Ribose Polymerase (PARP) cleavage assay. Cells were plated on 10 cm plates in a concentration range of $2-3 \times 10^6$ cells/plate and incubated overnight in 37°C in 5% CO₂. Cells were treated with SD8 at the IC₅₀ dose (as determined in proliferation assay) and 50 nM gemcitabine as a positive control. Cells were treated with 50 and 75 μ M SD8, for H2596 and H2009, respectively. H2030 and H2461 were both treated with 125 μ M SD8. Cells were lysed after 24 and 48 hours of treatment. Protein concentration was determined using the Bradford method and 50 μ g of protein were run on 10% SDS-PAGE gels and transferred to PVDF membrane and immunoblotted using anti-PARP antibody. H2596 cells treated with 25 nM gemcitabine were used as a positive control as this has been shown to induce PARP cleavage at this dose [14]. Induction of apoptosis was indicated by the presence of 2 bands at 116 kDa and 89 kDa corresponding to the intact and cleaved protein, respectively.

IMMUNOBLOTTING—Treated cells were washed with cold 1X phosphate-buffered saline (PBS), and lysates were prepared by scraping using 1X cell lysis buffer containing protease inhibitors and phosphatase inhibitors (Cell Signaling, Inc.). Lysates were immediately stored at -80°C until used. Protein concentration was quantified using the Bradford Method and 50 μ g of protein was run on 10% mini-SDS-PAGE gels, transferred to PVDF membrane and probed for PARP using a monoclonal antibody at a dilution of 1:1000 (Cell Signaling, Inc.) overnight at 4°C on a rocker. Mouse anti- β -Actin antibody (Sigma) was used as a loading control at a dilution of 1:20,000. Blots were washed 3 $\times$ 5 minutes with 1X tris-buffered saline with Tween-20 (TBS-T) and placed in anti mouse- and rabbit HRP-conjugated secondary antibody at a dilution of 1:3000 and 1:2500, respectively, for 1 hr at room temperature with gentle shaking. Blots were then washed 4 $\times$ 5 min and developed using a chemiluminescent substrate (Pierce).

DNA DECATENATION ASSAY—Reaction mixtures (20 μ L) containing 50 mM Tris-HCl (pH 8.0), 10 mM MgCl₂, 200 mM potassium glutamate, 10 mM dithiothreitol, 50 μ g/ml BSA, 1 mM ATP, 0.3 μ g of kinetoplast DNA (Topogen), 2 units of human TopoII (TopoII α from Topogen), and the indicated concentrations of drug (etoposide was from Sigma) were incubated at 37 °C for 10 min. Reactions were terminated by adding EDTA to 25 mM and the DNA products were analyzed by electrophoresis through vertical 1.2% agarose gels at 2 V/cm for 15 hours in TAE buffer. Gels were stained with ethidium bromide and photographed using an Eagle Eye II system (Stratagene).

DNA CLEAVAGE ASSAY—Reaction mixtures (20 μ L) containing 50 mM Tris-HCl (pH 8 at 23 °C), 10 mM MgCl₂, 10 mM DTT, 50 μ g/mL BSA, 1 mM ATP, 300 ng of relaxed

plasmid pBR322 DNA, 6 units of human TopoII, and the indicated concentrations of SD8 or etoposide were incubated at 37°C for 10 minutes. SDS was added to a concentration of 1%, and the reaction mixtures were further incubated at 37°C for 5 minutes. EDTA and proteinase K were then added to concentrations of 25 mM and 100 µg/mL respectively, and the incubation was continued for an additional 1 hour at 50°C. The DNA products were purified by extraction with phenol/chloroform/isoamyl alcohol (25:24:1, v/v) and then analyzed by electrophoresis through vertical 1.2% agarose gels at 2 V/cm for 15 hours in TAE buffer that contained 0.5 µg/mL ethidium bromide. After destaining in water, gels were photographed and quantified using an Eagle Eye II system.

RESULTS

The inhibitory effect of SD8 on the catalytic activity of TopoII was tested *in vitro* by assessing the decatenation of kinetoplast DNA (Fig. 1A). SD8 inhibited TopoII activity in a dose-dependent manner with an IC₅₀ of approximately 80 µM. SD8 was twice as potent as etoposide, a TopoII poison, in inhibiting the *in vitro* decatenation activity of TopoII. We also tested whether SD8 could act as a poison and stimulate the DNA cleavage activity of TopoII (Fig. 1C). In contrast to etoposide, which stimulated cleavage, SD8 had no effect on TopoII cleavage activity at the lower concentrations and DNA cleavage by TopoII was completely inhibited at the higher concentrations of SD8. These results demonstrate that SD8 does not act as a poison of human TopoII, but rather as a catalytic inhibitor.

The antiproliferative effects of SD8 on NSCLC cell lines, H2009 and H2030, and MM cell lines, H2596 and H2461, are shown in Fig. 2. There was no response in any of the cell lines to low micromolar doses of SD8 (data not shown). At higher doses, there was a dose-dependent effect on H2009 cells with an IC₅₀ of approximately 75 µM. H2030 cells were more resistant to SD8 with an IC₅₀ of approximately 130–140 µM. For MM cell lines, the IC₅₀ was approximately 125 µM and 100 µM for H2461 and H2596 cell lines, respectively. To demonstrate that SD8 had some degree of selectivity for cancer cells, we treated the non-transformed mesothelial cell line, LP9. The IC₅₀ for LP9 cells was approximately 150 µM, higher than the cancer cell lines tested.

Inhibition of TopoII has been shown to induce apoptosis in embryonic cells as well as cancer cells [1, 8]. Therefore, we wanted to see if SD8 induced apoptosis in our NSCLC and MM cells. Cells were treated with SD8 at the approximate IC₅₀ dose for each cell line and were assayed for apoptosis by the PARP cleavage assay (Fig. 3). Treatment with SD8 resulted in cleavage of PARP at 24 hrs and was increased at 48 hrs for all cell lines tested. For H2030 cells, apoptosis induction was seen only after 48 hrs of treatment. Interestingly, PARP cleavage was more pronounced in H2009 cells than H2461 cells treated with SD8 than those treated with gemcitabine at the indicated concentrations.

DISCUSSION

TopoII inhibition as a cancer therapy is a well established concept as evidenced by the long history with TopoII poisons. While these drugs are effective therapies for many types of cancer including NSCLC and MM, the DNA damage that occurs with TopoII poisons also

results in significant toxicity and the risk of secondary leukemia [2]. In this work, we have tested the anticancer activity of a novel TopoII inhibitor that functions as a catalytic inhibitor without inducing DNA strand breaks. While SD8 does demonstrate anticancer activity, the concentrations at which it inhibits proliferation may be difficult to achieve *in vivo*. The low potency is likely related to its limited efficacy in inhibiting TopoII enzymatic activity as the *in vitro* decatenation assay yielded a similar IC₅₀ (Fig. 1A).

Consistent with previously described TopoII inhibitors, SD8 inhibited growth of NSCLC and MM cells by induction of apoptosis [8, 11, 13]. The use of TopoII poisons such as etoposide, results in rapid and potent induction of apoptosis as well [13]. However, because the inhibition of a single TopoII enzyme can result in DNA breaks, TopoII poisons lack selectivity for cancer cells [6, 11]. Thus, the novel mechanism of action of SD8 will make it a more attractive mode of TopoII inhibition as it should be selective for cells that are actively proliferating and dependent upon TopoII enzyme activity. Indeed, our experiments show decreased activity of SD8 against nonmalignant cells.

While several other candidate TopoII catalytic inhibitors are currently in development, none have showed promise enough to proceed beyond phase I clinical trials [4, 5, 8, 10]. The catalytic inhibitor merbarone has been tested in phase II trials in both melanoma and gliomas and it has now been found to also induce DNA strand breaks therefore also acting as a TopoII poison [12]. Furthermore, this drug has been limited by renal toxicity. Fostriecin appeared safe in a phase I study, however, the pharmacokinetic data suggested that adequate *in vivo* levels were not attained and this agent has not been tested further [12]. Our data show that SD8 is a true catalytic inhibitor. Its mode of action is unique among catalytic inhibitors in that it directly interacts with Type-II topoisomerases to block its binding to DNA. Therefore SD8 does not induce DNA strand breaks even at very high doses. Because of this SD8 represents a novel compound that can serve as a lead agent for the development of new anticancer drugs with lower toxicity. Before SD8 can be tested in animal models and clinical trials, chemical modifications need to be made that both improve its potency as a TopoII catalytic inhibitor as well as its cytotoxicity. Current efforts are underway to make such modifications.

Acknowledgments

We thank Hans-Peter Fiedler for expertise and advice on the fermentation, isolation, and purification of SD8. KE thanks Gunda Georg of the University of Minnesota Department of Medicinal Chemistry for financial support. ME was supported by the Summer Research Training at the University of Minnesota Medical School (R25HL088728; C. Campbell, PI). MP was supported by 5T32HL07062 (Division of Hematology-Oncology-Transplant, University of Minnesota, G. Vercellotti PI).

References

1. Akimitsu N, Kamura K, Tone S, Sakaguchi A, Kikuchi A, Hamamoto H, Sekimizu K. Induction of apoptosis by depletion of DNA topoisomerase II α in mammalian cells. *Biochem Biophys Res Commun.* 2003; 307:301–7. [PubMed: 12859955]
2. Azarova AM, Lyu YL, Lin CP, Tsai YC, Lau JY, Wang JC, Liu LF. Roles of DNA topoisomerase II isozymes in chemotherapy and secondary malignancies. *Proc Natl Acad Sci U S A.* 2007; 104:11014–9. [PubMed: 17578914]

3. Burden DA, Osheroff N. Mechanism of action of eukaryotic topoisomerase II and drugs targeted to the enzyme. *Biochim Biophys Acta*. 1998; 1400:139–54. [PubMed: 9748545]
4. Davies SL, Bergh J, Harris AL, Hickson ID. Response to ICRF-159 in cell lines resistant to cleavable complex-forming topoisomerase II inhibitors. *Br J Cancer*. 1997; 75:816–21. [PubMed: 9062401]
5. de Jong RS, Mulder NH, Uges DR, Sleijfer DT, Hoppener FJ, Groen HJ, Willemse PH, van der Graaf WT, de Vries EG. Phase I and pharmacokinetic study of the topoisomerase II catalytic inhibitor fostriecin. *Br J Cancer*. 1999; 79:882–7. [PubMed: 10070885]
6. Fournel S, Genestier L, Rouault JP, Lizard G, Flacher M, Assossou O, Revillard JP. Apoptosis without decrease of cell DNA content. *FEBS Lett*. 1995; 367:188–92. [PubMed: 7796918]
7. Fridman R, Giaccone G, Kanemoto T, Martin GR, Gazdar AF, Mulshine JL. Reconstituted basement membrane (matrigel) and laminin can enhance the tumorigenicity and the drug resistance of small cell lung cancer cell lines. *Proceedings of the National Academy of Science USA*. 1990; 87:6698–6702.
8. Goodell JR, Ougolkov AV, Hiasa H, Kaur H, Remmel R, Billadeau DD, Ferguson DM. Acridine-based agents with topoisomerase II activity inhibit pancreatic cancer cell proliferation and induce apoptosis. *J Med Chem*. 2008; 51:179–82. [PubMed: 18163538]
9. Hande KR. Clinical applications of anticancer drugs targeted to topoisomerase II. *Biochim Biophys Acta*. 1998; 1400:173–84. [PubMed: 9748560]
10. Khelifa T, Beck WT. Merbarone, a catalytic inhibitor of DNA topoisomerase II, induces apoptosis in CEM cells through activation of ICE/CED-3-like protease. *Mol Pharmacol*. 1999; 55:548–56. [PubMed: 10051540]
11. Kizaki H, Onishi Y. Topoisomerase II inhibitor-induced apoptosis in thymocytes and lymphoma cells. *Adv Enzyme Regul*. 1997; 37:403–23. [PubMed: 9381984]
12. Larsen AK, Escargueil AE, Skladanowski A. Catalytic topoisomerase II inhibitors in cancer therapy. *Pharmacol Ther*. 2003; 99:167–81. [PubMed: 12888111]
13. Oizumi S, Isobe H, Ogura S, Ishida T, Yamazaki K, Nishimura M, Kawakami Y, Dosaka-Akita H. Topoisomerase inhibitor-induced apoptosis accompanied by down-regulation of Bcl-2 in human lung cancer cells. *Anticancer Res*. 2002; 22:4029–37. [PubMed: 12553028]
14. Patel MR, Jacobson BA, De A, Frizelle SP, Janne P, Thumma SC, Whitson BA, Farassati F, Kratzke RA. Ras pathway activation in malignant mesothelioma. *J Thorac Oncol*. 2007; 2:789–95. [PubMed: 17805054]
15. Pritchard KI, Messersmith H, Elavathil L, Trudeau M, O'Malley F, Dhesy-Thind B. HER-2 and topoisomerase II as predictors of response to chemotherapy. *J Clin Oncol*. 2008; 26:736–44. [PubMed: 18258981]
16. Schiller JH, Harrington D, Belani CP, Langer C, Sandler A, Krook J, Zhu J, Johnson DH. Comparison of four chemotherapy regimens for advanced non-small-cell lung cancer. *N Engl J Med*. 2002; 346:92–8. [PubMed: 11784875]
17. Schimana J, Fiedler HP, Groth I, Sussmuth R, Beil W, Walker M, Zeeck A. Simocyclinones, novel cytostatic angucyclinone antibiotics produced by *Streptomyces antibioticus* Tu 6040. I. Taxonomy, fermentation, isolation and biological activities. *J Antibiot (Tokyo)*. 2000; 53:779–87. [PubMed: 11079799]
18. Skubitz KM. Phase II trial of pegylated-liposomal doxorubicin (Doxil) in mesothelioma. *Cancer Invest*. 2002; 20:693–9. [PubMed: 12197225]
19. Vogelzang NJ, Porta C, Mutti L. New agents in the management of advanced mesothelioma. *Semin Oncol*. 2005; 32:336–50. [PubMed: 15988688]

Figure 1. Biochemical Assays

(A) *DNA decatenation assay*. An *in vitro* assay to assess the TopoII activity using kinetoplast DNA as a substrate. The DNA products were run on agarose gels. Disappearance of decatenated DNA, as the doses of SD8 increased, demonstrated the inhibition of the TopoII decatenation activity by SD8. The effect of etoposide, a TopoII poison, on the decatenation activity of TopoII is shown for comparison. (B) *DNA Cleavage Assay*. An *in vitro* assay used to assess the effect of SD8 on the DNA cleavage activity of human TopoII. In this assay, the DNA cleavage activity of TopoII was monitored by the generation of linear DNA. The relaxed plasmid DNA used as the substrate was supercoiled by ethidium bromide during electrophoresis.

Figure 2. Cell Proliferation Assay

(A) H2009 cells, (B) H2030 cells, (C) H2596 cells, and (D) H2461 cells, and (E) non-transformed LP9 cells were treated with SD8 at the indicated concentrations for 72 hours in a semi-automated assay for cell viability. Data is expressed as a percentage of untreated cells. These were done in triplicate. Error bars indicate the standard deviation. * indicates a significant p value as calculated by paired t test.

Figure 3. Apoptosis Assay

NSCLC and MM cells were treated with SD8 at IC₅₀ doses for 24 hours. Lysates were immunoblotted with anti-PARP antibody. Actin was used as a loading control. PARP cleavage was indicated by the presence of an 89 kDa fragment. The same cells were treated with 50 nM Gemcitabine for 24 hours for comparison. Mesothelioma cell line, H2596, was treated with 25 nM gemcitabine as a positive control [14]. SD8=SD8, C=vehicle treated cells, G=gemcitabine, + = H2596 cells treated with 25 nM gemcitabine.