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Inhibitors of the Lyase Activity of DNA Polymerase β

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Abstract

One focus of our NCDDG program is the DNA repair enzyme DNA polymerase β. This enzyme serves an important protective role by participating in the repair of lesions to DNA caused by environmental and metabolic events. However, this enzyme is also believed to help repair the damage caused by agents employed as antitumor agents, such as bleomycin and cisplatin. In this sense, transient inhibition of the enzyme concomitant with antitumor therapy might improve the effectiveness of certain DNA damaging antitumor agents. Accordingly, we have been identifying naturally occurring inhibitors of DNA polymerase β.

It is now known that there are two transformations mediated by polymerase β, namely a gap-filling polymerization from which the enzyme derives its name, as well as a lyase reaction that results in excision of the deoxyribose phosphate moiety at the site of the lesion. The lyase reaction is believed to be the rate-limiting step. This presentation summarizes the status of studies of identification of inhibitors of both activities.

Keywords: DNA polymerase β; lyase inhibitors; polymerase inhibitors; natural products; adjuvant chemotherapy; base excision repair.

Introduction

The present report details some recent activities carried out as part of a National Cancer Institute-sponsored National Cooperative Drug Discovery Program. Our program initiated its efforts in 1989; in addition to my laboratory at the University of Virginia, the present participants include the laboratories of Prof. David Kingston (Virginia Polytechnic Institute and State University) and Prof. John Lazo (University of Pittsburgh). Our industrial partner is Galileo Pharmaceuticals; Dr. Sekhar Boddupalli supervises this program.

The initial efforts of our NCDDG were focused on the discovery of DNA damaging agents. This included natural principles that mediated frank strand scission detectable in a cell free system, as well as those apparent in yeast strains selectively deficient in individual biochemical pathways utilized for DNA repair (Overhand & Hecht, 1994). Based on our early success in identifying the mechanism of action of camptothecin (Hsiang et al., 1985), and thereby enabling the discovery and development of topotecan (Kingsbury et al., 1991), we have also been interested in identifying novel inhibitors of DNA topoisomerase I, and more generally in the selective inhibition of DNA processing enzymes such as the topoisomerases.

In recent years, our program has evolved to consider the consequences of inhibiting specific DNA repair pathways and key mediators within the signal transduction cascade. In the latter area, we are presently focusing our efforts on Cdc25 phosphatases, proteins that are linked in a central fashion to the cellular processes of mitogenic growth, apoptosis, cell cycle progression and the transcription of steroid responsive genes (Lyon et al., 2002).

This report describes ongoing efforts within our NCDDG to identify and characterize inhibitors of the repair enzyme DNA polymerase β. DNA polymerase β is involved in the base excision repair (BER) pathway. The BER pathway is thus important in protecting cells from the effects of nucleoside alkylation or oxidation (Beard & Wilson, 2000). This pathway plays an important role in cancer cells, for example in repairing the DNA damage inflicted by antitumor agents such as cisplatin and bleomycin (Miller & Chinault, 1982; Seki & Oda, 1988; DiGiuseppe & Dresler, 1989; Fornace et al., 1989; Hoffman et al., 1995). As such polymerase β likely contributes to the mechanisms by which cancer cells exhibit resistance to chemotherapeutic agents. While DNA polymerase β is also required as a protective mechanism against environmental stresses by normal cells, it seemed possible that co-administration of polymerase β inhibitors as adju-

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vants to chemotherapy with DNA damaging agents might increase the potency of such agents, or allow lower doses to be employed to reduce the side effects of chemotherapy. The consequences of transient inhibition of polymerase β function in normal cells obviously needs to be considered as well.

Materials and methods

Plant materials

Dried plant materials were soaked repeatedly with hexanes until all of the hexanes soluble material had been dissolved. This procedure was repeated with methyl ethyl ketone, methanol and then water. Each of the solutions was concentrated separately to afford the respective crude extract.

DNA polymerase β inhibition assay

The standard reaction mixture (60 µL total volume) contained 62.5 mM 2-amino-2-methyl-1,3-propanediol (ammediol) buffer, pH 8.6, 10 mM $MgCl_2$, 1 mM dithothreitol, 0.1 mg/mL of bovine serum albumin, 6.25 µM dNTPs, 0.04 Ci/mmol [^{3}H]TTP and 0.25 mg/mL of micrococal nuclease-treated calf thymus DNA. The test samples were added to the incubation mixture in 6 µL of 1 : 1 DMSO-MeOH and the reaction was initiated by the addition of 4 µL of rat DNA polymerase β (6.9 units, 48,000 units/mg) (Date et al., 1988; Widen et al., 1988). The reaction mixture was incubated at 37 °C for 1 h and an aliquot was applied to a DEAE-cellulose paper filter. The filter was dried, washed with 0.4 M K_2HPO_4, pH 9.4, and then with 95% EtOH. The dried filter was used for determination of radioactivity.

DNA polymerase β lyase inhibition assay

3′-[^{32}P]-End labeling of an oligonucleotide substrate

A DNA oligonucleotide (36 nucleotides) containing an internal deoxyuridine at a single position on one strand was 3′-end radiolabeled using terminal deoxytransferase and [α-^{32}P]ddATP. The product was purified on a preparative polyacrylamide gel and then annealed to its complementary DNA oligonucleotide to form a duplex. This was accomplished by heating the solution at 70 °C for 3 min, followed by slow cooling to 25 °C.

Apurinic site preparation

The reaction mixture (200 µL total volume) contained 354 nM 3′-[^{32}P]-end labeled DNA duplex having a single deoxyuridine in 10 mM K Hepes buffer, pH 7.4, 50 mM KCl, 5 mM $MgCl_2$, 10 mg/mL of bovine serum albumin, 3 units of AP endonuclease and 2.4 units of uracil-DNA glycolase. The reaction mixture was incubated at 37 °C for 20 min, and the product was used directly in the dRP excision assay.

dRP-excision assay

The reaction mixture (5 µL total volume) contained 354 nM ^{32}P-end labeled duplex containing an apurinic site, 0.17 unit of rat DNA polymerase β and the test samples (crude extracts or fractions) dissolved in DMSO. The final DMSO concentration did not exceed 2%. The reaction mixture was incubated at 25 °C for 30 min, treated with 50 mM $NaBH_4$ and incubated at 25 °C for 10 min. After incubation at 75 °C for 20 min to destroy the excess $NaBH_4$, the reaction products were separated on a 20% denaturing polyacrylamide gel, scanned on a Molecular Dynamics phosphorimager and the data were analyzed using ImageQuant software.

Results

The DNA repair process in which DNA polymerase β participates is outlined schematically in Figure 1. Following excision of an altered nucleobase by DNA glycosylase, the ribose phosphate backbone on the damaged DNA strand is hydrolyzed at the phosphate ester 5′- to the site of damage by apurinic (AP)-endonuclease. Removal of the resulting 5′-phosphoribose moiety is then mediated by the lyase activity of DNA polymerase β, leaving a gap in the damaged strand. This gap is then filled by the polymerase activity of DNA polymerase β using the appropriate nucleoside 5′-triphosphate as a substrate. Resealing of the nicked strand by DNA ligase then completes the repair process (Beard & Wilson, 2000; Garcia-Diaz et al., 2001; Hübscher et al., 2002).

Pol β polymerase inhibitors

The polymerase activity of DNA polymerase β was the first to be identified, and was also the first for which an inhibitor has been sought. Most of the known inhibitors of this enzyme have been identified as part of our NCDDG program. Some of the natural products identified as inhibitors are shown in Figure 2.

As shown in the Figure, several structural classes of compounds are represented among those containing inhibitors of the polymerase activity. These include several anacardic acid derivatives, exemplified by **1** and **2** (Chen et al., 1998), triterpenoids (e.g., **3** and **4**) (Deng et al., 2000a, b), a novel diterpenoid denoted harbinatic acid (**5**) (Deng et al., 1999a), a new diterpenoid-substituted quinol that we have denoted chrysochlamic acid (**6**) (Deng et al., 1999b) and mispyric acid (**7**), a monocyclic triterpenoid with an unusual skeleton (Sun et al., 1999a).

The kinetic mechanism of polymerase β inhibition was studied in detail using three isolated triterpenes (**8**–**10**) (Sun et al., 1999b). All three compounds exhibited mixed type inhibition when the [^{3}H]TTP or DNA template-primer concentrations were varied. When the TTP concentration was varied, the inhibition pattern was intermediate between

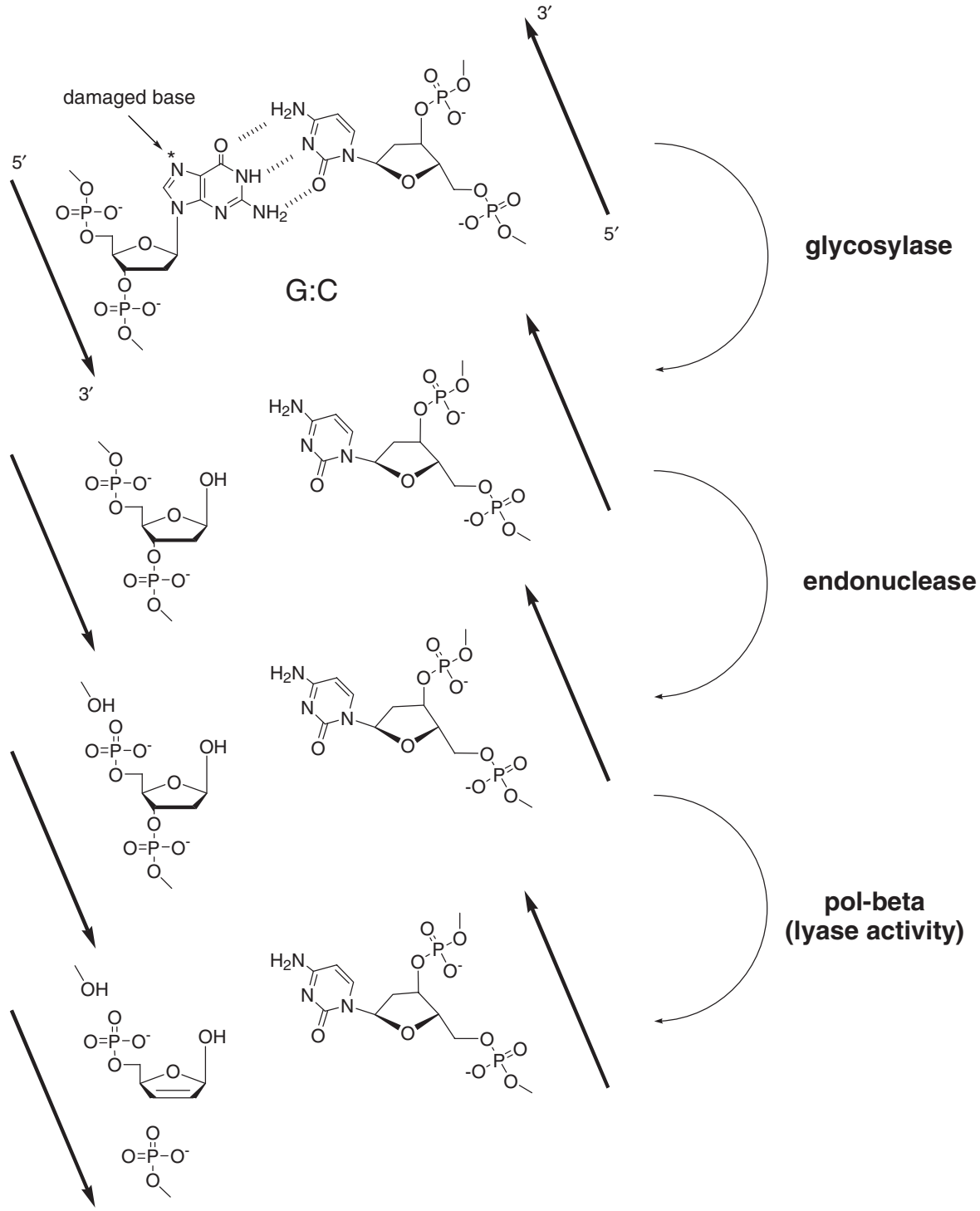

Figure 1. Base excision repair of DNA containing nucleobase damage.

noncompetitive inhibition and competitive inhibition. A similar pattern was obtained as a consequence of variation of DNA template-primer concentration.

The K_{is} (enzyme-inhibitor dissociation constant) and K_{ii} (enzyme-substrate-inhibitor dissociation constant) were obtained and are shown in Table 1, along with the IC_{50} values, which varied from 20–36 μM. For the TTP substrate, compounds **8**–**10** all exhibited higher K_{is} values than K_{ii} values, indicating that the compounds bound more tightly to the enzyme-TTP complex than to the enzyme alone. The reverse was true in the case of the DNA template-primer; the inhibitors all bound more strongly to polymerase β alone than to the complex of the enzyme with the DNA template-primer.

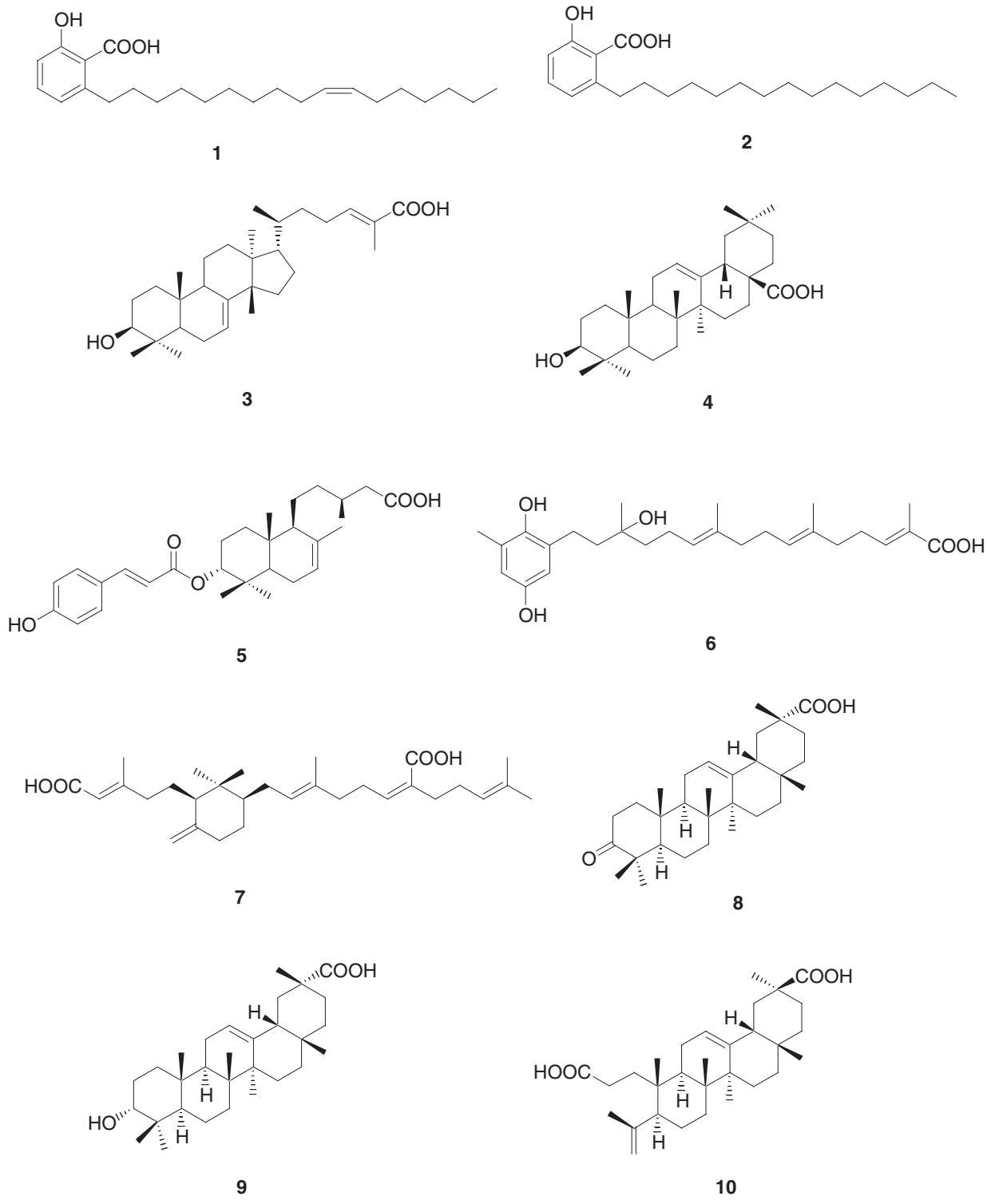

Figure 2. Inhibitors of the gap-filling polymerization activity of DNA polymerase β.

Also of interest was definition of the behavior of the polymerase β inhibitors in cell culture. The goal of our studies is to employ the polymerase β inhibitors as adjuvants to DNA damaging agents in chemotherapeutic regimens. Therefore, the initial experiments sought to define the ability of sublethal concentrations of the polymerase β inhibitors to potentiate the cytotoxicity of sublethal concentrations of DNA damaging agents such as bleomycin, whose DNA lesions are repaired by DNA polymerase β (Miller & Chinault, 1982; Seki & Oda, 1988; DiGiuseppe & Dresler, 1989). As shown

Table 1. IC_{50} Values and Kinetic Constants for DNA Polymerase β Inhibitors **8**–**10**.

		[^{3}H]TTP		DNA template-primer	
compound	IC_{50} (μM)	$^aK_{is}$ (μM)	$^bK_{ii}$ (μM)	$^aK_{is}$ (μM)	$^bK_{ii}$ (μM)
8	22	60	9.1	5.5	50
9	36	60	24	4.2	11
10	20	24	11	5.6	18

[a] Enzyme–inhibitor dissociation constant.
[b] Enzyme–substrate–inhibitor dissociation constant.

in Figure 3, incubation of cultured P388D_1 cells with 75 nM bleomycin alone, or with 50 μM mispyric acid (**7**) alone for 6 hours resulted in essentially no reduction in the number of viable cells relative to an untreated control. In contrast, when the cultured cells were treated with 75 nM bleomycin +50 μM mispyric acid (**7**), nearly a two-fold reduction in the number of viable cells was observed (Sun et al., 1999a). Analogous observations have been made for all of the polymerase β inhibitors studied in this fashion, which includes most of the species shown in Figure 2.

More extensive studies were carried out using anacardic acid derivatives **1** and **2**, both of which also potentiated the action of bleomycin in cultured P388D_1 cells and CCL9.1 cells. Anacardic acid (**1**) also strongly potentiated the action of bleomycin in P388D_1 cells when treatment was continued for periods of time up to 72 hr (Snow, 1995). The effects of bleomycin and compound **1** on unscheduled DNA synthesis were also studied. As shown in Table 2, compound **1** alone had little effect on unscheduled DNA synthesis which was determined as uptake of [^{3}H]thymidine into trichloroacetic acid-precipitable DNA. On the other hand, bleomycin caused a large increase in unscheduled DNA synthesis. As would be anticipated for an authentic inhibitor of the repair of bleomycin-damaged DNA, the addition of anacardic acid (**1**) to cultures also treated with bleomycin largely reduced the unscheduled DNA synthesis that had been induced by bleomycin (Snow, 1995).

Pol β lyase inhibitors

Recently , it was reported that removal of the deoxyribose phosphate moiety (Fig. 1) during the repair of nucleobase lesions also involves DNA polymerase β (Matsumoto & Kim, 1995; Matsumoto et al., 1998; Piersen et al., 1996; Prasad et al., 1998a, b; Deterding et al., 2000). Interestingly, the catalytic center responsible for this activity is localized within the 8 kDa N-terminal domain of polymerase β, and is spatially separated from the structural domain responsible for the (gap-filling) polymerase activity of the enzyme. The transformation mediated by the lyase is believed to involve

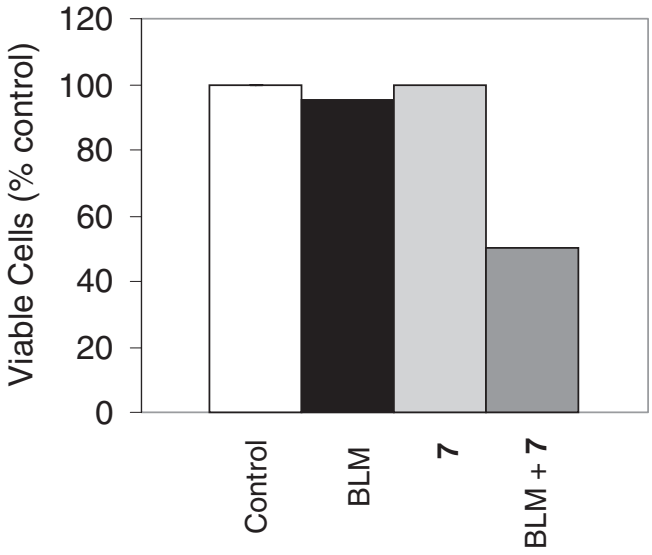

Figure 3. Potentiation of the cytotoxicity of bleomycin by mispyric acid (**7**). Suspension cultures of P388D_1 cells were maintained in the presence of 75 nM bleomycin, 50 μM mispyric acid, or the two compounds combined. After a 6-h incubation, viability was assessed by trypan blue exclusion.

Table 2. Effect of Anacardic Acid (**1**) on BLM-induced Unscheduled DNA Synthesis in Cultured P388D_1 Cells.

Compound	Induction of DNA Synthesis (%)
–	–
1 (50 μM)	10
bleomycin (50 μM)	103
1 + bleomycin (50 μM each)	31

an intermediate in which Lys72 of polymerase β forms an imine with the aldehyde moiety of the deoxyribose phosphate moiety in the damaged DNA (Fig. 4). Abstraction of a H atom from the carbon atom adjacent to the imine, with concomitant β-elimination of the DNA phosphate ester, would complete the transformation mediated by the lyase. Because the lyase activity of polymerase β is now known to be the rate-limiting step for this enzyme (Strauss et al., 1997; Prasad et al., 1998b; Beard & Wilson, 2000), it seemed logical to search for inhibitors of the lyase activity.

The assay used to survey plant and marine extracts for the presence of polymerase β lyase inhibitors is shown in Figure 5. This substrate, prepared enzymatically from a DNA duplex that originally contained deoxyuridine at the site of the formed deoxyribose phosphate lesion (cf. Fig. 1), was ^{32}P-radiolabeled at the 3′-end of the DNA strand which contained the deoxyribose phosphate moiety at its 5′-end. Deoxyribose phosphate excision by the lyase alters the mobility of the radiolabeled strand on a polyacrylamide gel (Fig. 6). Also shown in Figure 6 is the effect on the lyase reaction of a natural product (JD-II-85-3) previously isolated as an

inhibitor of the gap-filling activity of DNA polymerase β and found to inhibit the lyase reaction weakly as well.

In common with findings for inhibition of the gap-filling activity of DNA polymerase β, numerous types of compounds have also been found to inhibit the lyase activity of the enzyme. Several of these are illustrated in Figure 7. They include biscoumarins exemplified by compound **11**, a new triterpenoid (**12**) and plant sterols (e.g. **13**), all of which have been isolated in my laboratory at the University of Virginia. The Kingston laboratory at Virginia Polytechnic Institute and State University has isolated several new naturally occurring lyase inhibitors including triterpenoid **14**, acylated oleanane triterpenoid **15** (Chaturvedula et al., 2003) and neolignans **16**–**19**. These compounds are being characterized biochemically and biologically at the present time.

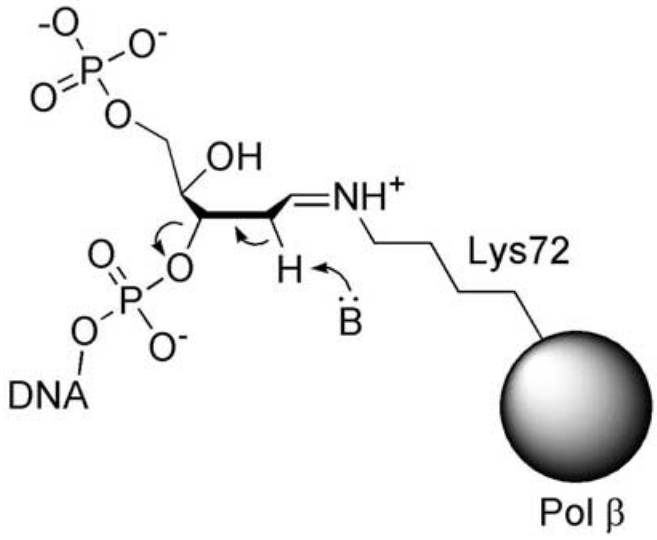

Figure 4. Putative covalent imine intermediate formed by polymerase β and its damaged DNA substrate in the deoxyribose phosphate excision (lyase) assay.

3′- TCCATGCAGTTGCCTTGCTATGGTGATCTCGACCCT-5′

5′- AGGTACGTCAACGGAACGA ^{R}ACCACTAGAGCTGGG-3′-^{32}P

Figure 5. DNA oligonucleotide substrate used to identify extracts putatively containing inhibitors of polymerase β lyase activity. ^{R}denotes the deoxyribose moiety (cf. Fig. 1).

DNA polymerase β inhibitors that also damage DNA

One series of compounds identified as inhibitors of the gap-filling polymerization assay of DNA polymerase β were bisalkylresorcinols (Deng et al., 1999b). Three of these (**20**–**22**) are shown in Figure 8. Compounds of this type had been isolated earlier from plant extracts using a bioassay to identify Cu^{2+}-dependent DNA cleaving agents (Barr et al., 1988; Scannell et al., 1988).

While the original concept in isolating inhibitors of DNA polymerase β had been to identify non-toxic compounds capable of potentiating the action of DNA damaging agents used clinically for antitumor therapy, the isolation of bisalkylresorcinols as polymerase β inhibitors suggested a new strategy. It seemed possible that a single compound might be able to damage DNA, and also inhibit the repair of the damage that it had inflicted. Proof of principle was sought using a number of 5-alkylresorcinols and bisalkylresorcinols (Starck et al., 2000). As shown in Figure 9, bisalkylresorcinol **20** (which was shown to relax supercoiled pSP64 plasmid DNA in the presence of Cu^{2+}) caused a 25% reduction in the number of viable P388D_1 cells when incubated with the cells for 18 h at 10 μM concentration in the presence of 40 μM Cu^{2+}. The same compound also potentiated the action of bleomycin in reducing the number of viable P388D_1 cells (Fig. 10). A synthetic 5-alkylresorcinol derivative that effected DNA relaxation more potently than **20**, and also inhibited DNA polymerase β, displayed stronger activ-

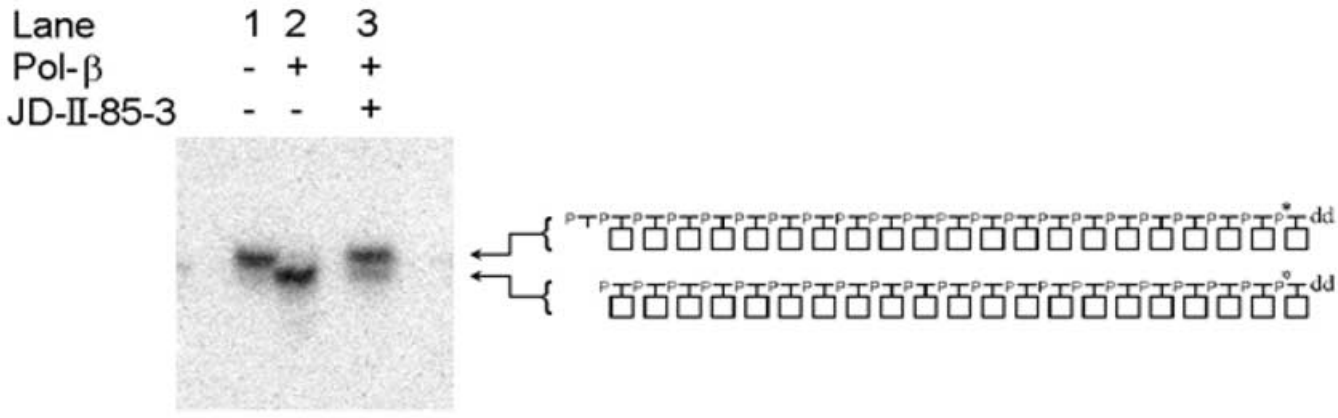

Figure 6. Polyacrylamide gel electrophoresis of the product formed by lyase treatment of the substrate shown in Figure 5. The effects of a lyase inhibitor (JD-II-85-3) are also shown.

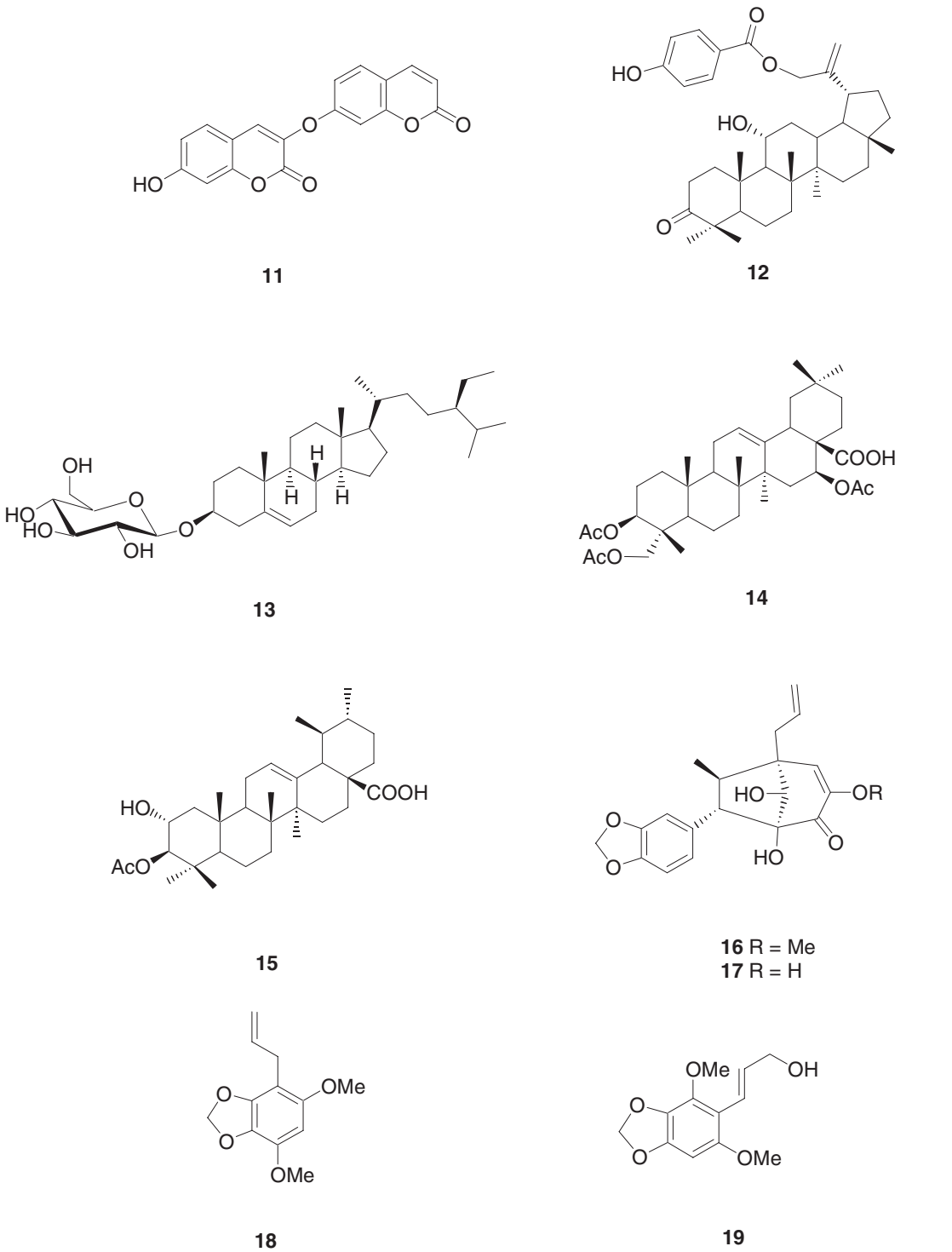

Figure 7. Inhibitors of the lyase activity of DNA polymerase β.

ity than **20** in these cell culture experiments (Singh et al., 1995).

In contrast to our efforts to identify non-toxic compounds that can potentiate the action of DNA damaging agents, it is anticipated that natural products that both damage DNA and inhibit repair of that damage will be cytotoxic. A search for novel natural products having both activities has met with some success, and these agents from the basis for a novel strategy for antitumor therapy.

Discussion

Many of the chemotherapeutic regimens currently employed for antitumor therapy include one or more agents that

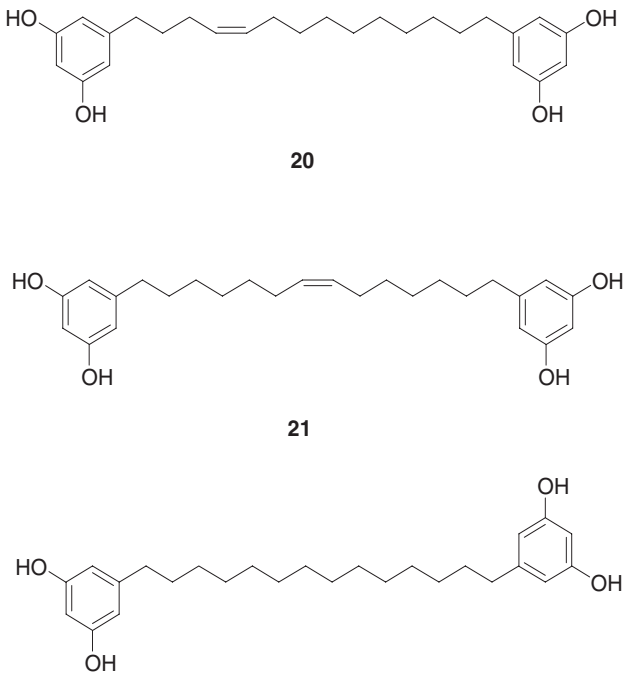

Figure 8. Bisalkylresorcinols isolated as inhibitors of the gap-filling activity of DNA polymerase β.

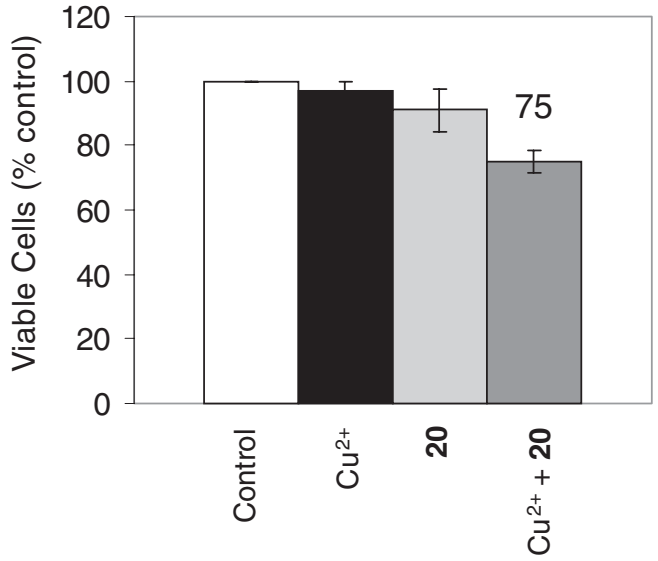

Figure 9. Effects of compound **20** plus Cu^{2+} on $P388D_1$ cells. The cells were treated as described for 18 hr and then assessed for viability by trypan blue exclusion. Compond **20** was employed at 10 μM concentration; Cu^{2+} at 40 μM concentration.

damage DNA (Sharpless & DePinho, 2002). Resistance to the action of these agents by cancer cells can limit the clinical utility of such agents. Base excision repair is the predominant mechanism that protects cells from DNA nucle-

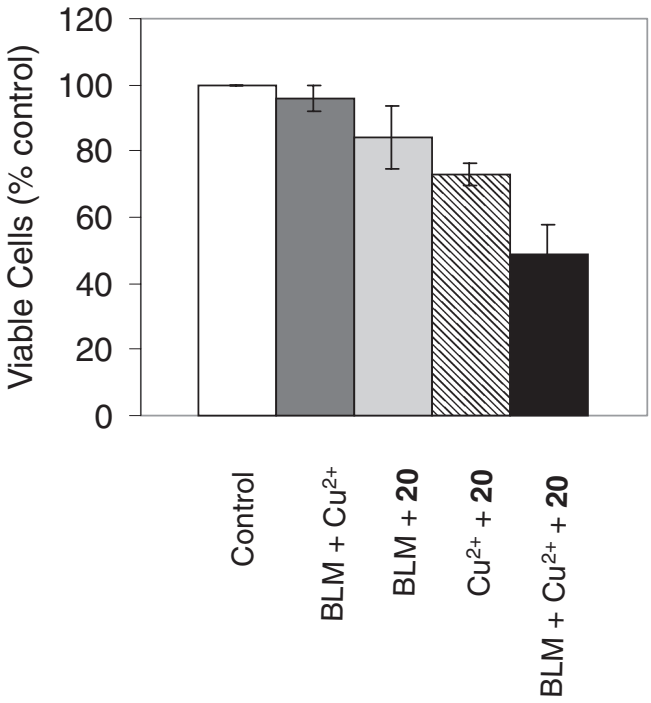

Figure 10. Effects of compound **20**, bleomycin and Cu^{2+} on $P388D_1$ cells. The cells were treated as described for 6 hr and then assessed for viability by trypan blue exclusion. Compound **20** was employed at 10 μM concentration, bleomycin at 75 nM concentration, and Cu^{2+} at 40 μM concentration.

obase damage resulting from oxidation or alkylation (Beard & Wilson, 2000). Logically, inhibitors of one or more steps in the multistep process of base excision repair might be expected to be of utility in potentiating clinically used agents that operate at the level of DNA damage. Anticancer agents such as bleomycin, cisplatin and monofunctional alkyating produce damage repaired through this pathway (Miller & Chinault, 1982; Seki & Oda, 1988; DiGiuseppe & Dresler, 1989; Fornace et al., 1989; Hoffmann et al., 1995).

As outlined in Figure 1, the base excision repair pathway involves at least five steps, two of which are mediated by DNA polymerase β. Polymerase β has 335 amino acid residues and is the smallest of the eukaryotic DNA polymermases (Hübscher et al., 2002). The enzyme consists of a single 39 kDa polypeptide chain; proteolysis can separate these into an 8 kDa N-terminal domain and a 31 kDa C-terminal domain. The N-terminal domain contains the rate-limiting lyase activity, and binds single-strand DNA monovalent metal cations as well as gapped double-strand DNA (Pelletier et al., 1996a, b; Beard & Wilson, 2000; Piersen et al., 2000; Maciejewski et al., 2000). The C-terminal domain contains the polymerase activity and mediates double-strand DNA binding (Idriss et al., 2002). There is strong evidence that both activities are required to support base excision repair *in vivo* (Podlutsky et al., 2001).

There are numerous natural products that have been shown to inhibit the gap-filling polymerization activity of DNA polymerase β, and the majority of these have been identified as part of our NCDDG program. Ten of these inhibitors are shown in Figure 2. While a number of structural classes are represented, it may be noted that all of the inhibitors show certain structural features. These include at least one ring, reasonably hydrophobic character, and the absence of any heteroatom other than oxygen. Every inhibitor of this type isolated to date also contains at least one carboxylic acid moiety. It is known that the C-terminal domain binds Mg^{2+} (Nguyen et al., 2000); the presence of a carboxylate moiety in each inhibitor suggests that electrostatic interaction of the carboxylate with the bound Mg^{2+} represents an important element of ligand affinity for the polymerase domain.

In contrast, inhibitors of the dRP lyase activity generally lack a carboxylate moiety (see, however, **14** and **15**). While triterpenoids are well represented in both classes of inhibitors, the lyase inhibitors include structurally smaller and less complex species (e.g., **18** and **19**). The lyase inhibitors have been identified fairly recently and there is presently limited information about their behavior in biochemical and biological systems.

Although not characterized in great detail, somewhat more information is available about the properties of those compounds that inhibit gap-filling polymerization. It has been shown, for example, that many of these compounds can potentiate the cytotoxicity of bleomycin in short and longer term cell culture (Snow, 1995) in $P388D_1$ and CCL9.1 cells. Compounds **1** and **2** have also been shown to potentiate the action of cisplatin and neocarzinostatin. Further, **1** and **2** can block unscheduled DNA synthesis induced by bleomycin, as well as gap-filling in a cell free assay that utilizes a discrete gapped oligonucleotide substrate (Snow, 1995). A more complete biological evaluation of these inhibitors is underway and should guide efforts to identify one or more polymerase β inhibitors suitable for use as an adjuvant in antitumor therapy. One obvious issue that is being addressed experimentally is the desirability of inhibiting both of the catalytic functions of DNA polymerase β. Several compounds that inhibit both loci are already in hand, although none of those identified to date inhibits both activities potently.

One new concept that has arisen from our studies is that of a DNA damaging agent that can inhibit the repair of the DNA lesions that it has formed. In addition to the prototype agents that we have described (Starck et al., 2000) more potent agents having this dual property have been isolated and will be reported in due course.

Acknowledgement

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