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Expanding the Scope of Human DNA Polymerase λ and β Inhibitors

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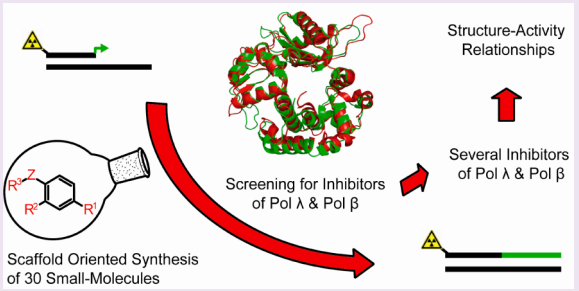
Supporting Information

ABSTRACT: The exact biological functions of individual DNA polymerases still await clarification, and therefore appropriate reagents to probe their respective functions are required. In the present study, we report the development of a highly potent series of human DNA polymerase λ and β (pol λ and β) inhibitors based on the rhodanine scaffold. Both enzymes are involved in DNA repair and are thus considered as future drug targets. We expanded the chemical diversity of the small-molecule inhibitors arising from a high content screening and designed and synthesized 30 novel analogues. By biochemical evaluation, we discovered 23 highly active compounds against pol λ. Importantly, 10 of these small-molecules selectively inhibited pol λ and not the homologous pol β. We discovered 14 small-molecules that target pol β and found out that they are more potent than known inhibitors. We also investigated whether the discovered compounds sensitize cancer cells toward DNA-damaging reagents. Thus, we cotreated human colorectal cancer cells (Caco-2) with the small-molecule inhibitors and hydrogen peroxide or the approved drug temozolomide. Interestingly, the tested compounds sensitized Caco-2 cells to both genotoxic agents in a DNA repair pathway-dependent manner.



Each living cell is exposed to DNA-damaging agents 24 hours a day. To maintain the genetic integrity of its genome, an elaborate set of sophisticated repair mechanisms have evolved. Base excision repair (BER), for example, is responsible for the accurate removal of DNA nucleobase damage generated by oxidation, hydrolytic reactions, or alkylation.[1−3] Errors in DNA damage repair pathways can lead to severe developmental defects, cancer, or even death.[1−5] Key enzymes in DNA metabolism are DNA polymerases. They are involved in the duplication of the genome, repair of DNA lesions, and DNA recombination.[3−5] To date 15 different DNA polymerases have been discovered in human cells.[3,5] The functions of some of these 15 enzymes are known, but for the majority, for example, repair enzymes, the exact roles still await clarification. Therefore, appropriate reagents to probe their respective functions are required. Given their fast mode of action, cell-permeable small-molecule inhibitors are ideally suited to interfere in this highly dynamic process. These molecular tools not only might be of great value for basic science but also may open up novel avenues for the treatment of diseases related to genome integrity.[2−4,6,7]

In this study, we focused on the inhibition of the nonreplicative human DNA polymerases β (pol β)[8] and λ (pol λ),[9] both members of the DNA polymerase X family.[2−5] The exonuclease-deficient pol λ (64 kDa) contains all the important residues required for DNA binding, nucleotide binding and selection, and catalysis of DNA polymerization, which are conserved in pol β (39 kDa), the smallest known human DNA polymerase. Hence, the 3D-structure and the primary sequence of the catalytic core in the C-terminal part of pol λ (residues 244−575) are highly homologous to pol β.[9,10] Because of its ability to remove the 5′-deoxyribose phosphate (dRP) generated after incision by an abasic (AP) endonuclease (dRP-lyase activity) and its DNA synthesis specificity for short gaps, pol β is the prime DNA polymerase involved in BER.[2−5,11,12] Additionally, pol β is able to associate with other downstream enzymes of the BER pathway like DNA ligase I, AP endonuclease, and XRCC1-DNA ligase III.[2,11] Extensive studies show that pol β is able to bypass several DNA lesions via translesion synthesis (TLS), for example, AP sites[13] and cisplatin adducts.[14] Pol λ, the other enzyme of interest here, is capable of synthesizing DNA *de novo* and template-dependent. Furthermore, it displays terminal deoxynucleotidyl transferase (TdT) as well as dRP-lyase activity.[15−19] Pol λ is implicated in V(D)J recombination,[20,21] TLS,[13,22,23] and BER.[15,24,25] Moreover, studies with eukaryotic cells and reactive oxygen species (ROS) indicate that pol λ functions as a backup for pol β in BER[26] and protects cells from oxidative damage.[24,27] There is also evidence that pol λ is required for cell cycle progression and is functionally connected to the S phase DNA damage response machinery in cancer cells.[27]

A recent investigation of pol β and λ expression patterns in 68 tumor samples indicated overexpression of either one in 30% of the assayed cases.[28] The regulation of these two DNA

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polymerases could be fundamental in cancer treatment, since many chemotherapeutic regimes in use depend at least in part on the artificial induction of DNA damage. The clinical efficacy of anticancer drugs like cisplatin and monofunctional alkylating agents is often reduced by cellular DNA repair mechanisms.[1−5,12,29] Consequently, both DNA polymerases involved in DNA repair are discussed as promising future drug targets, to reduce the dosage of DNA-damaging agents while improving their activity via inhibition of their respective DNA repair pathways.[2−4,12,29]

In order to discover and evaluate inhibitors of DNA polymerases, high content screening (HCS) strategies and *in vitro* enzyme assays were developed.[14,30−34] By application of a recently reported fluorescence based screening technique and radioactive primer extension (PEX) assays, several small-molecule inhibitors of human pol λ were discovered.[32] The rhodanine-based compounds, classified as a privileged drug scaffold,[35−38] were found to be the most active inhibitor class (Figure 1).

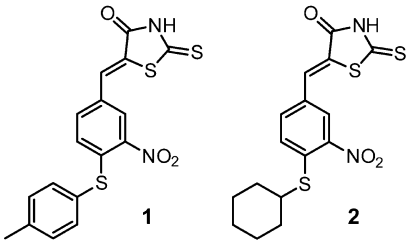

Figure 1. Chemical structures of the initial screening hits **1** and **2**.

The most potent pol λ inhibitor obtained was compound **1**, with a half-maximal inhibitory concentration (IC_{50}) of 5.9 μM for its DNA polymerization function and 4.5 μM for its TdT function. Interestingly, **1** is up to 10 times less active against the highly homologous pol β (IC_{50} = 64.4 μM)[32] and thus could be a useful probe for specifically investigating the biological functions of pol λ. Additionally, **1** did not inhibit (IC_{50} > 100 μM) the DNA polymerases 9°N, Therminator, Pfu, and Dpo4 (unpublished data). During the course of evaluating the inhibitory potential of the initial compound library, further rhodanine-based inhibitors were found whose properties are comparable with **1**.[32] So far a side-by-side comparison of **1** with the most active known pol λ inhibitor (epigallocatechin gallate[39]) has been investigated, and it was found that the rhodanines are currently the strongest known pol λ inhibitors. Furthermore, there were early indications that some compounds (e.g., **2**) also showed activity against pol β.[32] Based on these findings, systematic synthetic optimization has been undertaken by us in order to further expand the chemical diversity and to find novel and more potent small-molecule inhibitors of pol β and λ.

Because pol β and λ are implicated as prime targets for improving the response to genotoxic agents and the rhodanines showed moderate toxicity in human cervix carcinoma (HeLa S3) and hepatocellular carcinoma (Hep G2) cell lines,[32] we explored the cotreatment of these kinds of DNA-damaging agents with rhodanine probes. The anticancer regime was studied with the approved monofunctional alkylating drug temozolomide (TMZ) and the model ROS inducer hydrogen peroxide (H_2O_2) on the human colon carcinoma cell line Caco-2. This cell line is especially interesting since there is an urgent need for the development of novel approaches to enhance tumor cell cytotoxicity of chemotherapeutics for the treatment of colorectal cancers.[33,40−42]

RESULTS

Compound Design and Chemical Synthesis. To further expand the chemical diversity of pol λ and pol β small-molecule inhibitors, we designed and synthesized 30 (**3−32**) novel analogues of **1** and **2** (Table 1). Molecule **2** was resynthesized because of its highest activity against pol β in the initial screen.[32] For this compound series, **1** and **2** were subdivided into the previously described molecular scaffold.[32] The scaffold consists of three variable parts, R^1, R^2, and R^3, which are connected via an aromatic core and a variable Z-linkage (Table 1). To generate the next generation of small-molecule inhibitors, we further derivatized **1** and **2** in all these parts. Reportedly, the heterocyclic rhodanine, moiety A in R^1, proved to be highly important for the inhibitory activity against pol λ.[32] However, it could be shown that moiety A can be replaced in some cases by the 2,4-thiazolidinone, moiety B. Motivated by this fact, we investigated the synthesis of heterocyclic molecules **3−9** bearing moiety B and the further well-known pharmacophoric moieties C, D, E, F, G and H. The thioether-Z-linkage proved not to be essential for high activity toward pol λ and could be replaced by ester-, benzyl phenyl ether-, or diphenyl ether-Z-linkages.[32] Thus, this modification site was not in our main focus herein, and we only tested a sulfone as the Z-linkage (**10**). Importantly, we could show that the variation of the substituents in R^2 and R^3 can influence the inhibitory activity against pol λ. On the other hand there were indications that some of these changes in R^2 and R^3 boosted the inhibitory activity against pol β.[32] To further expand the compound series with modifications in part R^2 and R^3, we designed and synthesized analogues **11−30**. In the initial screen (*Z*)-5-(5-nitro-2-(*p*-tolylthio)benzylidene)-2-thioxothiazolidin-4-one also proved to be a pol λ inhibitor in the low micromolar range (IC_{50} = 10.0 μM).[32] For that reason, we "combined" this compound (apart from the nitro groups) with **1** to generate molecule **31**. Lastly, we investigated the inhibitory potential of the first compound with a heterocyclic aromatic core structure and created **32**.

For the chemical synthesis of the small-molecule entities **2−32**, we followed the reported synthesis strategy, which can be divided into two parts (Scheme 1).[32]

In the first part, we built up the precursor aldehydes **33−35** and **37−58** and for compound **8** a precursor acetophenone **36**. To synthesize the new precursors **34−57** in good to excellent yields, we applied the nucleophilic aromatic substitution (S_NAr) under basic conditions in dimethylformamide (DMF) to displace activated halides (F, Cl, or Br) at the aromatic core by aromatic or aliphatic thiols (Scheme 1A). To synthesize the 3-nitro-4-tosylbenzaldehyde precursor **58** in a three step sequence, we assigned a literature-known synthesis strategy[43] starting from 3-nitro-4-(*p*-tolylthio)benzaldehyde **33**.[32] Therefore, **33** was reduced with sodium borohydride ($NaBH_4$) in methanol to yield the (3-nitro-4-(*p*-tolylthio)phenyl)methanol intermediate in 98%. Next, the intermediate was refluxed together with H_2O_2 in acetic acid to give (3-nitro-4-tosylphenyl)methanol **59** in 61% yield. In the last step, **59** was oxidized with Dess−Martin periodinane (DMP) to furnish precursor **58** in 57% yield (Scheme 1**A**).

In the second part, we performed Knoevenagel condensations by fusing the precursor molecules together with varying heterocycles (Scheme 1B). In doing so, an exocyclic double

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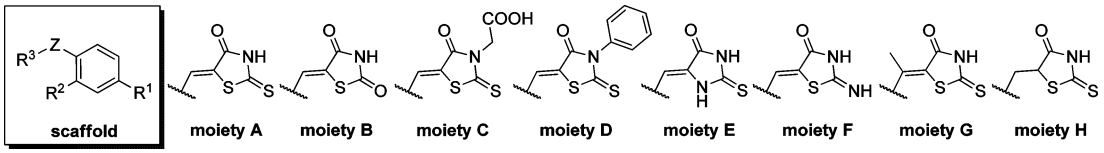
Table 1. Small-Molecules 1−32 and Their Biochemical Evaluation[a]



no.	R^1	R^2	R^3	Z	Pol λ conversion [%], 20 μM compound[a]	Pol λ conversion [%], 10 μM compound[a]	Pol λ IC_{50} [μM][a]	Pol β conversion [%], 50 μM compound[a]	Pol β IC_{50} [μM][a]
1	A	$-NO_2$	4-Me-Ph-	-S-	3[b]	8[b]	5.9[b]	92[b]	64.4[b]
2	A	$-NO_2$	cyclohexane-	-S-	4	19	<10	32	<50
3	B	$-NO_2$	4-Me-Ph-	-S-	4	26	<10	90	>50
4	C	$-NO_2$	4-Me-Ph-	-S-	80			88	
5	D	$-NO_2$	4-Me-Ph-	-S-	100			99	
6	E	$-NO_2$	4-Me-Ph-	-S-	91			93	
7	F	$-NO_2$	4-Me-Ph-	-S-	97			98	
8	G	$-NO_2$	4-Me-Ph-	-S-	5	19	<10	38	<50
9	H	$-NO_2$	4-Me-Ph-	-S-	46			100	
10	A	$-NO_2$	4-Me-Ph-	$-SO_2-$	23		<20	96	
11	A	-H	4-Me-Ph-	-S-	3	31	<10	37	<50
12	A	-F	4-Me-Ph-	-S-	2	14	<10	3	38.7
13	A	-Cl	4-Me-Ph-	-S-	2	9	5.7	82	>50
14	A	-Br	4-Me-Ph-	-S-	2	19	<10	87	>50
15	A	$-CF_3$	4-Me-Ph-	-S-	2	14	<10	7	28.1
16	A	-CN	4-Me-Ph-	-S-	2	11	<10	93	
17	A	$-NO_2$	4-Et-Ph-	-S-	3	14	<10	20	<50
18	A	$-NO_2$	4-F_3C-Ph-	-S-	2	18	<10	90	>50
19	A	$-NO_2$	4-F_3CO-Ph-	-S-	18		<20	83	>50
20	A	$-NO_2$	3-F,4-Me-Ph-	-S-	3	14	<10	90	>50
21	A	$-NO_2$	2,3,5,6,-F; 4-F_3C-Ph-	-S-	3	18	<10	76	>50
22	A	$-NO_2$	3-Me-Ph-	-S-	2	10	6.0	8	29.8
23	A	$-NO_2$	2-Me-Ph-	-S-	3	6	3.9	5	18.2
24	A	$-NO_2$	2,5-Me-Ph-	-S-	2	15	<10	31	<50
25	A	$-NO_2$	2-naphtyl-	-S-	3	19	<10	67	>50
26	A	$-NO_2$	cyclopentane-	-S-	17		<20	90	>50
27	A	-Br	3-F,4-Me-Ph-	-S-	2	5	4.0	84	>50
28	A	-H	2-Me-Ph-	-S-	4	21	<10	47	~ 50
29	A	-F	2-Me-Ph-	-S-	3	14	<10	50	~ 50
30	A	$-CF_3$	2-Me-Ph-	-S-	3	15	<10	37	<50
31	(*Z*)-5-(2,4-bis(*p*-tolylthio)benzylidene)-2-thioxothiazolidin-4-one				2	10	4.0	27	<50
32	(*Z*)-5-((4-bromo-5-(*p*-tolylthio)thiophen-2-yl)methylene)-2-thioxothiazolidin-4-one				4	17	<10	22	<50

[a]Averages of three independent experiments are shown (for details, see Supporting Information). [b]See ref 32.

bond is generated, whereas in theory two diastereomeres *E* and *Z* can be formed. Reportedly, the *Z*-configuration is thermodynamically more stable than the *E*-configuration.[44−46] For that reason, compounds **2**−**8** and **10**−**32** were synthesized under thermodynamic reaction control. We refluxed the precursor aldehydes **33**−**35** and **37**−**58** with rhodanine, thioazolidine-2,4-dione, thiohydantoine, rhodanine-3-acetic acid, 3-phenyl-2-thioxothiazolidin-4-one, or pseudothiohydantoin in a solution of anhydrous NaOAc in glacial acetic acid to obtain the isomeric pure compounds **2**−**7** and **10**−**32** in good to excellent yields (Scheme 1B).[32,47] The ^{1}H NMR spectra of compounds **2**−**5**, **7**, and **10**−**32** exhibited only one signal for the 5-methylidene proton in the range 7.50 to 7.80 ppm and for **6** at 6.50 ppm at lower field values than those expected for the *E* isomers,[48,49] which strongly indicates the formation of the more stable *Z* isomers. To attach rhodanine to the acetophenone precursor **36**, the reaction was performed according to Unangst et al.[50] in a mixture of NH_4OAc and toluene heated under reflux to yield **8** in 78% (Scheme 1B). Compound **8** shows again only one sharp signal for the 5-ethylidene methyl group in ^{1}H NMR implying *Z*-configuration. The exclusive formation of the thermodynamically stable *Z*-isomers of **2**−**8** and **10**−**32** is in agreement with various literature reports for similar compounds.[37,45,47−51] For the synthesis of small-molecule **9**, with the free rotatable pharmacophoric moiety H, we planned to hydrogenate the exocyclic double bond of **1**. Only a few methods have been reported to reduce the exocyclic double bond in 5-benzylidene-2-thioxothiazolidin-4-ones. Kikelj and Peterlin Mašič et al.[48,49] successfully adopted the Hantzsch ester on silica gel method.[52] We also used this chemoselective method and reduced **1** to the racemic compound **9** in a good yield (Scheme 1B).

Biochemical Evaluation and IC_{50} Determination. Holding novel small-molecule entities in hand, we tested

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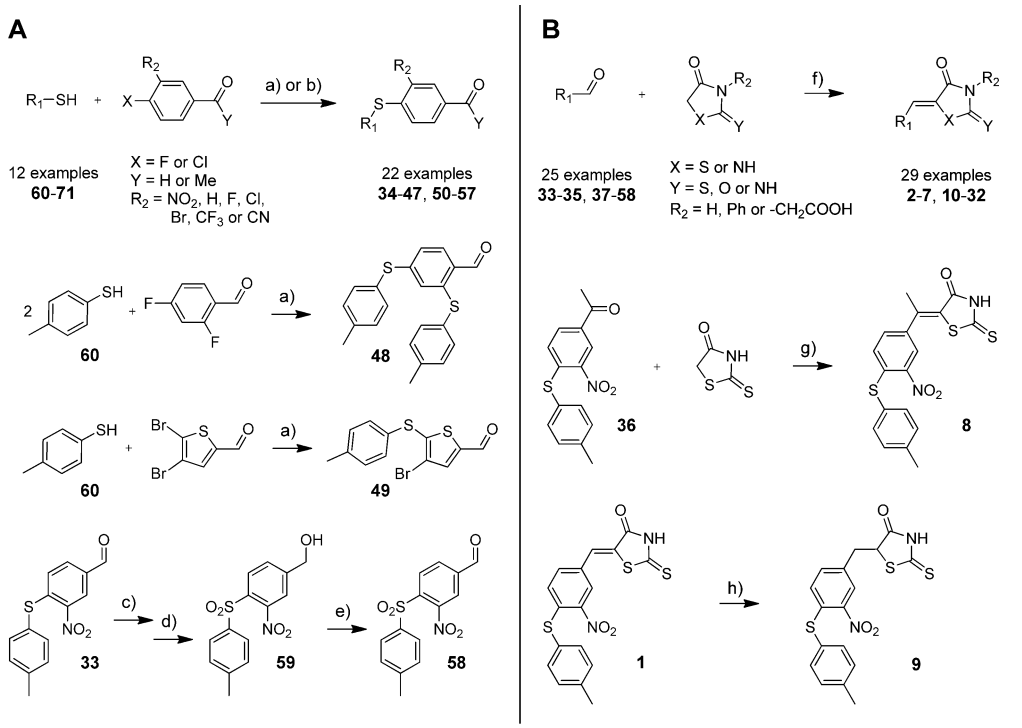
Scheme 1. Chemical Synthesis of Small-Molecules 2−32[a]



[a]Reagents and conditions: (A) Part one, (a) corresponding thiol, corresponding aldehyde (or acetophenone), K_2CO_3, DMF, 80 °C, up to 89%; (b) corresponding thiol, corresponding benzaldehyde, KOH, DMF, 0 °C to rt, up to 97%; (c) $NaBH_4$, methanol, rt, 1 h, 98%; (d) 30% H_2O_2, AcOH, reflux 61%; (e) DMP, CH_2Cl_2, rt, overnight, 53%. (B) Part two, (f) corresponding aldehyde, rhodanine (or rather thioazolidine-2,4-dione, thiohydantoine, rhodanine-3-acetic acid, 3-phenyl-2-thioxothiazolidin-4-one, pseudothiohydantoin), NaOAc (2.5 M in AcOH), reflux, overnight, up to 98%;[32,47] (g) NH_4OAc, toluene, reflux, overnight, 78%; (i) diethyl 2,6-dimethyl-1,4-dihydro-3,5-pyridinedicarboxylate (Hantzsch ester), activated silica gel 60, toluene, reflux for 24 h in the dark, 82%. For details, see Supporting Information.

them at 20 μM concentration in the radioactive pol λ PEX assay in comparison to compound **1** (Figure 2, Table 1). Therefore, a radioactively labeled 20-nucleotide primer strand was annealed to a 33-nucleotide template strand, and all four native dNTPs were added to start the reaction. After 30 min at 37 °C, the reaction was quenched and analyzed via polyacrylamide gel electrophoresis (PAGE) analysis (Supplementary Scheme S1A, Supporting Information). In doing so, we found four inactive compounds (**4**−**7**), four compounds (**9**, **10**, **19**, and **26**) with a moderate activity (15−50% conversion), and 23 highly active compounds (**2**, **3**, **8**, **11**−**18**, **20**−**25**, and **27**−**32**) with conversion below 10% (Figure 2A; Table 1). Next, we studied the 20 highly active small-molecules for their effect in pol λ PEX assay at 10 μM to get a further selection criterion. Interestingly, we found out that all these compounds had an IC_{50} value of less than 10 μM against pol λ (Figure 2B; Table 1). The exact IC_{50} value was determined for the five most active compounds (**13**, **22**, **23**, **27**, and **31**) with a turnover of ≤10% in the PEX assay (Figure 2C; Table 1). Compounds **13**, **22**, **23**, **27**, and **31** inhibited dose-dependently the polymerization function of pol λ with IC_{50} values of 5.7, 6.0, 3.9, 4.0, and 4.0 μM, respectively, and are thus equally or even more active than the lead compound **1** (IC_{50} = 5.9 μM).[32] Reportedly, human pol λ has a TdT activity, and its involvement in recombination events has been suggested.[53,54] Thus, to test the inhibitory potential of **13**, **22**, **23**, **27**, and **31**, we investigated TdT activity using the radioactive assay of single-stranded primer extension. All tested compounds inhibit this function in a low micromolar range comparable to **1** (IC_{50} = 4.5 μM)[32] (Figure 2D, see also Supplementary Scheme S1B, Supporting Information).

To test for selectivity and to identify inhibitors of pol β, we screened the small-molecule series at 50 μM concentration in the radioactive pol β PEX assay (Figure 3, Table 1). Therefore, PEX with the same sequence context was used as described before (Supplementary Scheme S1A, Supporting Information). In this manner, we identified ten compounds (**3**, **13**, **14**, **16**, **18**, **20**, **21**, and **25**−**27**) that selectively inhibited in a low micromolar range pol λ but not pol β. Compounds that were not (**4**−**7**) or were moderately (**9**, **10**, **19**, and **26**) active against pol λ showed no activity against pol β. The resynthesized compound **2** belongs to the ten compounds (**2**, **8**, **11**, **17**, **24**, and **28**−**32**) showing a moderate activity (15−50% conversion) against pol β. Interestingly, we discovered four highly active compounds (**12**, **15**, **22**, **23**) with a conversion below 10% (Figure 3A; Table 1). For the four small-molecule, the exact IC_{50} values were determined. We found out that **12**, **15**, **22**, and **23** dose-dependently inhibited the polymerization function of pol β with IC_{50} values of 38.7, 28.1, 29.8, and 18.2 μM (Figure 3B, Table 1). To further evaluate compound **23**, we used the reported pol β inhibitors betulinic acid,[55] oleanolic acid,[56] and lithocholic acid[57] in a side-by-side comparison using the radioactive PEX assay. We found that the reported pol β inhibitors are less active than **23** in this assay (Figure 3C).

Cellular Studies. Motivated by the reports that the two BER repair proteins pol β and λ were found to be overexpressed in colorectal cancer tissue[28] and are discussed

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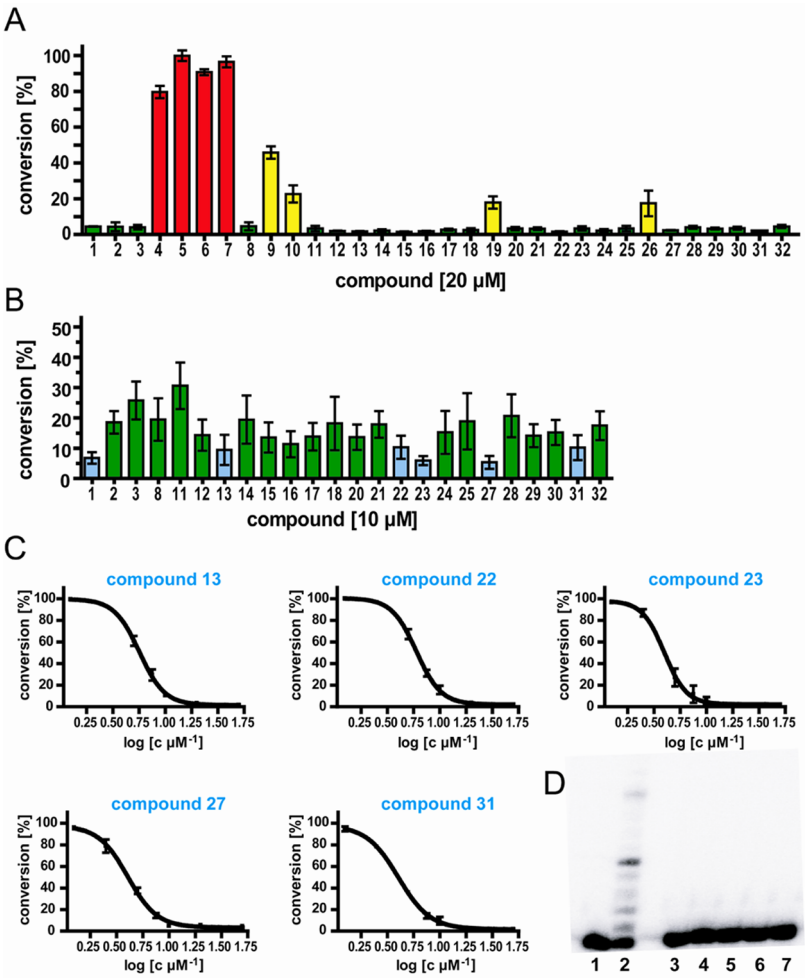

Figure 2. Evaluation of compounds listed in Table 1 toward pol λ. (A) Evaluation of the compounds at 20 μM in the pol λ PEX assay. Inactive compounds are shown in red. Compounds with moderate activity (15−50% conversion) are shown in yellow. Compounds with less than 10% conversion (green) were chosen to be analyzed at 10 μM in the pol λ PEX assay. (B) Evaluation of the compounds at 10 μM in the pol λ PEX assay. Compounds with a conversion below 50% are shown in green. Compounds with less than 10% conversion (blue) were chosen to determine their exact IC_{50} value. (C) Dose−response curves of synthesized compounds **13**, **22**, **23**, **27**, and **31**, which dose-dependently inhibited the polymerization function of pol λ with IC_{50} values of 5.7 ± 1.0, 6.0 ± 1.0, 3.9 ± 1.1, 4.0 ± 1.1, and 4.0 ± 1.0 μM. In general averages of at least three independent experiments and standard deviations are shown. (D) PAGE analysis showing the influence of **13**, **22**, **23**, **27**, and **31** on TdT function of pol λ. Lane 1, primer only; lane 2, DMSO control; lanes 3−7, compounds **13**, **22**, **23**, **27**, and **31** (each 10 μM). For details, see Supporting Information.

as appropriate cellular targets to overcome resistance of DNA-damaging agents,[2−4,12,29] we explored cotreatment of the human colorectal tumor cell line Caco-2 with genotoxic reagents TMZ or H_2O_2 in combination with small-molecule inhibitors **1** or **23**. To find suitable conditions for this proof-of-concept study, we first adopted the previously described 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay[40,58] and determined the half-maximal inhibitory concentration of the cell viability (EC_{50}) of **1**, **23**, TMZ, and H_2O_2. As shown in Supplementary Figure S1, Supporting Information, cell viability was suppressed dose-dependently by **1** (EC_{50} = 58.1 μM) and **23** (EC_{50} = 63.8 μM) after 96 h incubation and by H_2O_2 (EC_{50} = 540 μM) after 72 h incubation. Because of the low cellular response, the exact EC_{50} value of TMZ could not be determined (EC_{50} > 1000 μM). Afterward, cotreatment experiments were performed at concentrations that yielded responses below 30% cell death (Figure S1, Supporting Information), to study whether **1** or **23** can sensitize Caco-2 cells to H_2O_2 or TMZ.

The data shows (Figure 4) that **1** and **23** enhanced the sensitivity of human Caco-2 cells toward H_2O_2 and TMZ. Indeed, the major pathway for oxidative and monoalkylated DNA damage recognition and repair is the BER.[1−3,12,29] The additive or even synergistic effect of cell death induction in Caco-2, caused by inhibitor **1** and **23** together with the ROS inducer H_2O_2 or monoalkylating drug TMZ, suggests that both small-molecules act in a way that affects the BER pathway. To prove these facts in detail is particularly challenging, but these findings support our efforts to further develop the reported pol λ and β inhibitors in a cellular context.

DISCUSSION AND CONCLUSION

By systematic synthetic optimization, we could further expand the chemical diversity of rhodanine-based small-molecules. The scaffold oriented synthesis of drug-like compounds **2**−**32** was easily performed in two to four high-yielding steps starting from cheap and commercially available building blocks.

Because of the evaluation of **2**−**32** in radioactive PEX assays, we could not only discover several highly potent inhibitors of

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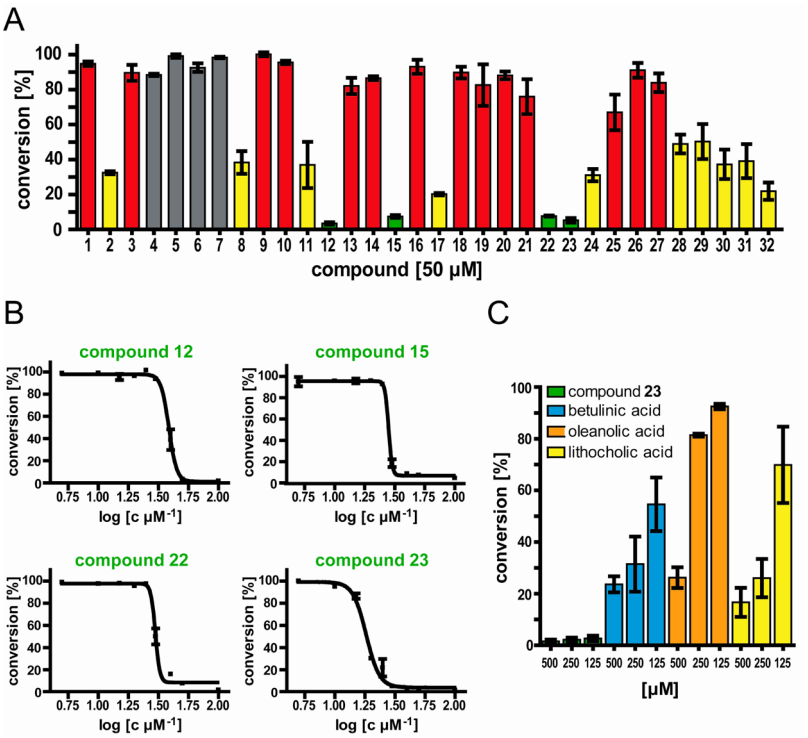

Figure 3. Evaluation of compounds listed in Table 1 toward pol β. (A) Evaluation of the compounds at 50 μM in the pol β PEX assay. Compounds inactive against pol β and pol λ are shown in gray. Inactive compounds, with a high conversion, are shown in red. Compounds with moderate activity (15−50% conversion) are shown in yellow. Interesting compounds with less than 10% conversion (green) were chosen to determine their exact IC_{50} values. (B) Dose−response curves of synthesized compounds **12**, **15**, **22**, and **23**, which inhibited dose-dependently the polymerization function of pol β with IC_{50} values of 38.7 ± 1.0, 28.1 ± 1.0, 29.8 ± 1.0, and 18.2 ± 1.0 μM. (C) Potency of compound **23** against pol β compared with betulinic acid,[55] oleanolic acid,[56] and lithocholic acid[57] using the same reaction conditions (500, 250, and 125 μM compound). In general, averages of at least three independent experiments and standard deviations are shown. For details, see Supporting Information.

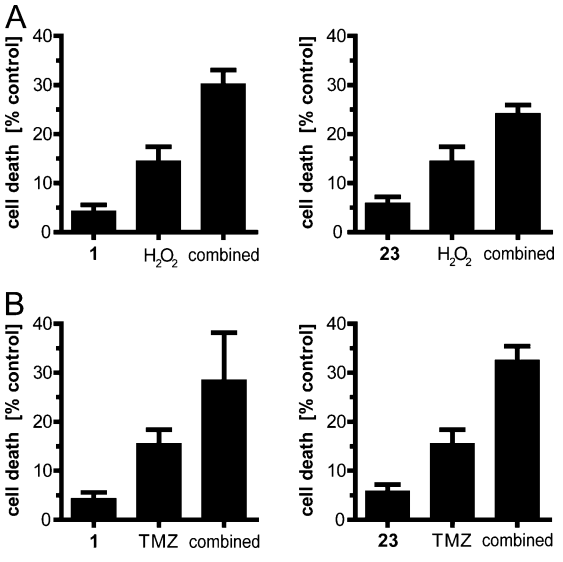

Figure 4. Compounds **1** and **23** sensitize colorectal cancer cells to the ROS inducer H_2O_2 and monoalkylating drug TMZ. Caco-2 cells were preincubated with 25 μM **1** or **23**. After 24 h, cells were treated with 125 μM H_2O_2 (A) or 500 μM TMZ (B) for 72 h. Then, cell death induction was measured by the MTT assay. Averages of at least seven independent experiments and standard deviations are shown. For details, see Supporting Information.

pol λ and β but also confirm and significantly extend the recently reported basic structure−activity relationships (SAR).

The molecular scaffold (Table 1), also ideally suited to establish SAR, consists of three variable parts R^1, R^2, and R^3, which are connected via an aromatic core and a variable linkage Z.[32] The study herein clearly demonstrates the extraordinary importance of the heterocyclic warhead for the effectiveness of the inhibitors against pol λ and pol β. We could show that the hydrogen atom in moiety A cannot be substituted. In line with the recently reported substitution of the hydrogen atom to an allyl group,[32] also the replacement by an acetic acid group (moiety C) or a phenyl group (moiety D) resulted in completely inactive compounds (**4**, **5**) against pol λ and β. Moreover, the endocyclic sulfur atom in moiety A proved to be important since the substitution with an NH group (moiety E) in compound **6** resulted in a loss of activity. In contrast, modifications at the exocyclic double bond (moieties G and H) were tolerated. In consequence of methylation of the double bond in **8** (moiety G), the high potency against pol λ was conserved, but resulted in a compound with moderate activity against pol β. The hydrogenation of **1** to **9** was attended by the change of the hybridization state of the exo- and endocyclic carbon atoms (sp^3 to sp^2). Pol λ could be inhibited by **9**, but with a lower effectiveness than **1**. The replacement of the exocyclic sulfur of moiety A via an oxygen atom (moiety B) or an NH group (moiety F) resulted in indifferent findings. Moiety F containing **7** was completely inactive against both family X DNA polymerases. Interestingly, by substitution of moiety A with moiety B, we generated **3** showing the same inhibitory properties as lead compound **1**, but with a 2,4-thiazolidinone warhead.

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In the initial screening, (*Z*)-5-(5-nitro-2-(*p*-tolylthio)-benzylidene)-2-thioxothiazolidin-4-one proved to be a pol λ inhibitor in the low micromolar range (IC_{50} = 10.0 μM).[32] For that reason, we "combined" this compound (apart from the nitro groups) with **1** and generated the very potent pol λ inhibitor **31** with an IC_{50} value of 4.0 μM. Inhibitor **31** was also moderately active against pol β.

As mentioned above, the thioether used as Z linkage proved not to be essential for high activities or selectivity and could be replaced by ester, benzyl phenyl ether, or diphenyl ether linkages,[32] and so herein we installed only the sulfone linkage in compound **10**. By oxidation of the thioether to the sulfone Z linkage, the pol λ selective inhibitor **10** with a lower activity than **1** was generated.

Comparing compounds **11**−**30**, it is evident that the variation of the substituents in position R^2 and R^3 of the scaffold is also able to influence the activity against pol λ and pol β. With the introduction of several substituents in position R^2 (like H, F, Cl, Br, CF_3, CN, NO_2, or MeO[32]), high inhibitory activity was maintained against pol λ without exception. Interestingly variation of the R^2 substituent to Cl (**13**), Br (**14**), and CN (**16**), beside the known NO_2 group (**1**),[32] led to compounds that are able to discriminate between pol λ and pol β. By analyzing **11**, **12**, **15**, **28**−**30**, we found that the activity rose considerably against pol β in the specified order H ≤ F < CF_3. As we reported previously, R^3 belongs to the scaffold and therefore it cannot be waived. Looking at the compound series with variations in R^3, we found that all tested compounds (except R^3 = 2-pyridine[32]) were able to inhibit pol λ. It is noteworthy that the R^3 modifications 4-F_3C-Py-[32] or 4-F_3C-Ph- to 4-F_3CO-Ph- and cyclohexane to cyclopentane led to moderately active pol λ inhibitors. A particularly interesting discovery is that the alkylation pattern of the aromatic ring in R^3 not only affects the activity against pol λ but also against pol β. A close look at **1**, **17**, **22**−**24**, and **28**−**30** reveals that the IC_{50} value against pol λ decreases in the same order (4-Me-Ph > 3-Me-Ph > 4-Me-Ph), as the activity against pol β is modulated (4-Me-Ph ≪ 4-Et-Ph = 3-Me-Ph < 4-Me-Ph). Lastly, we analyzed **32**, the first compound with a heterocyclic aromatic core structure. As a result of the bioisosteric thiophene ring, the activity against pol λ was preserved, but the selectivity dropped.

In general, rhodanines are classified as nonmutagenic,[59] and a long-term study on the clinical effects of the rhodanine-based epalrestat demonstrated that it is well tolerated by patients.[60] Because chemotherapies depend frequently in part on the artificial induction of DNA damage, consideration of targeting DNA repair capacity is an ongoing concern in improving responses to treatments. Like other gene products involved in DNA damage repair, the regulation of pol β and λ, which are overexpressed in cancer tissue,[28] could be fundamental in cancer treatment.[2−4,12,29] The herein reported cellular studies support this notion, as **1** and **23** enhanced the sensitivity of human colorectal cancer cells toward the genotoxic H_2O_2 and TMZ considerably. The additive or even synergistic effect suggest that **1** and **23** have the potential to reduce the dosage of DNA damaging reagents, while improving their activity via inhibition of their respective DNA repair pathways. For this reason, the discovered small-molecules not only might be of great value for basic science but may also serve as lead structures for the development of novel avenues for the treatment of diseases related to genome integrity.

In conclusion, we report the discovery and development of a highly potent inhibitor series based on a rhodanine scaffold arising from a fluorescence based HCS.[32] We expanded the chemical diversity of pol λ and pol β small-molecule inhibitors and designed and synthesized 30 (**3**−**32**) novel analogues.

By biochemical evaluation of the small-molecule entities, we discovered 23 highly active compounds against pol λ (**2**, **3**, **8**, **11**−**18**, **20**−**25**, **27**−**32**) with IC_{50} values less than 10 μM. Interestingly, 10 of these small-molecules (**3**, **13**, **14**, **16**, **18**, **20**, **21**, **25**−**27**) selectively inhibited pol λ in a low micromolar range but not pol β. The exact IC_{50} values were determined for the five most active compounds. Compounds **13**, **22**, **23**, **27**, and **31** dose-dependently inhibited the polymerization function of pol λ with IC_{50} values of 5.7, 6.0, 3.9, 4.0, and 4.0 μM and are thus equally or even more active than lead compound **1** (IC_{50} = 5.9 μM).[32] To our knowledge, the herein reported small-molecules are currently the strongest inhibitors for pol λ. Furthermore, we discovered 14 small-molecules (**2**, **8**, **11**, **12**, **15**, **17**, **22**−**24**, **28**−**32**) that target pol β. The exact IC_{50} value was determined for the four most active compounds. Compounds **12**, **15**, **22**, and **23** inhibited dose-dependently the polymerization function of pol β with IC_{50} values of 38.7, 28.1, 29.8, and 18.2 μM. To further evaluate pol β inhibitors, we compared **23** with betulinic acid,[55] oleanolic acid,[56] and lithocholic acid[57] in a side-by-side comparison using the radioactive pol β PEX assay and found that the reported pol β inhibitors are less active. Out of the *in vitro* data, we could additionally extend and confirm the recently reported SAR.[32]

In addition, we explored whether the discovered compounds sensitize cancer cells toward DNA-damaging reagents. Thus, we cotreated human colorectal cancer cells (Caco-2) with the discovered inhibitors and ROS inducer H_2O_2 or the approved drug TMZ. Importantly, the tested small-molecules **1** and **23** were pharmacologically active and sensitized Caco-2 cells toward both genotoxic agents. The resulting data indicates that the compounds act in a way that influences the BER pathway and supports our efforts to further develop the reported pol λ and β inhibitors *in vitro* and in a cellular context.

METHODS

Full experimental details are given in the Supporting Information.

ASSOCIATED CONTENT

Supporting Information

Schematic of PEX assays for polymerase and TdT inhibitor activity of pol β and λ, full experimental details, measured cell death curves of compounds **1** and **23**, H_2O_2, and TMZ, and ^{1}H NMR spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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