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Synthesis and Biological Evaluation of Cardiac Glycosides for Cancer Therapy by Targeting the DNA Damage Response

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Cardiac glycosides (CGs) are bioactive compounds originally used to treat heart diseases, but recent studies have demonstrated their anticancer activity. We previously demonstrated that *Antiaris toxicaria* 2 (AT2) possesses anticancer activity in KRAS mutated lung cancers via impinging on the DNA damage response (DDR) pathway. Toward developing this class of molecules for cancer therapy, herein we report a multistep synthetic route utilizing k-strophanthidin as the initial building block for determination of structure–activity relationships (SARs). A systematic structural design approach was applied that included modifications of the sugar moiety, the glycoside linker, stereochemistry, and lactone ring substitutions to generate a library of *O*-glycosides and *MeON*-neoglycosides derivatives. These molecules were screened for their anticancer activities and their impact on DDR signaling in KRAS mutant lung cancer cells. These results demonstrate the ability to chemically synthesize CG derivatives and define the SARs to optimize AT2 as a cancer therapeutic.

Introduction

The DNA damage response (DDR) plays an important role in maintaining the genome stability and is critical for the survival of cancer cells under high replication stress.[1] When DNA damage occurs, DDR activates a series of biochemical events that act in concert to preserve the integrity of genomic DNA by recruiting and activating factors such as the checkpoint kinases ATR and Chk1 to initiate the DNA damage repair; however, if the damage extent is too severe, DDR initiates signals to induce programmed cell death such as apoptosis.[2] This cellular response to DNA damage and replication stress can be exploited for the development of newer targeted cancer therapeutics. The first clinically approved DDR-targeting drug that has been approved for treatment of BRAC1 and BRAC2 mutations in several cancers was Olaparib which targets poly (ADP-ribose) polymerases (PARP) to induce synthetic lethality in BRCA mutated cancers.[3] This has since seen an incline in the number of small molecules under preclinical and clinical studies targeting DDR.

Cardiac glycosides (CGs) have been found to exhibit anticancer properties[4] with additional studies showing a reduced probability of tumor reoccurrence.[4e,5] CGs are traditionally used to treat heart disease by inhibiting the Na^+/K^+ ATPase transmembrane channel.[6] Activation of this enzyme triggers several downstream pathways that can result in cell death by apoptosis.[4g] This observation is indicative of the CGs' involvement in more complex cellular signaling that cause selective reduction of certain tumors.[7] While the mechanism for their anticancer properties is not completely elucidated, several hypotheses have been proposed which include inhibiting the synthesis of HIF-1α,[8] altering Ca^{2+} signaling[9] and modulating signal transduction pathways.[6a] In our previous report, we showed that CGs can be used in tandem with chemotherapy to increase sensitivity of KRAS mutant lung cancer.[4h] We identified AT2 molecule (Figure 1) as one of the most potent CGs from a library screening of over 800 compounds.[4h] The inhibition activity towards the DDR pathway was evidenced by the reduced phosphorylation of Chk1 in the presence of camptothecin (CPT), a topoisomerase 1 inhibitor that activates the DDR. The results revealed a relationship between the DDR pathway and CGs via suppressing the expression of UHRF1.[4h] These studies further showed that the activity of the CGs likely occurred independent of the involvement of Na^+/K^+ ATPase. These findings are consistent with

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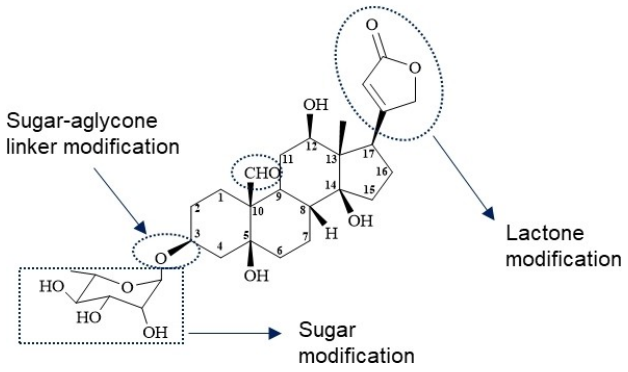


Figure 1. SAR approach for modification of AT2 molecule.

previous reports that CGs exhibit anticancer properties by targeting the DDR pathway.[10]

Structurally, CGs are molecules comprised of a steroid core bound to a sugar moiety at the C3 position and a lactone moiety at the C17 position. Several CGs such as digoxin, digitoxin, ouabain, oleandrin, isolanid, and UNBS1450 have been reported to possess anticancer properties.[6a] While the benefits of their utility has been recognized, their cardiotoxicity still poses a significant challenge.[5] This toxicity has been found be related to the increase in concentration of CGs that consequently increases the calcium levels.[11]

The objective of this report is to develop efficacious CGs that can inhibit the proliferation of cancer cells through targeting the DDR signaling while demonstrating reduced toxicity to normal cells. While the initial CGs were extracted and isolated from plants species,[12] the structural modification of these compounds is always limited and challenging. Conversely, synthetic CGs present an opportunity for further derivatization of the scaffold that would consequently result in lead compounds with enhanced anticancer properties. Each of the structural motifs/features/functional groups in the AT2 scaffold play a crucial role that influences the bioactivity of the compound. The sugar moiety affects the solubility and cellular permeability.[6c] Therefore, modification of the sugar group would influence the pharmacokinetics of these compounds. It has been shown that the glycoside linker and its stereochemistry have influence and drive the cytotoxicity of the CGs.[13] Nagorny and co-workers recently demonstrated that the lactone ring is not only essential in enhancing anticancer activity due to its unsaturation, but also the presence of heteroatoms and its orientation are important as well.[14] In spite of these findings, it is challenging to compare the differences in structural features and biological assays in different cellular models. To the best of our knowledge, there is still a limited number of publications that provide an extensive analysis of the structure activity relationships (SARs) of CGs in a single cellular model.[14–15]

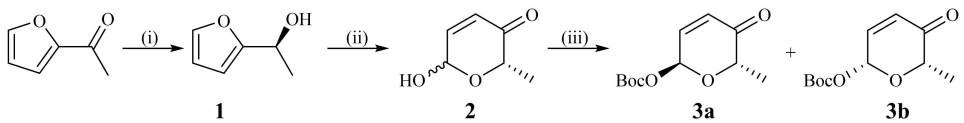
In this study, we undertake an SARs design approach by exploiting the AT2 scaffold for further derivatization (Figure 1). We report a complete multi-step synthesis of AT2 analogs. A systematic structural modification of the sugar moiety, glycoside linker, stereochemistry, and lactone ring were performed to generate a library of *O*-glycosides and *MeON*-neoglycosides. We also assessed their SAR by evaluating their potency on inhibiting the DDR and cell growth of the A549 non-small cell lung cancer (NSCLC) cells. In parallel, we analyzed cell growth inhibition of top derivatives in a non-transformed MCF10 A cells.

Results and Discussion

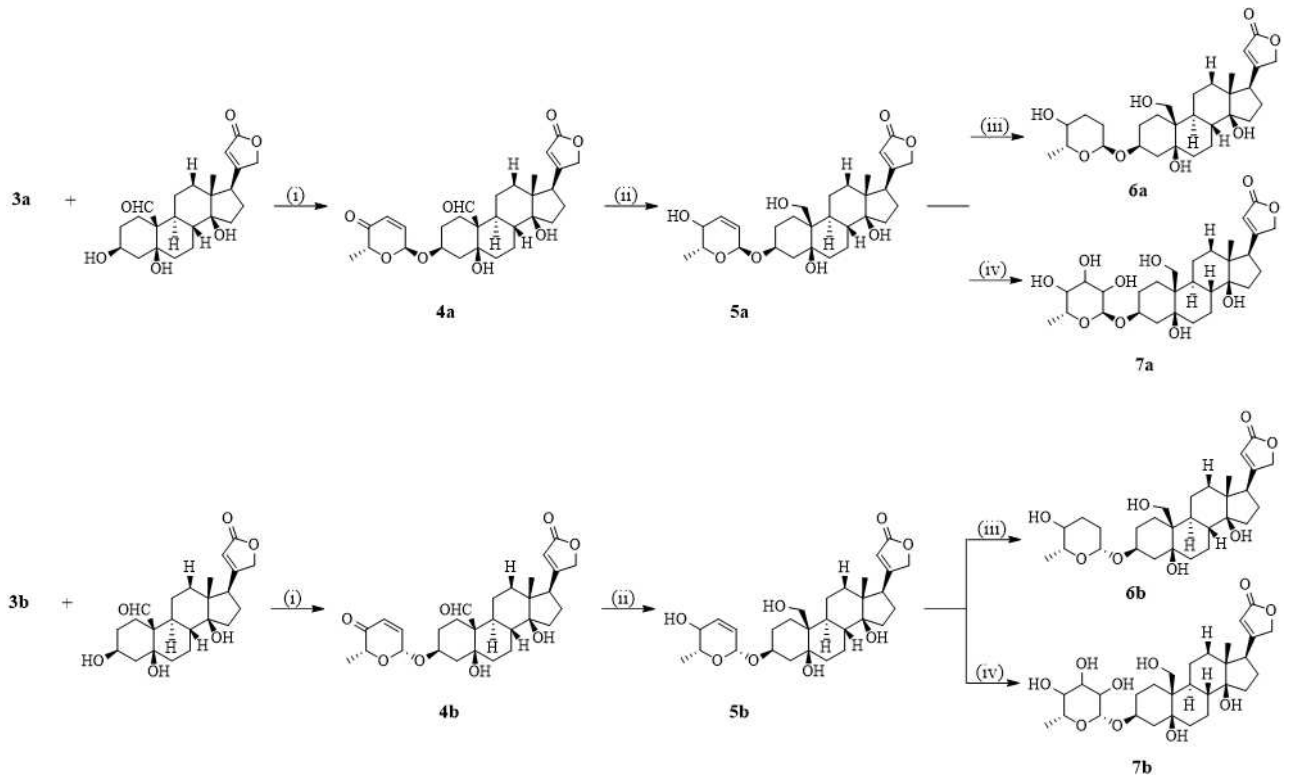
Chemistry

The *O*-glycoside and *MeON*-neoglycoside derivatives were synthesized from precursors modified from commercially available substrates, 2-acetyl furan and k-strophanthidin. The sugar moiety precursor was synthesized from 2-acetyl furan as previously reported by Bajaj *et al.* (Scheme 1).[16] Briefly, the ketone was catalytically reduced to selectively prepare (S)-1-(furan-2-yl)ethan-1-ol, **1**. The furfural alcohol was subjected to Achmatowicz oxidation in the presence of N-bromosuccinimide to produce a pair of diastereomers of **2** (85% yield). Compound **2** was carried into the following step without separation. Boc-protection of the obtained mixture was performed at low temperature (−78 °C) and the diastereomers were isolated in a 2.5 : 1 ratio of **3a** : **3b** (67% total yield).

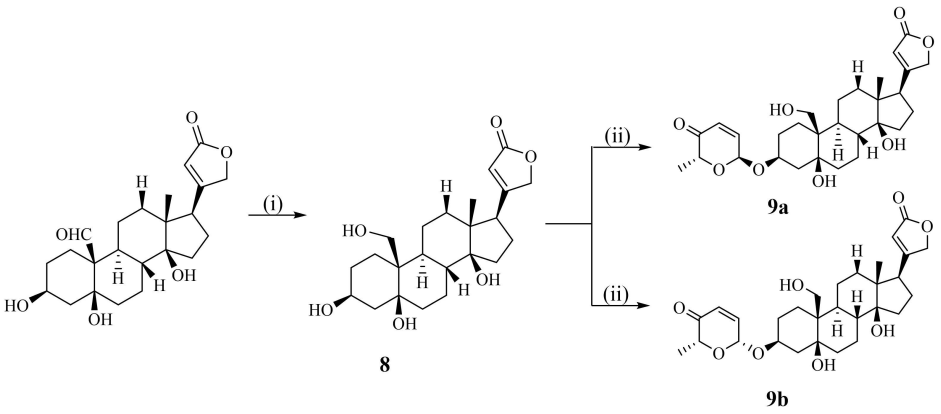
A series of *O*-glycosides were subsequently synthesized (Scheme 2) via glycosidation using a $Pd_2(dba)_3CHCl_3$ catalyst (2.5 mol%) with triphenyl phosphine to achieve **4a** and **4b**. A Luche reduction was then performed using $NaBH_4$ and $CeCl_3$ to obtain **5a** and **5b**. This reaction resulted in the reduction of the aldehyde on the glycoside backbone in addition to the ketone on the sugar moiety. Compounds **5a**/**5b** was subjected to dihydroxylation conditions and converted to **7a**/**7b**. Conversely, when treated with IPNBSH, the alkene moiety in **5a**/**5b** was reduced to afford **6a**/**6b**. In order to have comparable structural similarities to **5a**/**5b**, **6a**/**6b** and **7a**/**7b**, hydroxyl-analogs of **4a**/**4b** were synthesized (Scheme 3). In brief, k-strophanthidin was treated to $NaBH_4/CeCl_3$ and the aldehyde was consequently



Scheme 1. Synthesis of sugar moiety precursors (**3a** and **3b**). (i) Noyori (S, S), CTAB, HCO_2Na, DCM, H_2O, RT, 24 h; (ii) NBS, $NaHCO_3$/NaOAc, THF/H_2O, 0 °C, 2 h; and (iii) $(Boc)_2O$, DMAP, DCM, −78 °C, 24 h.



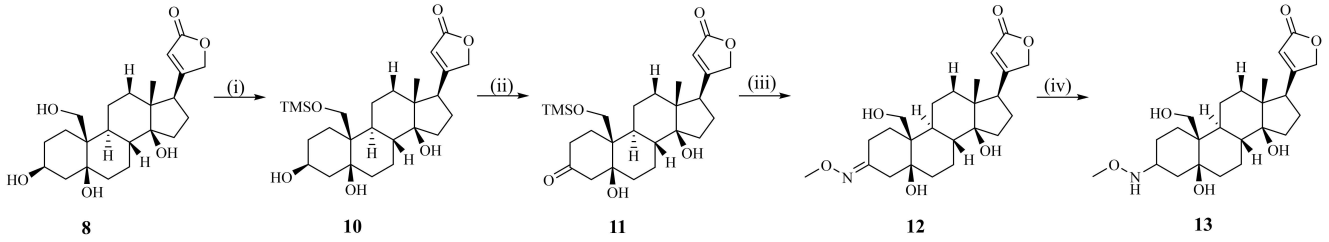
Scheme 2. Preparation of *O*-glycoside analogs. (i) $Pd_2(dba)_3CHCl_3$, PPh_3, DCM/THF, 0 °C, 24 h; (ii) $NaBH_4$, $CeCl_3$, MeOH, DCM, −78 °C, 2 h; (iii) IPNBSH, Et_3N, NMM, 0 °C – RT, 24 h; and (iv) OsO_4, NMO, t-BuOH/acetone, 0 °C, 4 h.



Scheme 3. Synthesis of **4a** and **5a** derivatives. (i) $NaBH_4$, $CeCl_3$, MeOH, DCM, −78 °C, 2 h; and (ii) $Pd_2(dba)_3CHCl_3$, PPh_3, DCM/THF, 0 °C, 24 h.

reduced to obtain precursor **8**. This intermediate underwent glycosidation to produce **9a**/**9b**.

It was previously determined that *MeON*-neoglycosides exhibit anticancer properties.[17] Therefore, we set out to modify the glycoside linker to methoxylamine in order to examine its inhibition activity. The initial synthetic pathway for this precursor followed the oxidation of the secondary alcohol on k-strophanthidin using pyridinium chlorochromate (PCC) and then the product was treated with methoxylamine. The challenge with this reaction route is that the aldehyde and the ketone functional groups on the obtained oxidised K-strophanthidin were both reactive with methoxylamine hydrochloride in the second step. This would consequently result in diglycosidation of the steroid backbone. However, we explored an alternative pathway to address this issue (Scheme 4). K-strophanthidin was reduced using $NaBH_4$ and $CeCl_3$ at −78 °C and a quantitative yield of compound **8** was obtained. Intermediate **8** was subjected to trimethyl silyl chloride to selectively protect the primary alcohol **10**. The resulting product (**10**) was then oxidized using PCC in CH_2Cl_2 to produce trimethyl silyl ketone (**11**). Intermediate **12** was subsequently synthesized following the treatment of **11** with methoxylamine



Scheme 4. Synthesis of Neoglycoside precursor (**13**). (i) TMSCl, Et_3N, pyridine, 0 °C (3 h) – RT (15 h); (ii) PCC, DCM, 2 h; (iii) CH_3ONH_3HCl, pyridine, MeOH, RT, 2 h; (iv) t-$BuNH_2BH_3$, aq HCl, CH_3CH_2OH, dioxane, 0 °C, 2.5 h.

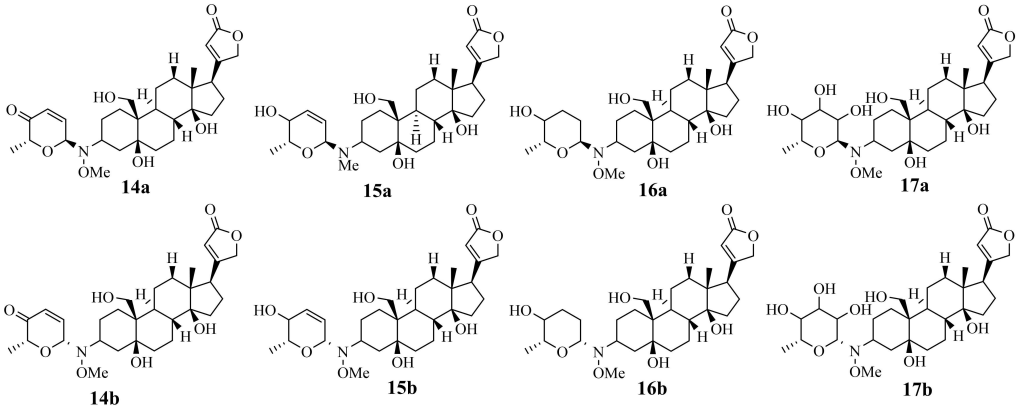
hydrochloride. The NMR analysis of the obtained product **12** indicated the absence of the trimethyl silyl hydroxyl group. This is consistent with the previous studies that reported the cleavage of silyl group under basic conditions.[18] The methoxylimine precursor was further reduced in the presence of borane *tert*-butylamine to obtain the *MeON*-neoglycoside precursor, **13**.

With the neoglycoside precursor (**13**) in hand, the *MeON*-neoglycosides (**14a**–**17a** and **14b**–**17b**, Scheme 5) were then synthesized by glycosidation coupling reaction with Boc-pyranone (**3a**/**3b**) and the subsequent reactions shown in the synthesis of *O*-glycosides (Scheme 2). Other derivatives of *O*-glycosides (**19a**–**22a** and **19b**–**22b**) were synthesized by modifying the lactone ring as shown in Scheme 6. In brief, the lactone modification was performed by aldol condensation of k-strophanthidin with benzaldehyde to synthesize the benzylidene intermediate (**18**). This product was further coupled with Boc-pyranone (**3a**/**3b**) to produce **19a**/**19b** which underwent Luche reduction to yield **20a**/**20b** and finally, olefin protonation and osmium tetroxide dihydroxylation reactions were performed to obtain **21a**/**21b** and **22a**/**22b**, respectively.

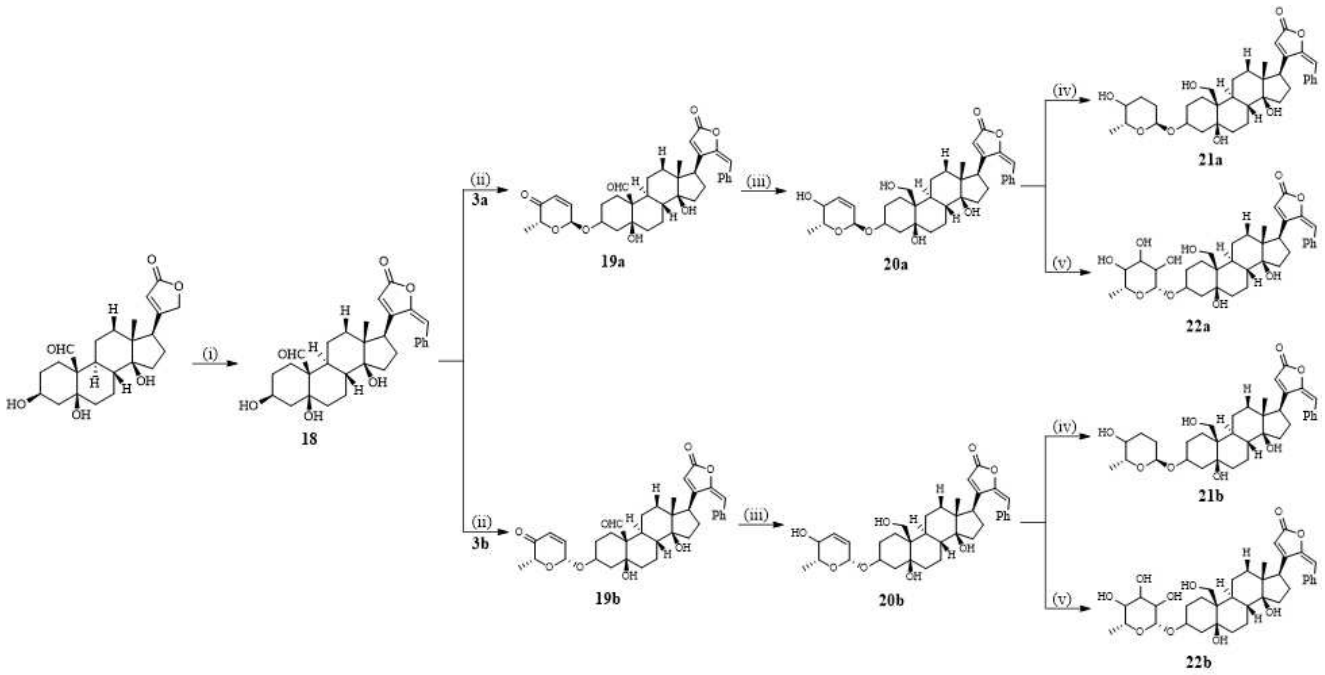
Biological activity of synthesized compounds

Effects of CGs on DDR in KRAS mutated lung cancer A549 cells

Our previous work demonstrated AT2, a typical CGs, sensitized KRAS mutant lung cancer cells to chemotherapy through inhibiting the DNA damage response (DDR).[4h] Therefore, we evaluated the inhibition of the DDR signaling in NSCLC A549 cells induced by camptothecin (CPT) after pre-treatment with the CG derivatives. The ratio of phosphorylated Chk1 (pChk1) normalized to total Chk1 indicated the activation of DDR response. CPT treatment induced several folds of increase in the pChk1/Chk1 ratio (Figures 2–3), which is indicative of the activation of DDR in these cells. However, co-treatment with AT2 significantly reduced CPT-induced Chk1 phosphorylation (Figures 2–3), which is consistent with our previous report on the inhibitory activity of AT2 on DDR.[4h] Compounds **14a/b**, **15a/b**, **17a/b**, **6b**, **7b**, **9a** or **19b** treatment only moderately reduced CPT-induced Chk1 phosphorylation, much weaker than the effect of AT2. On the other hand, **5b**, **k-stro**, **k-stro-ph** (**18**), **k-stro(OH)-ph** (reduced version of **18**), **19a**, **20a/b**, **21a/b and 22a/b** did not reduce and in some cases even increased the activation of DDR (Figures 2–3). However, compounds **4a/b**, **5a**, **6a**, **7a**, and **k-stro** showed strong inhibition on Chk1 phosphorylation, although still slightly weaker than AT2 (Figures 2–3). The effects of these compounds on the protein level of UHRF1 mirror those on pChk1, which is consistent with our previous



Scheme 5. Summary of *MeOH*-neoglycoside compounds (**14a**–**17a** and **14b**–**17b**).



Scheme 6. Preparation of *O*-glycoside-benzylidene analogues of AT2. (i) PhCHO, MeOH, 70 °C, 6 h; (ii) **3a**/**3b**, $Pd_2(dba)_3 \cdot CHCl_3$, PPh_3, DCM/THF, 0 °C, 24 h; (iii) $NaBH_4$, $CeCl_3$, MeOH, DCM, −78 °C, 2 h; (iv) IPNBSH, Et_3N, NMM, 0 °C – RT, 24 h; and (v) OsO_4, NMO, t-BuOH, acetone, 0 °C, 4 h.

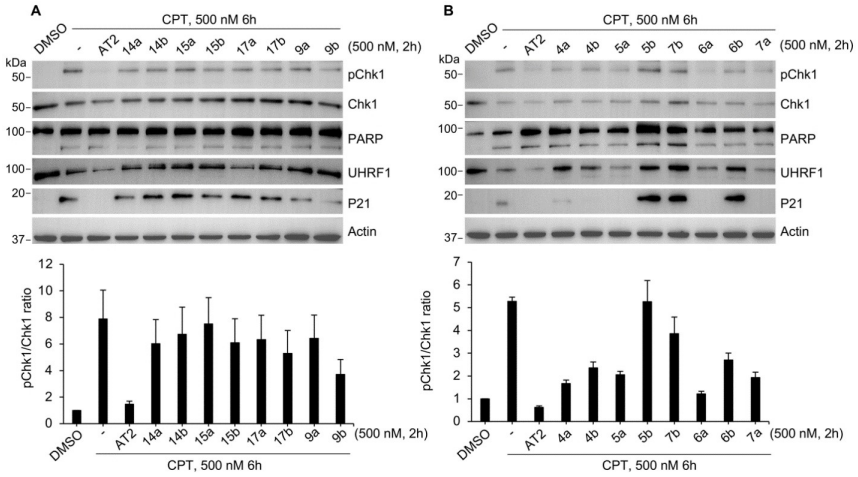


Figure 2. Effects of AT2 analogs on the DDR activation in A549 cancer cells. KRAS mutant A549 lung cancer cells were treated with 500 nM CGs for 2 h before adding 500 nM of CPT for another 6 h. The expression levels of the indicated proteins were assessed by Western blotting using specific antibodies. The relative band intensities of the pChk1 to total Chk1 blots were quantitated by the NIH Image J software and normalized to that in DMSO group. Data represent average and standard deviation of pChk1/Chk1 ratio from 3 replicates.

observations.[4h] UHRF1 is a double strand break repair factor that functions in the P53-dependant DNA damage. The influence of CGs on both UHRF1 and Chk1 phosphorylation emphasizes the significance of their role in DNA damage repair. These results suggest that generally *O*-glycoside analogs retained the DDR inhibition activity of AT2, whereas the neoglycosides largely lost their activities in inhibiting the DDR activation.

To further validate these results, we selected **4a/b**, **5a**, **6a** and **7a** to confirm their effects on the DDR activation. The result show that compound **5a**, **6a**, and **7a** demonstrated nearly identical activities in inhibiting Chk1 phosphorylation as AT2 (Figure 4A–B). Compounds **4a** and **4b** showed weaker effect than other compounds, but still significantly reduced CPT-induced Chk1 phosphorylation (Figure 4A–B), similar as to the previous results. While there might be some difference in terms of the function of the compounds in reducing CPT-induced Chk1 phosphorylation, our data are in general consistent and support the effect of these newly synthesized compounds in inhibiting chemotherapy-induced DNA damage

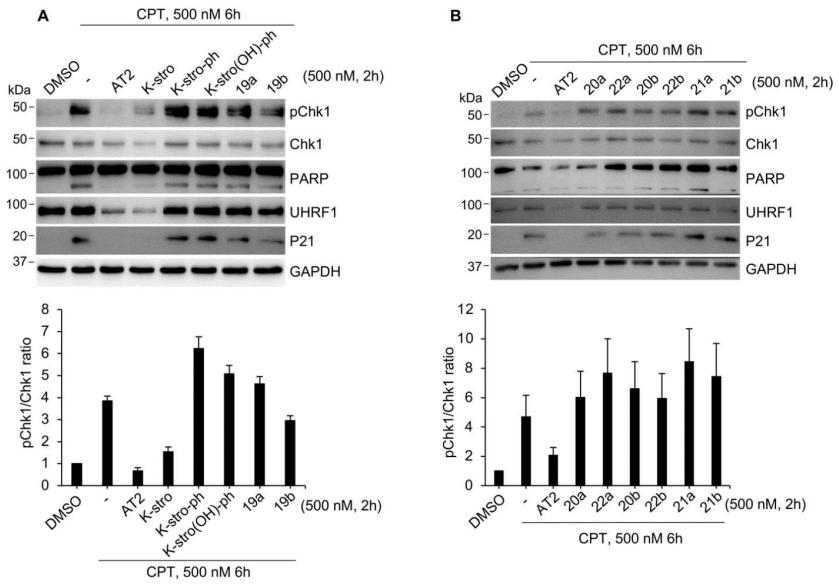


Figure 3. Effects of synthesized AT2 analogs on the DDR activation in A549 cancer cells. Experimental procedures and data analysis were the same as in Figure 2.

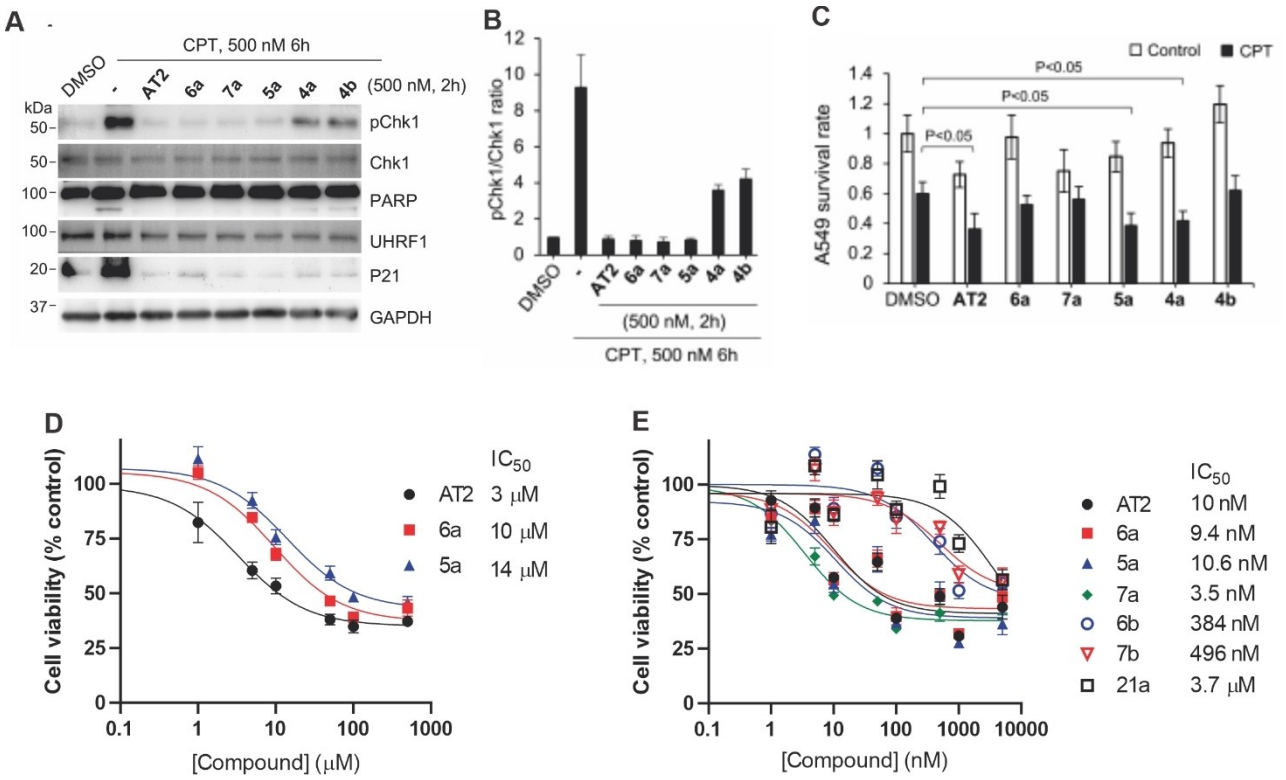


Figure 4. Cytotoxicity of CGs in cancer cells and non-cancerous cells. (**A**) KRAS mutant A549 lung cancer cells were treated with 500 nM CGs for 2 h before adding 500 nM of CPT for another 6 h. The expression levels of the indicated proteins were assessed by Western blotting using specific antibodies. **(B)** The relative band intensities of the pChk1 to total Chk1 blots were quantitated by the NIH Image J software. Data represent average and standard deviation of pChk1/Chk1 ratio normalized to that in DMSO group from 3 replicates. The arrow indicates the band of pChk1. **(C)** A549 cells were treated with 500 nM CGs for 2 h and then added 500 nM CPT for another 6 h, replated into drug free media and cultured for 10–14 days, and surviving colonies were analyzed. Data represent average and standard deviation from 3 replicates. The cytotoxicity of CGs was investigated using MTT assay on **(D)** non-tumorigenic epithelial cell line, MCF10 A and **(E)** Non-small cell lung cancer cell line, A549. The cells were treated with indicated CGs at different concentrations for 12 h and cell viability was measured from 5 replicates. IC_{50} values were calculated in GraphPad prism 8.

response activation. From these results, we found **5b**, **6b** and **7b** exhibiting the opposite effects of their isomers. The α-oriented glycosides (**5a**–**7a**) exhibited greater activity than the β-oriented CGs (**5b**–**7b**). This indicated that the inhibitory activity was driven by the orientation of the sugar moiety. At the same time, the slight differences observed in the α-oriented CGs can be attributed to the substituents on the sugar moiety. The *MeON*-neoglycosides and the lactone modified *O*-glycosides displayed poor activity compared to the non-modified *O*-glycoside.

The effect of CGs on potentiating CPT dependent cancer cell growth inhibition

Having established the effects on DDR signalling, we investigated if these synthesized compounds could further enhance the anticancer effect of CPT. For this purpose, we performed clonogenic survival assay in A549 cells in the presence of CPT with or without CG analogs treatments. The results show that AT2 enhanced the growth inhibitory effect of CPT (Figure 4C), which is consistent with our previous report.[4h] Compound **4b** did not enhance or reduce the cell growth compared with CPT alone. Compounds **6a** and **7a** only weakly enhanced the effect of CPT, but not statistically significant. However, compounds **4a** and **5a** further enhanced the growth inhibitory effect of CPT to a level similar to that of AT2 (Figure 4C). In conclusion, the α-oriented *O*-glycoside exhibited more cytotoxicity than the β-oriented glycosides or the lactone modified *O*-glycoside. Overall, the types and orientations of the sugar moiety are essential for manipulating efficiency of lung cancer chemotherapy treatment.

The evaluation of selective cytotoxicity of CGs in cancer cells

The clinical limitation and narrow therapeutic window of CGs is often related to cytotoxicity to normal tissues independent of whether treatment is in the context of cardiac disease or cancer.[19] Therefore, it is essential to balance the CGs' toxicity to normal cells with efficacy versus cancer cells. The analysis of cellular proliferation is an effective measure of these and can be used to compare relative impacts on cellular proliferation. We therefore assessed the novel CG analogs for toxicity to normal versus cancer cells in comparison to the parental AT2 compound. For this purpose, we first selected compounds **5a** and **6a** to investigate the effect of these single agents on the viability of a normal breast epithelial cell line, MCF10 A, by performing the cell viability assay. The results show that both **5a** and **6a** were 3—4 folds less toxic than the parental compound, AT2 (Figure 4D), indicating that these analogs are less toxic to normal cells than the parent compound.

Subsequently, we asked how these agents inhibit cancer cell proliferation. For this purpose, we selected CGs that both showed the capacity of inhibiting DDR closer to AT2 and the ones showing no effects on the DDR activation and evaluated the regeneration of A549 cells after the CG treatment. As shown in Figure 4E, the α-oriented *O*-glycosides (**5a**, **6a** and **7a**) exhibited strong cytotoxicity against A549 cancer cells with IC_{50} values that were nearly 3 orders of magnitude lower than in non-transformed MCF10 A cells, indicating that these CG analogs are more effective at killing cancer cells than normal cells. In addition, the β-oriented glycosides or the lactone modified *O*-glycoside (**6b** and **7b**) were much less toxic to A549 cancer cells (Figure 4E). Given that these α-oriented *O*-glycosides compounds similarly enhanced the anticancer effect of CPT as did by AT2, the toxicity differences between non-malignant and cancer cell lines suggest the potential to use these agents clinically to sensitize cancer cells to chemotherapy while reducing their toxicity to normal cells.

Conclusion

In summary, we have synthesized and evaluated the effects of novel AT2 and its derivatives on the DDR signaling. We further evaluated the chemotherapy enhancing effects of newly synthesized analogs in KRAS mutant lung cancer cells, as well as their toxicities in cancer cells and non-transformed epithelial cells. These results demonstrate the possibility of identifying novel CG analogs that retain the DDR inhibitory effect while showing reduced toxicity to normal cells.

Experimental Section

Chemistry

Materials and instrumentation. All reagents and chemicals were supplied from commercial sources and used without further purification. Reactions containing air-sensitive, and moisture sensitive chemicals were carried out under a nitrogen pressure. Product purification was performed on a Biotage isolera automated flash chromatography (Biotage OS 852 M Operating system) using pre-packed silica columns. ^{1}H-NMR and ^{13}C-NMR spectra were recorded on Bruker AV NMR spectrometer at 300 MHz and 75 MHz, respectively. NMR spectra were analyzed using MestReNova licensed by MetreLab Research. The mass was determined using the Agilent 1290 Infinity II LC – 6545 Q-ToF Mass spectrometers.

Synthetic procedure and characterization of AT2 derivatives

(3S,5S,8R,9S,10S,13R,14S,17R)-5,14-dihydroxy-13-methyl-3-(((2S,6R)-6-methyl-5-oxo-5,6-dihydro-2H-pyran-2-yl)oxy)-17-(5-oxo-2,5-dihydrofuran-3-yl)hexadecahydro-10H-cyclopenta[a]phenanthrene-10-carbaldehyde (4a). A solution of **3a** (152 mg, 0.67 mmol) and k-strophanthidin (270 mg, 0.67 mmol) in 4:1 DCM/THF (6 mL) was cooled to 0 °C under N_2 atmosphere. $Pd_2(dba)_3CHCl_3$ (17.3 mg, 0.02 mmol) and PPh_3 (17.3 mg, 0.02 mmol) were added, and the reaction was stirred for 24 h at 0 °C. The crude reaction mixture was concentrated and purified by using 5% CH_3OH in CH_2Cl_2. Yield 100 mg (39%) ^{1}H NMR (300 MHz, DMSO-d_6) δ 10.01 (s, 1H), 7.09–6.88 (m, 1H), 6.05 (d, J = 10.1 Hz, 1H), 5.42 (d, J = 4.2 Hz, 1H), 5.06–4.73 (m, 2H), 4.50 (q, J = 6.9 Hz, 1H), 4.27 (d, J = 2.0 Hz, 1H), 2.84–2.55 (m, 1H), 2.17–1.88 (m, 4H), 1.76 (d, J = 15.6 Hz, 2H), 1.59 (t, J = 11.1 Hz, 5H), 1.36 (s, 3H), 1.24 (d, J = 6.7 Hz, 4H), 1.09–0.91 (m, 1H), 0.72 (s, 3H). ^{13}C NMR (75 MHz, DMSO-d_6) δ 208.58, 196.85, 176.24, 173.89, 145.20, 126.42, 116.35, 91.61,

83.54, 73.86, 73.15, 72.81, 69.81, 56.05, 54.29, 49.87, 49.08, 36.52, 35.22, 31.29, 26.26, 24.50, 24.02, 23.99, 21.80, 18.58, 17.54, 15.49, 15.18. m/z = 537.2099 [M + Na] and 515.2257 [M + H].

(3S,5S,8R,9S,10S,13R,14S,17R)-5,14-dihydroxy-13-methyl-3-(((2R,6R)-6-methyl-5-oxo-5,6-dihydro-2H-pyran-2-yl)oxy)-17-(5-oxo-2,5-dihydrofuran-3-yl)hexadecahydro-10H-cyclopenta[a]phenanthrene-10-carbaldehyde (4b). The procedure is as described for **4a** above. Yield 170 mg (67%) ^{1}H NMR (300 MHz, DMSO-d_6) δ 10.02 (d, J = 1.3 Hz, 1H), 7.08–6.92 (m, 1H), 6.07 (dt, J = 10.2, 2.3 Hz, 1H), 5.50–5.29 (m, 1H), 5.08–4.82 (m, 2H), 4.61–4.44 (m, 1H), 4.27 (d, J = 1.3 Hz, 1H), 2.84–2.63 (m, 1H), 2.20–1.92 (m, 2H), 1.82–1.47 (m, 5H), 1.42–1.15 (m, 4H), 0.79–0.66 (m, 3H). ^{13}C NMR (75 MHz, DMSO-d_6) δ 208.58, 196.84, 176.23, 173.89, 145.20, 126.42, 116.34, 91.60, 83.54, 73.86, 73.15, 72.81, 69.81, 56.05, 54.29, 49.87, 49.08, 36.52, 35.22, 31.29, 26.26, 24.50, 24.01, 21.80, 18.58, 17.54, 15.49, 15.18. LC–MS: m/z = 537.2333 [M + Na].

4-((3S,5S,8R,9S,10R,13R,14S,17R)-5,14-dihydroxy-3-(((2S,6R)-5-hydroxy-6-methyl-5,6-dihydro-2H-pyran-2-yl)oxy)-10-(hydroxymethyl)-13-methylhexadecahydro-1H-cyclopenta[a]phenanthren-17-yl)furan-2(5H)-one (5a). A solution of $CeCl_3$ (83.4 mg, 0.2 mmol) in CH_3OH (1 mL) was added to a solution of **4a** (107 mg, 0.21 mmol) in CH_2Cl_2 (1 mL) and cooled to −78 °C. $NaBH_4$ (11.7 mg, 0.31 mmol) was added to the mixture and the reaction was stirred for 1 h at −78 °C. The crude reaction mixture was warmed to rt, diluted with ethyl acetate and quenched with saturated sodium bicarbonate. The organic layer was extracted with ethyl acetate, dried with anhydrous Na_2SO_4, and evaporated in *vacuo*. The crude reaction purified by using 5% CH_3OH in CH_2Cl_2. Yield 70 mg (70%). ^{1}H NMR (300 MHz, DMSO-d_6) δ 5.91 (d, J = 3.1 Hz, 1H), 5.56 (s, 1H), 5.28 (d, J = 4.9 Hz, 1H), 4.97–4.89 (m, 2H), 4.25 (d, J = 13.9 Hz, 1H), 4.11 (s, 1H), 4.00–3.80 (m, 1H), 2.73 (s, 2H), 2.01 (t, J = 13.7 Hz, 3H), 1.83–1.33 (m, 11H), 1.31–1.04 (m, 5H), 0.76 (s, 3H). ^{13}C NMR (75 MHz, DMSO-d_6) δ 176.45, 176.26, 173.91, 134.93, 126.73, 125.45, 116.32, 116.23, 93.58, 84.02, 83.70, 75.00, 74.67, 74.13, 73.17, 67.96, 67.87, 62.88, 54.94, 50.10, 49.35, 49.18, 42.86, 38.32, 38.17, 37.09, 31.87, 26.31, 25.36, 20.79, 18.19, 17.93, 15.75, 15.65. LC–MS: m/z = 541.2381 [M + Na].

4-((3S,5S,8R,9S,10R,13R,14S,17R)-5,14-dihydroxy-3-(((2S,6R)-5-hydroxy-6-methyltetrahydro-2H-pyran-2-yl)oxy)-10-(hydroxymethyl)-13-methylhexadecahydro-1H-cyclopenta[a]phenanthren-17-yl)furan-2(5H)-one (6a). A solution of **5a** (105 mg, 0.2 mmol) in NMM (1 mL) was cooled to 0 °C. IPNBSH (262 mg, 1.02 mmol) and Et_3N (45 μL) were added, and the reaction was stirred for 4 h at 0 °C. The reaction temperature was gradually increased to rt over 15 h. The crude reaction was diluted with ethyl acetate and quenched with saturated sodium bicarbonate. The organic layer was extracted with ethyl acetate, dried with anhydrous Na_2SO_4, and evaporated in *vacuo*. The crude reaction purified by using 5% CH_3OH in CH_2Cl_2. Yield 30 mg (60%). ^{1}H NMR (300 MHz, DMSO-d_6) δ 5.83 (d, J = 2.0 Hz, 1H), 5.12 (dd, J = 6.2, 2.5 Hz, 1H), 4.93 (d, J = 2.3 Hz, 1H), 4.10–4.00 (m, 2H), 3.80 (dd, J = 11.5, 2.8 Hz, 1H), 3.62–3.54 (m, 3H), 3.17 (dd, J = 5.3, 2.3 Hz, 1H), 2.73 (s, 2H), 2.02 (d, J = 9.5 Hz, 3H), 1.89–1.49 (m, 10H), 1.42–1.11 (m, 10H), 0.77 (d, J = 2.4 Hz, 3H). ^{13}C NMR (75 MHz, DMSO-d_6) δ 176.19, 173.78, 125.31, 116.26, 93.57, 83.96, 74.94, 74.76, 67.94, 62.93, 54.77, 50.11, 49.29, 42.77, 31.81, 26.29, 17.86, 15.69. LC–MS: m/z = 543.2588 [M + Na].

4-((3S,5S,8R,9S,10R,13R,14S,17R)-5,14-dihydroxy-3-(((2R,6R)-5-hydroxy-6-methyltetrahydro-2H-pyran-2-yl)oxy)-10-(hydroxymethyl)-13-methylhexadecahydro-1H-cyclopenta[a]phenanthren-17-yl)furan-2(5H)-one (6b). The procedure is as described for **6a** above. Yield 50 mg (75%) ^{1}H NMR (300 MHz, DMSO-d_6) δ 5.90 (d, J = 3.4 Hz, 1H), 5.36–5.20 (m, 1H), 5.03–4.79 (m, 2H), 4.11 (d, J = 2.2 Hz, 1H), 4.02–3.83 (m, 2H), 2.72 (s, 1H), 2.32 (d, J = 4.9 Hz, 4H), 2.11–1.92 (m, 2H), 1.78–1.46 (m, 5H), 1.44–1.17 (m, 7H), 1.07 (s, 1H), 0.76 (d, J = 2.6 Hz, 3H). ^{13}C NMR (75 MHz, DMSO-d_6) δ 176.19, 173.78, 125.31, 116.26, 93.57, 83.96, 74.94, 74.76, 67.94, 62.93, 54.77, 50.11, 49.29, 42.77, 31.81, 26.29, 17.86, 15.69. LC–MS: m/z = 543.2919 [M + Na].

4-((3S,5S,8R,9S,10R,13R,14S,17R)-5,14-dihydroxy-10-(hydroxymethyl)-13-methyl-3-(((2S,6R)-3,4,5-trihydroxy-6-methyltetrahydro-2H-pyran-2-yl)oxy)hexadecahydro-1H-cyclopenta[a]phenanthren-17-yl)furan-2(5H)-one (7a). NMO (92.5 mg, 0.79 mmol) dissolved in H_2O (0.5 mL) was added to a solution of **5a** (95.5 mg, 0.185 mmol) in 1:1 t-BuOH/Acetone (1 mL) was cooled to 0 °C. Crystalline OsO_4 (47.0 mg, 0.185 mmol) was added to the reaction mixture and the reaction was stirred for 4 h at 0 °C. The crude reaction was quenched with $Na_2S_2O_3$, extracted with ethyl acetate, dried with anhydrous Na_2SO_4, and evaporated in *vacuo*. Yield 72.5 mg (71%). ^{1}H NMR (300 MHz, DMSO-d_6) δ 5.92 (d, J = 1.8 Hz, 1H), 4.87 (s, 1H), 4.14 (ddt, J = 16.0, 8.8, 4.6 Hz, 3H), 3.84–3.76 (m, 1H), 3.75–3.54 (m, 4H), 3.47–3.33 (m, 1H), 2.86 (d, J = 8.0 Hz, 1H), 2.31–1.18 (m, 23H), 0.91 (s, 3H). ^{13}C NMR (75 MHz, DMSO-d_6) δ 174.15, 173.08, 113.76, 82.26, 74.60, 71.20, 64.65, 61.74, 57.41, 50.70, 47.76, 46.72, 39.52, 37.35, 36.98, 35.78, 33.83, 32.61, 28.89, 24.08, 23.80, 20.85, 18.57, 16.74, 15.95, 12.19, 10.35. LC–MS: m/z = 577.2420 [M + Na + 2H].

Methods for *in vitro* studies

Cell culture and reagents

A549 and MEF cells were cultured in DMEM with 10% FBS and 1% penicillin/streptomycin (HyClone). MCF-10A cells were cultured in DMEM: F12 (1:1) (HyClone, Thermo Fisher Scientific, Waltham, MA, USA) supplied with 1% penicillin/streptomycin (HyClone), 5% horse serum (HyClone), 0.1% insulin solution (Millipore/Sigma, St. Louis, MO, USA), 0.01% cholera toxin (Millipore/Sigma), 0.025% hydrocortisone (ACROS/Thermo Fisher) and 0.02% epidermal growth factor (Life Technologies/Invitrogen, Carlsbad, CA, USA). Antibodies used in this study are as follows: PARP (#9542S) and pChk1 (Ser-345, #2348S) were purchased from Cell Signaling Technology (Beverly, MA, USA). Anti-human LC3B (#NB100-2220) antibody was obtained from Novus Biologicals (Centennial, CO, USA). Anti-ATR (#SC-1887), anti-UHRF1 (#SC-373750) and anti-Chk1 (#SC-56291) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-GAPDH (#60004) was from Proteintech (Rosemont, IL, USA). HRP conjugated secondary antibodies were purchased from Thermo/Invitrogen. DMSO (D8418) was purchased from Millipore/Sigma (St. Louis, MO, USA). Thiazolyl blue tetrazolium bromide (MTT) was purchased from BioTechne/Tocris (#5224). Camptothecin (CPT, 276721000) was purchased from Acros organics (Geel, Belgium).

Western blotting

Western blotting was performed as previously described with modifications.[20] Cells were harvested by trypsinization, followed by centrifugation at 800 g for 5 min in a tabletop centrifuge. Cell pellets were washed with PBS once and lysed in NP-40 lysis buffer (100 mM Tris-HCl, pH 7.6, 150 mM NaCl, 1% NP-40, and protease and phosphatase inhibitors including 1 mM Na_3VO_4, 1 mM PMSF, 1 mM DTT, 10 μg/mL aprotinin, 1 μg/mL leupeptin, 1 μg/mL pepstatin A, and 10 mM sodium fluoride) on ice for 30 min. Structure-bound proteins were extracted by sonication using three pulses, each 10 s on/off at 1% amplitude. Following sonication, samples were centrifuged at 13,000 g for 10 min at 4 °C. The protein concentration was determined by the Pierce® BCA Protein Assay Kit (Pierce, #23225). Equal amounts of total proteins (~50 μg) were

boiled with SDS sample buffer for 5 min, ran on a gradient (6%–20%) SDS-polyacrylamide gel and transferred to PVDF membranes. The membranes were blocked in 5% BSA in 1X TBST for 1 h, washed six times with 1X TBST for 5 min, incubated with primary antibodies (1:1000) at 4 °C overnight, washed in 1× TBST for 8 min 5 times, and incubated in secondary antibodies conjugated with HRP in 1× TBST for 1 h at room temperature. The membranes were washed in 1× TBST for 8 min 5 times, reacted with ECL solution and visualized by the Tanon 5200 Imager system (Tanon, Shanghai, China).

Cell viability assay (MTT assay)

In addition, the cytotoxicity of compounds on cell proliferation was also determined by MTT assay on cancerous and non-cancerous cell lines, A549 and MCF10 A, respectively. Briefly, 5×10^3 cells/well were seeded in 96-well plates, grown at 37 °C for 12 h, and treated with increasing dosages of CGs for 12 h. Then 10 μL MTT solution (5 mg/mL) was added to each well, incubated at 37 °C for 3 h. After carefully removing the media with needle, 100 μL of DMSO was added to each well and incubate at RT for 15 minutes. The absorbance was determined at 570 nm from 5 replicates using a microplate reader (Synergy TM HT, BioTEK, USA). Absorption in the blank well was subtracted and that in the DMSO control was set as 100%, and others were normalized accordingly. The IC50 was calculated by the GraphPad Prism program.

Clonogenic survival assay

The relatively long-term cytotoxicity of compounds on A549 cells was determined as previously reported.[22] Briefly, 5000 A549 cells were plated into 6-well plates in triplicate, treated with DMSO or 200 nM selected CGs for 2 h, added 200 nM CPT for another 6 h, washed and cultured in drug-free full media for 10–14 days or until cell colonies were clearly visible. Cells were washed with phosphate buffered saline, fixed (acetic acid-methanol solution, 1:7, *vol*/*vol*) at room temperature for 5 min, and stained with 0.1% crystal violet in methanol for 15 min at room temperature in a hood, the plates were then gently rinsed under tap water, and placed upside down to allow air drying. The surviving colonies will be dissolved in 1% SDS and the absorbance at 570 nm will be measured by a microplate reader (Synergy TM HT, BioTEK, USA).

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Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available in the supplementary material of this article.

Keywords: Cardiac glycosides · DNA damage response · Chk1 phosphorylation · Anticancer agents

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