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The fungal natural product (1*S*, 3*S*)-austrocortirubin induces DNA damage in HCT116 cells via a mechanism unique from other DNA damaging agents

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ABSTRACT

Screening a series of natural product-based tetrahydroanthraquinones led to the identification of a novel molecule, (1*S*,3*S*)-austrocortirubin (**2**), which acts via inducing DNA damage. Compound **2** has a GI₅₀ of 3 μM against HCT116 and induces apoptosis. Mechanism of action studies indicate that it causes significant DNA damage during G0/G1, S, and G2 cell cycle phases. Cells are stopped at the G2/M phase checkpoint, and do not reach mitosis due to large amounts of DNA damage. Thus, compound **2** exhibits a unique mechanism of action, one that is distinct from doxorubicin, despite the high degree of structural homology between these two quinone-based structures.

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Natural product scaffolds are present in over 50% of pharmaceutical drugs.¹⁻³ Natural product-derived molecules have been extensively studied for their biological activity against cancer,⁴⁻¹² making them excellent lead structures in the development of new therapeutic compounds. Doxorubicin (Dox) is an anthracycline natural product (Figure 1) that acts via a well understood mechanism of action whereby it inhibits DNA synthesis by intercalating into double-stranded DNA.^{13, 14} The limited number of rotatable bonds in the multi-ring system seen in anthracycline molecules makes Dox and its derivatives structurally rigid, and provides a flat surface capable of intercalating into DNA via π -stacking. Dox stabilizes the DNA-topoisomerase II complex, which prevents DNA transcription and replication and causes DNA damage.^{15, 16} This mechanism of action generally causes the most impact on the G2 phase of the cell cycle. As a chemotherapeutic antibiotic, Dox is the drug of choice for treatment of leukemia and solid tumours.¹⁷

Taxanes such as paclitaxel (PAC) and docetaxel are microtubule stabilizers that are utilized in the clinic (Figure 1) and they play an important role in chemotherapy regimens. Microtubules are important cytoskeleton components as they maintain the cell shape and polarity, while regulating cell signalling and mitosis.¹⁸ Microtubules are made up from α - and β -tubulin, which form non-covalent polymers that produce cylindrical microtubule structures. During the mitosis stage of cell division (M-phase), formation of the mitotic spindle, which

is composed of microtubules, enables the transport of daughter chromosomes into the dividing cells. As a microtubule stabilizer, PAC has the highest impact on M-phase of the cell cycle as it induces polymerization of tubulin into microtubules, stabilizes their structure and inhibits de-polymerization back into tubulin or forward into the mitotic spindle.¹⁹ Another well established drug that impacts the cell cycle via binding to DNA is cisplatin (CIS)²⁰ (Figure 1). CIS is used to treat more than 50% of cancer patients, making it the most widely used oncological therapy.²¹⁻²⁵ The side-effect profile and resistance development to CIS has driven the development of new therapies. Although CIS is not considered to be highly selective,^{20, 22, 26, 27} it is believed to perform most of its cellular damage during S-phase of the cell cycle, i.e., the time during which the cell synthesizes DNA.

Figure 1. Structures of doxorubicin (Dox), paclitaxel (PAC), and cisplatin (CIS).

Herein we describe the cytotoxicity and mechanism of action of fungal natural product analogues that all belong to the

austrocortirubin structural class (Figures 2).²⁸ Compounds **1–15** where obtained from the Davis open-access library housed at Compounds Australia, Griffith University.³³ The compounds (1*S*,3*R*)-austrocortirubin (**1**),²⁹ (1*S*,3*S*)-austrocortirubin (**2**),^{30, 31} 1-deoxyaustrocortirubin (**3**),^{30, 31} and austrocortinin (**4**)³² are all known fungal natural products. In previously published work by Davis *et al.*, compound **2** was used as a natural product scaffold in the parallel solution-phase synthesis of a small library of N-substituted tetrahydroanthraquinones (**5–15**).²⁸ In the current studies, screening of this unique library²⁸ identified that compounds **2**, **9**, and **11** were cytotoxic at low micromolar concentrations and induced DNA damage in the human colon cancer cell line, HCT116. We then showed that compound **2**, the most potent member of the series, behaves similarly to Dox by arresting cells at G2/M phase, however it shows similarities to PAC and CIS as well. Thus, compound **2** appears to have a primary mechanism of action that is distinct from all three chemotherapeutics (Dox, PAC, and CIS).

Figure 2. Cytotoxicity of compounds **1–15** at 50 μ M, compared to DMSO treatment (vehicle control).

Testing the cytotoxicity of the 15 membered library against HCT116 cells indicated that compounds **1**, **2**, **3**, **9** and **11** were the most effective in killing cancer cells at the concentration of 50 μ M (Figure 2). Analysis of the SAR showed that incorporation of long alkyl side chains on the benzoquinone (compounds **5–8**) produced essentially inactive molecules (Figure 2). Attaching an aromatic side chain to an extended alkyl

side chain improved activity (compounds **9** and **11**). However, the nature of the aromatic group was critical, where the inclusion of a hydroxyl moiety on the side chain phenyl (**10** versus **9**) led to reduced activity, whereas the inclusion of a chlorine atom increased activity (**11**). The length of the side chain was also important for cytotoxicity, where the deletion of a single carbon significantly decreases activity (**13** versus **9**). Attaching a morpholine unit in place of the aromatic ring system also decreased cytotoxicity of the molecule (**12**), as did the incorporation of a pyrrolidine (**15**). Surprisingly replacement of the methyl ether on **2** with an N-methyl moiety (**14**) led to significantly reduced cytotoxicity. Inversion of the quaternary centre (**1**), removal of the secondary hydroxyl (**3**) or aromatization of the cyclohexyl moiety (**4**) all decreased cytotoxicity relative to compound **2** (Figure 2 and Table 1). These data indicate that not only is the incorporation of an aromatic side chain important, but appropriate stereochemistry of both alcohols on the cyclohexyl ring is also critical.

Examination of the GI_{50} values for compounds **1**, **2**, **3**, **9**, and **11**, showed that the most potent compounds were **2**, **9** and **11**, with GI_{50} = 4 μ M, 16 μ M, and 10 μ M respectively (Table 1). Compounds **1** and **3** had GI_{50} values above 20 μ M (supplementary data).

Compounds	GI_{50} in HCT116 cells
Doxorubicin	19.9 $\pm$ 1.9 nM
Paclitaxel	2.7 $\pm$ 0.2 nM
Cisplatin	1.5 $\pm$ 0.5 μ M
2	3.7 $\pm$ 0.6 μ M
9	16.0 $\pm$ 2.0 μ M
11	10.1 $\pm$ 2.2 μ M

Table 1. GI_{50} values of doxorubicin, paclitaxel, cisplatin, compounds **2**, **9**, and **11** against HCT116 human colon cancer cell line. Experiments were performed in triplicate, and the average GI_{50} values are shown with the standard deviation.

Since compound **2** had the lowest GI_{50} we compared its mechanistic behavior to Dox, because of its similar core structure, and to PAC and CIS, because their mechanisms of action are well established. Evaluation of **2** in an Annexin V-based apoptosis assays showed that 15 μ M of compound **2** was highly effective at inducing apoptosis (Figure 3). Indeed, 75% of treated HCT116 cells were in early apoptosis stage within 48 hrs of treatment. These data indicate that compound **2**, similar to Dox and PAC, is inducing cancer cell death via apoptotic pathways.

Treatment of HCT116 cells with drug at 5–10 fold over their GI_{50} values and evaluation of their impact on cell cycle distribution produced interesting results (Figure 4A). Since Dox's primary mechanism is via inhibition of protein synthesis by blocking transcription, it is expected to trap cells in the G2/M phase. As shown, Dox produced a dramatic decrease in cells with S phase, and a large increase in cells that were in G2/M phase compared to DMSO treatment (vehicle control). In contrast, treatment with PAC produced an increase in the number of cells in both S and G2/M phase relative to the control. Compound **2**, similar to PAC, induced a significant increase of cells in the S and G2/M phase compared to DMSO. Interestingly, despite being reported as acting primarily on S-phase cells, CIS increased the number of cells in G2/M phase but not in S-phase. These data suggest that cells treated with compound **2** make it through the S-phase checkpoint. The large increase in S phase seen when cells are treated with **2** suggests that it is targeting DNA synthesis and causing significant DNA damage. Trapping

cells in the G2/M phase indicates that compound **2** is either damaging the protein synthesis mechanism of the cell (perhaps by inducing DNA damage) or trapping cells in mitosis, like PAC.

Figure 3. Annexin V-based apoptosis analysis. After indicated treatments HCT116 cells were stained with Annexin V-FITC/7AAD and analysed by flow cytometry for apoptosis. All values presented are averages from at least three independent experiments.

Measuring cell cycle status does not indicate whether the compounds are inducing DNA damage. Thus, evaluating DNA damage induced by each molecule at each phase of the cell cycle provides valuable mechanistic information (Figure 4B). Staining cells with the DNA damage marker γ H2AX provides evidence of cells that have DNA damage associated with them (γ H2AX+) versus no damage (γ H2AX-) (Figure 4B). Compound **2** induced DNA damage at all phases of the cell cycle: G0/G1, S, and G2/M phase. Perhaps not surprising the highest amount of DNA damage seen upon treatment with **2** was in S phase, with over 90% of cells indicating DNA damage in this phase, which is similar to the effect of Dox treatment. These data strongly support the hypothesis that **2** intercalates DNA, causing damage. Evaluation of DNA damage caused by treatment of **2** at the G2/M phase showed that over 50% of cells had DNA damage, similar to Dox. By comparison, Dox showed ~ 50% of cells had DNA damage in both S-phase and G2/M phase, but essentially no DNA damage during G0/G1 phase. As expected, PAC demonstrated little DNA damage as its primary mechanism involves stabilizing cells in mitosis. Finally, CIS showed the most significant DNA damage effect during G0/G1 phase, however, as is established by others, CIS is not cell cycle selective. The significant amount of DNA damage induced when compound **2** is exposed to cells strongly suggests it's mechanism is different from that of Dox, and involves producing DNA damage rather than inhibiting protein synthesis.

Figure 4. A) Cell cycle analysis. After indicated treatments HCT116 cells were stained with PI for cell cycle analysis by flow cytometry. The percentages of cells in the G0/G1, S, and G2/M phases of cell cycle are indicated in bar graphs. All values presented are average $\pm$ SEM of at least three independent experiments. B) DNA damage analysis. Treatments with compound **2**, PAC, or Dox under indicated concentrations for 24 h caused DNA damage in HCT116 cells. Treated cells were stained with DNA damage marker γ -H2AX and PI, and then analysed by flow cytometry. Data presented are averages of at least three independent experiments. C) G2/M DNA damage checkpoint activation analysis. After indicated treatments, HCT116 cells were stained with Histone H3 (phospho, Ser-10)/PI and analysed by flow cytometry. All values presented are average $\pm$ SEM of at least three

independent experiments. Differences between indicated data and DMSO control are represented with *P* values (*, *P* < 0.05; **, *P* < 0.01; ***, *P* < 0.005; and §, *P* < 0.001).

Since measuring the G2/M phase does not differentiate between compounds that inhibit protein synthesis by blocking transcription (i.e. Dox) versus compounds that stabilize microtubules and trap compounds in M phase (i.e. PAC), we evaluated the mitotic index of all the compounds (Figure 4C).

Analysis of the G2/M DNA damage checkpoint activation provided the ratio of cells undergoing mitosis, where the mitotic index is calculated from the number of cells undergoing mitosis versus the total number of cells. PAC gave the anticipated results, where it is well known to trap cells at M-phase. Indeed, 24 hrs of PAC treatment led to a 3-fold increase in HCT116 cells undergoing mitosis compared to DMSO-treated cells (Figure 4C). In contrast Dox and compound **2** treatments (24 hrs) produced no cells in M phase, indicating that in the earlier analysis (Figure 4A), all the G2/M phase cells were arrested in G2 phase and essentially none were in M-phase. As seen in our data above, CIS traps the cells in M phase, showing no selectivity for a specific cell cycle phase. Our data indicate that similar to Dox treatment, when HCT116 cells are treated with compound **2**, they are too damaged to continue past the G2/M checkpoint (Figure 5). The G2/M checkpoint analyzes cells for their size and DNA replication and stops cells that are not ready for cell division. Extensive damage to DNA and proteins will drive cells into apoptosis. Taken together the DNA damage and the G2/M checkpoint activation analysis indicate that compound **2** exhibits a new mechanism, whereby it causes S/G2 phase cell cycle arrest, with complete mitosis inhibition, and extensive DNA damage in all three other cell cycle phases (G0/G1, S, and G2 phases). The extensive DNA damage observed in S-phase when treating cells with **2**, and the significant DNA damage seen in G0/G1 and G2 phase, point to **2** having a mechanism that is distinct from the three well established DNA damaging chemotherapeutics used as positive controls.

Figure 5. Cell cycle phases, where cisplatin acts primarily in the S-phase via DNA damage, Dox acts in G2 phase, and paclitaxel acts in M phase. Compound **2** causes DNA damage in G0/G1, S, and G2 phase, and like cisplatin, its primary impact is in S phase.

In conclusion, screening a new series of natural product-based tetrahydroanthracenes led to the identification of a novel molecule that acts via inducing DNA damage. We have shown that adding short side chains to the benzoquinone increases cytotoxicity, so long as the two hydroxyl moieties are incorporated with the appropriate stereochemistry on the cyclohexyl ring. Elimination of both or even a single hydroxyl,

or inversion of stereochemistry of the tertiary hydroxyl eliminates biological activity. Interestingly a 3-atom linker with a phenyl or *para* chloro phenyl moiety also can give enhanced cytotoxicity, although this activity was linker and aromatic group specific. Investigation of the lead compound's mechanism of action (compound **2**) in cancer treatment showed that this molecule acted by inducing significant DNA damage during G0/G1, S, and G2 cell cycle phases, and cancer cells were halted before reaching the M phase. Our data indicate that this molecule has a unique mechanism of action, one that is distinct from Dox, PAC, or CIS despite the similar tetrahydroanthracene structure found in Dox, and the potential DNA damaging properties reported for PAC or CIS. Given our preliminary data, further investigation of this compound may provide a mechanistically novel chemotherapeutic.

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Abbreviations

DMSO, dimethyl sulfoxide; GI₅₀, inhibitory concentration (50%), Dox, Doxorubicin; PAC, paclitaxel; CIS, cisplatin

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Supporting information

Supporting information includes experimental details for biological assays and imaging experiments. This material is available free of charge at <http://pubs.bmcl.org>.

References and notes

- Kingston, D. G. I. *J. Nat. Prod.* **2010**, *74*, 496.
- Lachance, H. *J. Med. Chem.* **2012**, *55*, 5989.
- Cragg, G. M.; Newman, D. J. *Biochimica et Biophysica Acta- General Subjects* **2013**, *1830*, 3670.
- Blunt, J. W.; Copp, B. R.; Keyzers, R. A.; Munro, M. G. H.; Prinsep, M. R. *Nat. Prod. Rep.* **2014**, *31*, 160.
- Evidente, A.; Kornienko, A.; Cimmino, A.; Andolfi, A.; Lefranc, F.; Mathieu, V.; Kiss, R. *Nat. Prod. Rep.* **2014**, *31*, 617.
- Ashley, R. E.; Osheroff, N. *Chem. Res. Toxicol.* **2014**, *27*, 787.
- Otrubova, K.; McGuire, K. L.; McAlpine, S. R. *J. Med. Chem.* **2007**, *50*, 1999.
- Lin, C.-C.; Tantisantisom, W.; McAlpine, S. R. *Org. Lett.* **2013**, *15*, 3574.
- Singh, E. K.; Ravula, S.; Pan, C.-M.; Pan, P. S.; Vasko, R. C.; Lapera, S. A.; Weerasinghe, S. V. W.; Pflum, M. K. H.; McAlpine, S. R. *Bioorg. Med. Chem. Lett.* **2008**, *18*, 2549.
- Singh, E.; Ramsey, D. M.; McAlpine, S. R. *Org. Lett.* **2012**, *14*, 1198.
- Ardi, V. C.; Alexander, L. D.; Johnson, V. A.; McAlpine, S. R. *ACS Chem. Biol.* **2011**, *6*, 1357.
- McConnell, J. M.; Alexander, L. D.; McAlpine, S. R. *Bioorg. Med. Chem. Lett.* **2014**, *24*, 661.
- Crooke, S. T.; Duvernay, V. H.; Galvan, L. P., A. *Mol. Pharmacol.* **1973**, *14*, 290.
- Gabbay, E. J.; Grier, D.; Fingerle, R. E.; Reimer, R.; Levey, R.; Pearce, S. W.; Wilson, W. *Biochemistry* **1976**, *15*, 2062.
- Fornari, F. A.; Randolph, J. K.; Yalowich, J. C.; Ritke, M. K.; Gewirtz, D. A. *Mol. Pharmacol.* **1994**, *45*, 649.

16. Momparler, R. L.; Karon, M.; Siegel, S. E.; Avila, F. *Cancer Res.* **1976**, *36*, 2891.
17. Blum, R. H.; Carter, S. K. *Amer. J. Intern. Med.* **1979**, *80*, 249.
18. Perez, E. A. *Mol. Cancer Ther.* **2009**, *8*, 2086.
19. Rohena, C. C.; Mooberry, S. L. *Nat. Prod. Rep.* **2014**, *31*, 335.
20. Rosenberg, B.; Vancamp, L.; Trosko, J. E.; Mansour, V. H. *Nature* **1969**, *5191*, 385.
21. Jakupec, M. A.; Glanaski, M.; Arion, V. B.; Hartinger, C. G.; Keppler, B. K. *Dalton Trans.* **2008**, 183.
22. Strodal, B.; Davey, M. *Life* **2007**, *11*, 696.
23. Siegel, R.; Naishadham, D.; Jemal, A. A *Cancer Journal for Clinicians* **2012**, *62*, 10.
24. Allardyce, C. S.; Dyson, P. J. *Platinum Metals Rev.* **2001**, *45*, 62.
25. Ang, W. H.; Daldini, E.; Scolaro, C.; Scopelliti, R.; Juillerat-Jeannerat, L.; Dyson, P. J. *Inorg. Chem.* **2006**, *45*, 9006.
26. Gasser, G.; Ott, I.; Metzler-Nolte, N. *J. Med. Chem.* **2011**, *54*, 3.
27. Demarcq, C.; Bunch, T. R.; Creswell, D.; Eastman, A. *Cell Growth Differ.* **1994**, *5*, 983.
28. Choomuenwai, V.; Andrews, K. T.; Davis, R. A. *Bioorg. Med. Chem. Lett.* **2012**, *20*, 7167.
29. Elsworth, C.; Gill, M.; Ten, A. *Aust. J. Chem.* **1999**, *52*, 1115.
30. Gill, M.; Smrdel, A. F.; Strauch, R. J.; Begley, M. J. *J. Chem. Soc., Perkin Trans* **1990**, *1*, 1583.
31. Gill, M.; Steglich, W. *Prof. Chem. Org. Nat. Prod.* **1987**, *51*, 1.
32. Archard, M. A.; Gill, M.; Strauch, R. J. *Phytochemistry* **1985**, *24*, 2755.
33. Simpson, M.; Poulsen, S.-A. *ACS Chem. Biol.* **2014**, *9*, 28.