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Synthesis, reactive oxygen species generation and copper-mediated nuclease activity profiles of 2-aryl-3-amino-1,4-naphthoquinones

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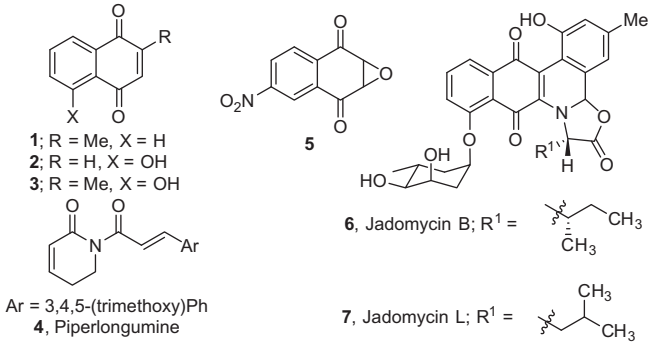
ABSTRACT

Here we report a series of 2-aryl-3-amino-1,4-naphthoquinones that generated reactive oxygen species (ROS) such as superoxide and hydrogen peroxide upon incubation in pH 7.4 under ambient aerobic conditions. ROS generation from these compounds was sensitive to structural modifications at the 3-amino position and a 2-aryl substituent promoted ROS generation. A number of these compounds were found to induce DNA damage in the presence of Cu(II) without any added reducing agent. Our data suggests that 2-aryl-3-amino-1,4-naphthoquinones' propensity to produce ROS correlated well with its DNA damage inducing ability. 2-Phenyl-3-pyrrolid-1-yl-1,4-naphthoquinone (**22**) was found to damage DNA at 1 μM suggesting that these compounds may have therapeutic relevance in targeting cancers which overexpress Cu(II)

Reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2), superoxide (O_2^-) and hydroxyl radical (·OH) are generated during immune response to combat pathogens.[1–5] Together, these reactive species cause extensive damage to biomacromolecules such as lipids, DNA and proteins.[1–5] Certain cancers have been shown to be sensitive to the toxic effects of ROS suggesting that compounds that generate ROS may have therapeutic potential.[2,6–8] For example, 1,4-naphthoquinones such as menadione (**1**), juglone (**2**) and plumbagin (**3**) are known to generate ROS through redox cycling; menadione has shown potent anti-proliferative activity against various cancers including breast and bladder cancers.[9–14]



Piperlongumine (**4**), a small molecule ROS generator was found to selectively kill cancer cells and not normal cells.[15] Recently, we reported that 2,3-epoxy-1,4-naphthoquinones such as **5** were glutathione-activated sources of ROS;[16] compounds which were better generators of ROS were superior inhibitors of human leukaemia THP1 cell proliferation. Thus, development of new ROS generators is of therapeutic interest. The jadomycins are a family of 2-aryl-3-amino-1,4-naphthoquinone-based natural products produced by the soil microbe *Streptomyces venezuelae* ISP5230 which have anti-bacterial, anti-cancer, and anti-viral activities.[17–39] Recently, several members of the jadomycin family were reported to induce DNA damage, a biomarker for ROS, under diverse conditions.[39] For example, **6** cleaved supercoiled plasmid DNA in the presence of $Cu(OAc)_2$ without the requirement of an added reducing agent[27,40] and nuclease activity **6** was significantly inhibited in the presence of scavengers of ROS suggesting the intermediacy of ROS. We proposed 2-aryl-3-amino-1,4-naphthoquinones as candidates for ROS generation; systematic modification of both the amine and the aryl substituent could help tune ROS generating properties (Fig. 1).

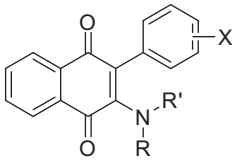


Figure 1. Proposed 2-aryl-3-amino-1,4-naphthoquinone scaffold for ROS generators and Cu(II)-mediated DNA cleaving agents.

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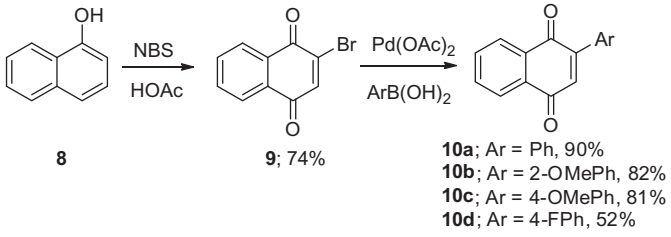
Development of new Cu(II)-mediated DNA damaging agents is of potential therapeutic interest as clinical studies show that several cancers including breast cancers are associated with elevated levels of copper in comparison with their normal counterparts.[41–49] Jadomycin B (**6**) was found to inhibit proliferation of two breast ductal carcinoma;[21,37] other examples of compounds that display Cu(II)-mediated DNA cleavage activities are prodigiosin and tambjamine E both natural products with potent anti-tumour activity.[50–53] Here, we synthesized and evaluated ROS generation and copper-mediated nuclease activity of a series of 2-aryl-3-amino-1,4-naphthoquinones.

2-Aryl-3-amino-1,4-naphthoquinones can be obtained in two steps from 2-bromo-1,4-naphtoquinone (**9**), which in turn was prepared from 1-naphthol (**8**) and *N*-bromosuccinimide in acetic acid in 74% yield (Scheme 1). Pd(II)-catalyzed Suzuki coupling with phenylboronic acid produced **10a** in 90% yield (Scheme 1).[54–56]

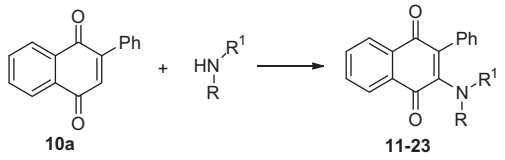
Addition of ammonia to **10a** produced compound **11** in 50% yield.[57] The ^{n}propylamine, ^{n}butylamine, and *N*,*N*-dimethylaminoethylamine derivatives **12**–**14** were prepared in yields ranging from 64% to 78% (Table 1, entries 2–4).

The allylamine and benzylamine derivatives **15** and **16** were isolated in 60% and 80% yield, respectively (Table 1, entries 5 and 6). In order to test the effect of modulating electronics of the aryl ring of benzylamine, compounds **17** and **18** were synthesized (Table 1, entries 7 and 8). Next, aniline derivatives **19**–**21** were prepared in 25–58% yields (Table 1, entries 9–11).[58] Addition of secondary amines pyrrolidine and piperidine gave **22** and **23** in 77 and 86% yields, respectively (Table 1, entries 12 and 13).



Scheme 1. Synthesis of **9** and **10a–d**.

Table 1
Synthesis of **11–27**



Entry	Ar	R	R^1	Product	Yield
1	Ph	H	H[a]	**11**	50
2	Ph	H	^{n}Pr[a]	**12**	64
3	Ph	H	^{n}Bu[a]	**13**	78
4	Ph	H	$CH_2CH_2NMe_2$[a]	**14**	72
5	Ph	H	Allyl[a]	**15**	60
6	Ph	H	Bn[a]	**16**	80
7	Ph	H	(4-OMePh)CH_2[a]	**17**	81
8	Ph	H	(4-Cl)$PhCH_2$[a]	**18**	63
9	Ph	H	Ph[b]	**19**	53
10	Ph	H	4-OMePh[b]	**20**	58
11	Ph	H	4-NO_2Ph[b]	**21**	25
12	Ph		R^1RNH = Pyrrolidine[a]	**22**	77
13	Ph		R^1RNH = Piperidine[a]	**23**	86
14	2-OMePh		R^1RNH = Pyrrolidine[a]	**24**	33
15	4-OMePh		R^1RNH = Pyrrolidine[a]	**25**	57
16	4-FPh		R^1RNH = Pyrrolidine[a]	**26**	67
17	H		R^1RNH = Pyrrolidine[a]	**27**	68

[a] Reaction was conducted in dioxane at rt.
[b] Reaction was conducted in acetic acid and water at 90 °C.

Next, we evaluated **11**–**23** for their ability to generate hydrogen peroxide using a reported xylenol orange-based colorimetric method for the estimation of hydrogen peroxide.[59–62] All compounds were found to generate H_2O_2 after incubation for 1 h but to varying extents (Fig. 2). Compounds **11**–**21** produced <2 μM H_2O_2 during 1 h with no clear relationship between peroxide yield and electron donating versus electron withdrawing substituents (Fig. 2). However, amongst the compounds tested, the pyrrolidine derivative **22** produced the highest amount of peroxide (7.6% yield) in this time period; while the yield of H_2O_2 from piperidine **23** was diminished (~2%). An independent fluorescence assay using Amplex Red/horseradish peroxidase was conducted and we found a comparable amount of H_2O_2 was generated in the case of **22** (1.45 μM, 6% yield). The formation of hydrogen peroxide was also inferred by the use of catalase, an enzyme that catalyzes the decomposition of hydrogen peroxide. Incubation of **22** in pH 7.4 after 1 h followed by treatment of this reaction mixture with catalase showed complete disappearance of color attributable to H_2O_2 in the xylenol orange assay.

Having identified that a 3-pyrollidin-1-yl substituent resulted in the most efficient H_2O_2 generation, we proposed to study the effect of changing the electronics around the 2-aryl ring on hydrogen peroxide generation, compounds **10b**–**10d** were prepared using a reported methodology (Scheme 1). Compounds **24**–**26** were prepared by addition of pyrrolidine to **10b–10d** (Table 1, entries 14–16). The analogue **27** was prepared by the reaction of 1,4-naphthoquinone and pyrrolidine in 68% yield (Table 1, entry 17). Hydrogen peroxide yields from **24** to **26** during 1 h were comparable with that of **22** (Fig. 2). However, yield of H_2O_2 was diminished in the case of **27** suggesting that an aryl substituent was necessary for generation of ROS (Fig. 2).

1,5-Dihydroflavins are examples of compounds that can spontaneously react with oxygen to generate hydrogen peroxide (Scheme 2).[63–65] Molecular orbital calculations suggest that the lone pair on the nitrogen bearing the ethyl group is of higher energy and hence susceptible for electron transfer to oxygen to generate superoxide, which can then further react to form hydrogen peroxide.

When **22** was incubated in pH 7.4 buffer, we found evidence for superoxide generation using a luminol-based chemiluminescence assay (Fig. 3);[66] luminescence due to superoxide was completely quenched in the presence of superoxide dismutase, an enzyme that catalyzes the decomposition of superoxide. A similar assay was conducted for selected analogues of **22**; analogues that generated higher amounts of H_2O_2 also produced higher amounts of superoxide (Fig. 3).

Pyrrolidine and *N*-methylpyrrolidine were incubated under similar conditions and no evidence for the generation of superoxide and/or hydrogen peroxide was found suggesting that the adjacent quinone was necessary for ROS generation. Based on these results, we proposed the following mechanism (Scheme 3). Transfer of an electron from **22** to oxygen produces the radical cation **22a** with resonance forms **22b** and **22c**. We inferred that the

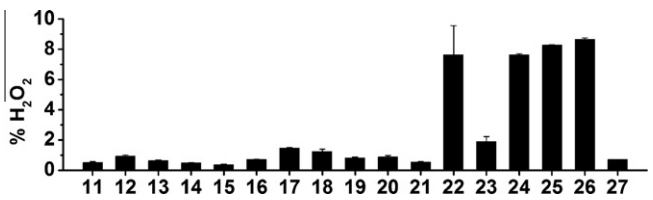
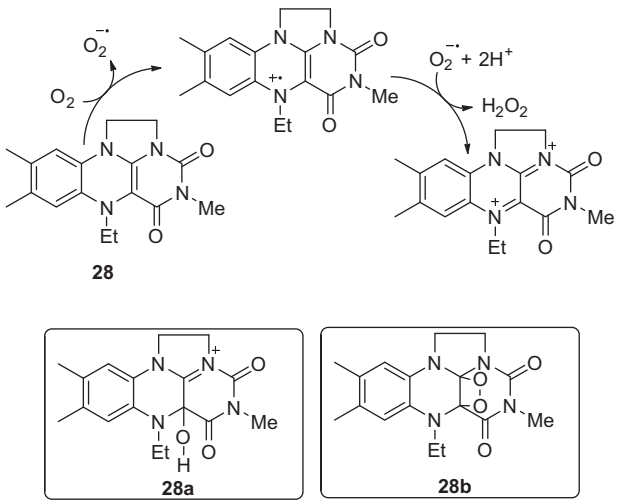


Figure 2. Hydrogen peroxide yields of **11**–**27** during incubation in pH 7.4 buffer as determined by a xylenol-orange based colorimetric assay.



Scheme 2. Reported mechanism of superoxide and hydrogen peroxide generation from 1,5-dihydroflavins.

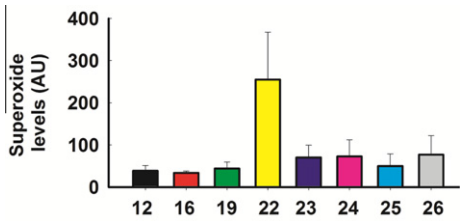


Figure 3. Superoxide yields during incubation of selected compounds in pH 7.4 buffer as determined by a luminol-based chemiluminescence assay.

carbon radical **22b** was an important contributor as removal of a stabilizing aryl ring (as in **27**) resulted in diminution of ROS generation (Fig. 2). Modifications to the aryl ring did not significantly affect ROS generation (**22**, **24**–**26**, Fig. 2); this observation can be rationalized as no significant change in the electronic absorption amongst these pyrrolidine analogues suggesting similar conjugation of the aryl ring with the pyrrolidin-1-yl group (λ_{max} = 490–493 nm). LC/MS (ESI) analysis of **22** incubated in pH 7.4 for 1 h showed the formation of a new product with an *m*/*z* of 322.14, which corresponds to the addition of an 'OH' group, perhaps **22e**, which could be generated from **22d** (Scheme 3). The alcohol **22e** is similar to **28a**, which was previously reported as a product of decomposition of **28** (Scheme 2).[63–65] Upon incubation for 6 h, LC/MS analysis showed complete disappearance of **22** with additional products with *m*/*z* of 358.05 (consistent with **22g**) and 343.11 (consistent with **22h**). Again, these intermediates are comparable with reported compounds **28b** and **28a**, respectively generated during decomposition of **28** (Scheme 3).[63–65] Our data suggests that ROS generation by 2-aryl-3-amino-1,4-naphthoquinones was sensitive to structural modifications to the amine: a secondary amine generated higher amounts of ROS in comparison with a primary amine. However, this observation is consistent with reported structure-activity relationship studies of jadomycins, which show that the identity of the substituent adjacent to the tertiary amine played an important role in determining biological activity.[37]

Next, we evaluated the DNA damage inducing ability of selected compounds **12**, **16**, **19**, **22**–**26** using a reported pBR322 supercoiled plasmid DNA-based assay (see Supplementary data).[27,67] None of the compounds tested showed detectable levels of DNA damage in the absence of Cu(II). However, in the presence of Cu(II), we found significant levels of supercoiled DNA cleavage at 25 μM (Fig. 4). The pyrrolidine-based compounds **22**, **24**–**26** were superior DNA cleaving agents in comparison with other compounds tested.

A concentration course of copper-mediated nuclease activity of **22** was conducted and significant DNA damage was seen at concentrations as low as 1 μM (lane 2, Fig. 5) and nearly all DNA was cleaved at 10 μM of **22** (lane 6, Fig. 5). Such concentrations of DNA cleavage may have physiological relevance as packing of DNA in the form of nucleosomes enhances DNA damage induced by hydrogen peroxide and Cu(II).[68,69] Nuclease activity data analysis revealed that compounds generating higher amounts of ROS were better DNA cleaving agents in the presence of copper. For example, compounds **22**, **24**–**26** produced higher amounts of hydrogen peroxide (Fig. 2) in comparison with other analogues tested and were better DNA cleaving agents (Fig. 4). Hydrogen peroxide in the presence of Cu(II) has been reported to induce nicks in

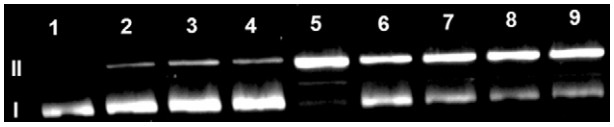
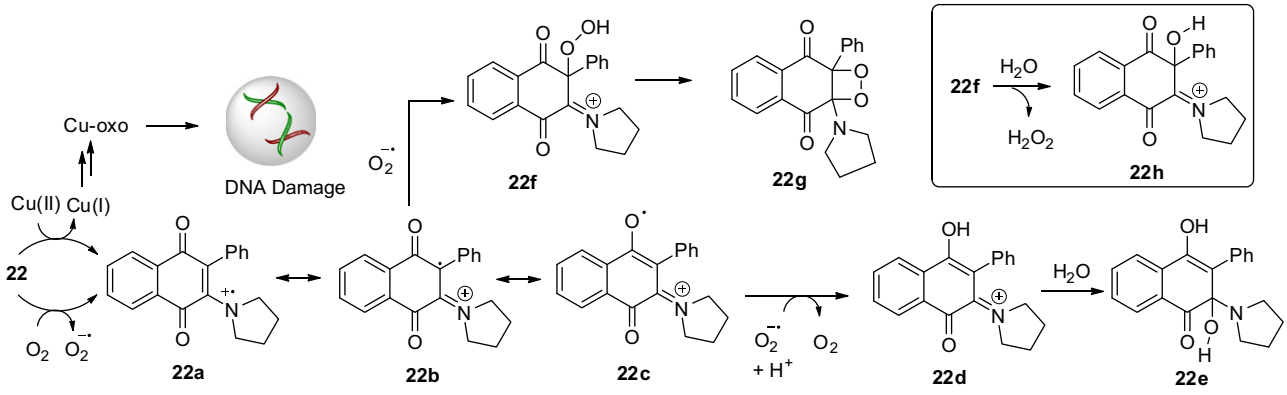


Figure 4. Determination of nuclease activity of selected jadomycin analogues at 25 μM in the presence of equimolar amounts of $Cu(OAc)_2$ using pBR322 supercoiled plasmid DNA. Reaction mixtures (20 μL total volume) contained 1:1 compound: Cu(II) 100 ng Form I DNA in 10 mM MOPS buffer, pH 7.4, and were incubated at 37 °C for 24 h. Lane 1, DNA only; Lane 2, **12**:Cu(II); Lane 3, **16:**Cu(II); Lane 4, **19:**Cu(II); Lane 5, **22:**Cu(II); Lane 6, **23:**Cu(II); Lane 7, **24:**Cu(II); Lane 8, **25**:Cu(II); Lane 9, **26:**Cu(II). Nicked DNA is represented by II.



Scheme 3. Proposed mechanism of superoxide and hydrogen peroxide generation from **22**. MS (ESI) for **22e**: $[M+H]^+$: calcd, 322.14; found, 322.22; **22g**: $[M+Na]^+$: calcd, 358.11; found, 358.05; **22h**: $[M+Na]^+$: calcd, 343.12; found, 343.10.

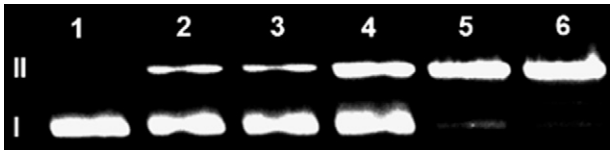


Figure 5. Representative gel image of supercoiled plasmid pBR322 DNA (form I) cleavage by **22** in the presence of equimolar amounts of Cu(II): Reaction mixtures (20 μL) contained 100 ng of form I DNA in 10 mM MOPS buffer, pH 7.4 and were incubated at 37 °C for 24 h: Lane 1, DNA; Lane 2, 1 μM **22**:Cu(II); Lane 3, 2.5 μM **22**:Cu(II); Lane 4, 5 μM **22**:Cu(II); Lane 5, 7.5 μM **22**:Cu(II); Lane 6, 10 μM **22**:Cu(II); Nicked DNA is represented by II.

supercoiled plasmid DNA;[70] the presence of chelating agents such as EDTA inhibited the nuclease activity of H_2O_2.[71] We found that nuclease activity of **22** was completely abrogated in the presence of EDTA suggesting that Cu(II) is necessary for any observed DNA damage.[27] However, under our assay conditions, when H_2O_2 was incubated with Cu(II), we found no significant nick induction at concentrations less than 20 μM (data not shown). Thus, like jadomycin B, the major pathway for DNA damage induced by **22** involves electron transfer to Cu(II) to produce Cu(I), which then reacts with oxygen to produce a copper-oxo species (Scheme 3). In addition to this mechanism of nick induction, production of hydroxyl radicals by Cu(I) has also been proposed in the case of Jadomycin L to mediate DNA damage. From our data, it appears that the 2-aryl-3-amino-1,4-naphthoquinones' propensity to transfer an electron to Cu(II) to produce Cu(I), and oxygen to produce superoxide are closely correlated.

Acknowledgments

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2012.04.009.

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