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New Chemical Tools for the assessment of Hemolytic Anemia induced by Naphtoquinones

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Abstract

A new series of 2-(3-hydroxy-4-oxonaphtalen-1-(*4H*)-ylidene) acetonitriles and 2-hydroxy-4-arylene naphtalen-(4*H*)-one **3a-h** were assessed for their antibacterial activity against four strains of pathogenic bacteria, namely *Pseudomonas aeruginosa* and *Escherichia coli which are* grams (-), and *Enterococcus faecalis* and *Staphylococcus aureus* which are gram (+). The obtained data showed no *in vitro* activity for most synthesized compounds, at the exception of compounds **3e** and **3f** which exhibited some antibacterial activity against *S. aureus* and *E. faecalis*, probably due to the presence of either a chlorine or nitro substituent. Taken together, these data sustain further the suggestion that Lawsone and other 1,4-naphtoquinones are generators of an ROS specie such as hydrogen peroxide H_2O_2 and superoxide, a still under investigation mechanism but known to induce cytotoxicity, and particularly hemolytic anemia and nephrotoxicity.

Keywords: Lawsone, 2-hydroxynaphtoquinomethanes, antibacterial activity, reactive oxygen specie.

Introduction

Naphthoquinones (NQs) and their derivates play an important role in several biological processes, such as photosynthesis, respiratory chain-dependent energy production, allelopathy, and defense [1–3]. During the last decades, a large number of natural and synthetic NQs have been studied for their antitumoral [4], antiparasitic [5], and antimicrobial [6–9] activities. Lawsone (Figure 1) in particular attracted recently much attention since it is one of the most used hair-dyes worldwide.

The classic mechanism of NQs toxicity is to produce reactive oxygen species (ROS). NQs can be reduced to semiquinones by NADPH–cytochrome P-450 reductase or to hydroquinones by DT-diaphorase NAD(P)H: quinoneoxireductase (EC 1.6.99.2). In the presence of oxygen, reduced NQs auto-oxidize, producing superoxide anion radical. This redox cycling of quinones promotes oxidative stress conditions, resulting in destabilization of lysosomes, release of cathepsin D, and mitochondrial membrane potential decrease [10,11]. Furthermore, the capacity to produce free radicals is influenced by the nature and the position of substituents in the quinone molecule [12]. Cell membranes are often permeable to NQs, which, once present in the cell, may form adducts with DNA, leading to abasic sites and single or double strand breaks [12–14]. NQs also react with proteins and lipids, destroying their functionality, thereby causing cell death [15].

Many people have investigated the influence of different substituents in various positions of NQs molecules [16-18]. In the case of lawsone (2-hydroxy, 1, 4-naphtoquinone), previous studies have had conflicting results *in vitro* and *in vivo*. Some considered that lawsone is a non-redox cycling compound *in vitro* [19-22], since direct exposure of lawsone to isolated erythrocytes and hepatocytes did not cause oxidative effects [21, 23]. On the other hand, it provoked anemic response with an oxidative damages profile and toxicity effects to the kidney and gastrointestinal tract following repeated oral dosing [24-26]. Even though an oxidative damage profile is reported to be induced by lawsone *in vivo*, the kind of ROS (reactive oxygen species) formation has not been fully resolved yet [27-29]. Munday have screened many naphtoquinones [25], showing that in the case of hydroxy and amino naphtoquinones, the mechanism of hemotoxicity seems to involve the enzyme NAD(P)H:quinone acceptor oxidoreductase (NQO1), which reduces naphthoquinones to the corresponding hydroquinones. The latter substances then undergo concomitant autoxidation, generating ‘active oxygen’ species that cause oxidative damage to erythrocytes, with consequent haemolysis. No definitive conclusion can however be drawn.

Here, we report the antibacterial evaluation of a new series of 2-hydroxynaphto-quinomethanes 3a-h, and which we suggest as new tools to be used to further investigate the redox cycling of naphtoquinones (Figure 1) leading to nephrotoxicity.

Materials and Methods

Chemicals

Commercial lawsone was purchased from Sigma-Aldrich and was used without further purification.The derivatives of 2-(3-hydroxy-4-oxonaphtalen-(*4H*)-ylidene) acetonitriles and 2-hydroxy-4-arylene naphtalen-(*4H*)-one 3a-h were prepared as follows [30]. A solution of the active methylene 2 (1.92 mmol) in ethanol (8 ml) was added at 40°C to a stirred solution of sodium 1,2-naphthoquinone-4-sulfonate 1 (0.50 g, 1.92 mmol) in H_2O (22 ml). The mixture was stirred at 40°C for 15 minutes before 25% aqueous NaOH (0.50 ml) was added dropwise. After 2 hours of stirring at 40°C, the solution was acidified (37% HCl) to give a solid that was collected, dried under vacuum and identified as 2-hydroxynaphthoquinomethane 3 in 37-92% yield. Exact structures and isomeric forms were proven by spectroscopic studies and molecular modelling. All used chemicals were reagent grade.

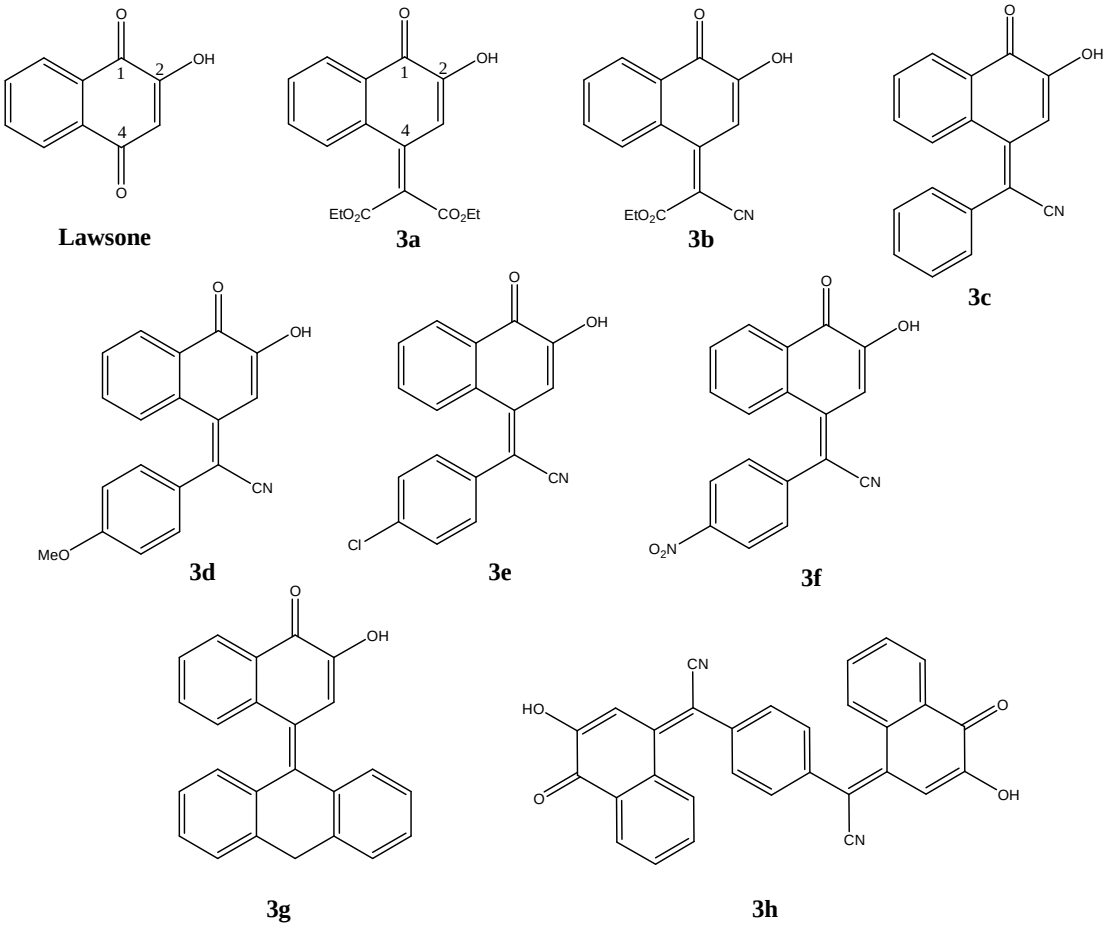

Figure 1. Structures of the synthesized 2-hydroxynaphtoquinomethanes

Microorganisms

The evaluation of the antibacterial activity of the natural Lawsone [37] and its analogs 3a-h was performed against four strains of pathogenic bacteria, namely *Pseudomonas aeruginosa* and *Escherichia coli which are* grams (-), and *Enterococcus faecalis* and *Staphylococcus aureus* which are gram (+).

The evaluation was carried out by disc diffusion on agar medium, based on the recommendations of the National Clinical Committee Laboratory Standards (NCCLS) [33, 35]. The activity evaluation was thus determined by measurement of the inhibitory zone diameter after incubation at 37°C during 18-24 hr [38]. Ciprofloxacin and gentamycin were used as positive controls. The choice of these drugs was based on the broad range of antibiotic activity with respect to the used strains.

The evaluation of the minimal inhibitory concentration (MIC) of the active compounds was determined by use of the broth multiple dilutions method, based on the recommendations of the NCCLS [34-36]. A serie of dilutions from 4.096mg/mL to 0.016mg/mL in DMSO were prepared [38]. The effect of DMSO at the concentration of 5% was checked and eliminated. The data points constitute the mean of replicates. All of our tests were performed three times. The concentration with a prominent decrease in turbidity was visually determined as the MIC. Colorimetric readings with or without Resazurin were not performed [42].

One of the strong points in the NCCLS recommendations is reproducibility, thing that we have also checked with other compounds. The results were in complete agreement with those described in literature.

Results and Discussion

The disc diffusion method showed that beside the positive controls and the commercial lawsone, only compounds **3e** and **3f** showed a mild activity. Compound **3f** was active toward the two gram (+) bacteria whereas **3e** was active toward *S.aureus* only. Lawsone is known for its antibacterial activity **[39, 40]** and possesses a wider spectrum of activity than our new compounds, as shown in Table 1. The activity presented by **3e** and **3f** is thought to be due to the presence of either the nitro or chlorine substituent, since modification of either one result in complete abrogation of the activity. Such a behavior is documented **[41]**.

Table 1. Screening for antibacterial activity of the synthetic derivatives 3a-h using the disc diffusion method

Products (2.048 mg/disc)	Diameter of the inhibitory zone (mm)			
	P. aeruginosa	*E. coli*	*S. aureus*	*E. faecalis*
Lawsone	06.00±00.00 [a]	20.50±00.71	16.80±00.71	13.50±00.71
3a	06.00±00.00 [a]	06.00±00.00 [a]	06.00±00.00 [a]	06.00±00.00 [a]
3b	06.00±00.00 [a]	06.00±00.00 [a]	06.00±00.00 [a]	06.00±00.00 [a]
3c	06.00±00.00 [a]	06.00±00.00 [a]	06.00±00.00 [a]	06.00±00.00 [a]
3d	06.00±00.00 [a]	06.00±00.00 [a]	06.00±00.00 [a]	06.00±00.00 [a]
3e	06.00±00.00 [a]	06.00±00.00 [a]	16.00±02.82	06.00±00.00 [a]
3f	06.00±00.00 [a]	06.00±00.00 [a]	17.00±02.82	14.50±00.70
3g	06.00±00.00 [a]	06.00±00.00 [a]	06.00±00.00 [a]	06.00±00.00 [a]
3h	06.00±00.00 [a]	06.00±00.00 [a]	06.00±00.00 [a]	06.00±00.00 [a]
Ciprofloxacin (5×10^{-3} mg/disc)	31.50±00.70	33.60±03.68	28.80±00.35	30.00±00.00
Gentamycin (15×10^{-3} mg/disc)	20.50±03.53	24.00±00.00	25.50±00.21	28.00±02.12

[a]: no inhibitory zone was observed

It has already been reported that the Agar and broth tests could show considerable discrepancies. On this basis, we screened all compounds for MIC on liquid medium (Table 2). Such data suggest that analogs 3e and 3f present no interesting activity, and that they are probably completely inactive toward gram (-) bacteria (i.e. *P. aeruginosa* and *E. coli*) in the used concentration interval. The best CMI was obtained with compound 3e (0,032 mg/ml) and 3f (0.064 mg/ml) with respect to *S. aureus*. This activity was increased up to sixteen times that of Lawsone for compound 3e and up to eight times that of Lawsone (0.512 mg/ml) for compound 3f. Finally, compound 3f exhibited an activity four times higher than that of Lawsone (0.256 mg/ml) against *E. faecalis*.

According to our results, all synthesized compounds were inactive at the exception of two (3e and 3f), which presented an antibacterial activity higher than that of Lawsone at the same concentration. This suggests that grafting of an acetonitrile group (or other active methylenes) on position 4 of the naphtoquinone moiety leads to an abrogation of the activity, and thus suggests that the 4-position ketone functionality is necessary for the activity. The activity obtained in the case of compounds 3e and 3f could be argued. But we believe it to be due to the presence of either the chlorine or nitro substituent, since removing it simply abrogates the activity. It is plausible to think that either of these substituents may be involved

in a redox cycle, which would lead to an activity.

Table 2. Minimal Inhibitory Concentrations (MIC) of the synthetic compounds 3a-h

Compound	MIC (mg/ml)			
	P. aeruginosa	*E. coli*	*S. aureus*	*E. faecalis*
Commercial Lawsone	2.048	1.024	0.512	2.048
3a	≥ 2.048	≥ 2.048	≥ 2.048	≥ 2.048
3b	≥ 2.048	≥ 2.048	≥ 2.048	≥ 2.048
3c	≥ 2.048	≥ 2.048	≥ 2.048	≥ 2.048
3d	≥ 2.048	≥ 2.048	≥ 2.048	≥ 2.048
3e	≥ 2.048	≥ 2.048	32.10^{-3}	≥ 2.048
3f	≥ 2.048	≥ 2.048	64.10^{-3}	256.10^{-3}
3g	≥ 2.048	≥ 2.048	≥ 2.048	≥ 2.048
3h	≥ 2.048	≥ 2.048	≥ 2.048	≥ 2.048
Ciprofloxacin	2.10^{-3}	$0.5.\ 10^{-3}$	$0.25.\ 10^{-3}$	8.10^{-3}
Gentamycin	$0.5\ 10^{-3}$	$0.008\ 10^{-3}$	$0.25\ 10^{-3}$	$0.5\ 10^{-3}$

As previously mentioned, the so far believed mechanism of oxidative stress involves a first reduction of a quinone (with or without the presence of a hydroxyl in position 2) to a semiquinone. This could undergo further reduction to a naphtalenediol which was shown by Wright *et al*. to be cytotoxic only in case it could be recycled back into a quinine [43]. The semiquinone could however act as a pro-oxidant by donating a second hydrogen (or electron since the semiquinone has a low pKa), leading finally to a quinone Q. The toxicity generally arises from the quinone alkylating glutathione or protein thiols via a Michael addition or via an enzymatic redox cycling process. Other mechanisms have likewise been suggested, involving extracellular autoxidation [44], or some combination of intra/extracellular transport and autoxidation [45]. Miller *et al*. had earlier investigated the cytotoxicity of the serie 1,4-NQ, menadione, 2,3-dimethyl-1,4-NQ. All have shown cytotoxicity with very different potencies though [46]. These results were in conflict with those obtained by Tikkanen *et al*. [16] who investigated the influence of substituents, such as hydroxy, methyl or both moieties in different positions of NQs molecules. Upon metabolic activation, naphthazarin (5,8-dihydroxy-1,4 naphthoquinone) (NTZ) and plumbagin (2-methyl-5-hydroxy-1,4-naphthoquinone) induced frameshift mutations in *Salmonella typhimurium* strain TA98. Hakura *et al*. [17] demonstrated that 4-amino-1,2-naphthoquinone was mutagenic in TA97, TA100, and TA102 strains. A recent study was conducted by Henriques *et al.* [18] showing the mutagenic activity of aminohydroxy-substituents in this class of compounds. To such an end, the authors compared the activities of 1,4- naphthoquinone, 5-amino-8-hydroxy-1,4-naphthoquinone and 2,8-dihydroxy-5-amino-1,4-naphthoquinone on the yeast *Saccharomyces cerevisiae*, *Salmonella, and* V79 Chinese hamster lung fibroblast cells. Meyer *et al*. reported an antimicrobial activity of some diospyrin derivatives, all being either 1,4-Qs or 1,4-HQ, against Mycobacterium tuberculosis [47]. Munday *et al*. have screened a whole plethora of 1,2- and 1,4-naphtoquinones, producing different results [25]. Thus, it seems that the presence of the hydroxyl group in position 2 decreases the activity, but does not abrogate it. The presence of a keto- group on position 4 seems necessary therefore for the activity. In our case, the designed compounds possess a hydroxyl group on position 2, with a converted keto into alkene group on position 4. We reasoned that upon first reduction, the newly synthesized compounds should produce a naphtol moiety which won't be recycled into a keto form. This would bring further proof of a redox cycle in the center of the mechanism. If however, the most prevalent mechanism involves the cytochrome P-450 monooxygenase system, it would in theory lead to a mutagenic metabolite, which can be shown. The question of the possible correlation between antibacterial and cytotoxic activity remains however unsolved, although

Lindequist suggested no relationship [48]. Further proof of it remains to be demonstrated. Quinones are very abundant in nature and continue to show promise as antitumor agents, indicating that their mutagenecity and carcinogenicity are worthy of future investigation.

Conclusion

In conclusion, the modification of the keto group in position 4 of lawsone produced some non-active compounds toward gram(+) and gram(-) bacteria. These newly synthesized compounds can be used to acertain the redox cycling mechanism by which lawsone and other hydroquinones are cytotoxic, a question that is so far unresolved.

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[38] *Method of diffusion on solid medium*: In this method the inoculum of 1.10^8 UFC (0.5 McFarland/DO=0.08-0.1) diluted with the 100th is sown on a Mueller-Hinton gelose box 3-4 mm of thickness. After repeated dryings, Wattman paper discs of 0.6 diameter containing 10µL of a solution of the compound to be tested in 100% of DMSO, are applied in the box. The activity is determined by the measurement of the inhibitory zone diameter in mm after incubation at 37°C during 18-24hr. Ciprofloxacin and gentamycin were used as reference antibiotics. Pure solvent was used as negative control. This activity is considered starting from a diameter of 6 mm or higher, and is classified as follows:
Very Strong activity: diameter ≥ 30 mm; Strong activity: diameter between 21-29 mm; medium activity: diameter between 16-20 mm; Weak activity: diameter between 11-15 mm; Small or no activity: diameter ≤ 10 mm.
Minimal Inhibitory concentration on liquid medium: The products that have shown an activity were subjected to an evaluation for their activity using the method of dilutions to estimate the Minimal Inhibitory Concentration (MIC). A serie of dilutions going from 2.048-4 µg/ml of DMSO 5% was prepared in a 96 well sterile microchip. To each well was introduced 100µL of substance to be tested. Subsequently, 100µL of an inoculum containing 10^6 UFC was added in each well. The well used as negative control was prepared using the inoculum alone. Ciprofloxacin and Gentamycin were used as antibiotics of reference. The plate was incubated at 37°C during 18-24hr. Minimal Inhibitory Concentration MIC was considered as the weakest concentration to which no turbidity was eye-observed.
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