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Antimutagenic and antioxidant properties of plumbagin and other naphthoquinones

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ABSTRACT

The structure–function relationships of the naphthoquinone phytochemicals, plumbagin, juglone, and menadione, have been studied with regard to antimutagenic and antioxidant activities. Antimutagenicity of these compounds was assessed by the Ames test and RNA polymerase B (*rpoB*)-based rifampicin resistance assay. Antioxidant potential was evaluated by radical scavenging assays and reducing power measurement. Protection of cells and DNA against gamma radiation-induced oxidative damage was assayed by survival analysis and gel electrophoresis profiling, respectively. On the 1,4-naphthoquinone nucleus, plumbagin possesses 5-hydroxyl and 2-methyl functional groups, whereas juglone has only the 5-hydroxyl and menadione only the 2-methyl group. Plumbagin showed strong antimutagenic (against ultraviolet and ethyl methanesulfonate) and antioxidant activities, whereas juglone displayed only strong antimutagenic, and menadione only strong antioxidant activities. Thus, these two functional groups (5-OH/2-CH_3) play important roles in the differential bioactivity of naphthoquinones. *Escherichia coli*, microarray analysis showed upregulation of the genes *rep* (replication/repair), *ybaK* (tRNA editing), *speE* (spermidine synthesis), and *yjfC* (glutathionyl spermidine synthesis) by plumbagin or juglone, and *sodC* (superoxide dismutase), *xthA* (oxidative repair), *hycB* (electron carrier between hydrogenase 3 and fumarate dehydrogenase), and *ligA* (formation of phosphodiester bond in DNA) by plumbagin or menadione. Studies with *E. coli* single-gene knockouts showed that *ybaK and speE*, reported to prevent mistranslation, are likely to be involved in the antimutagenicity displayed by juglone, and *sodC* to be involved in the antioxidant activity of menadione.

1. Introduction

Mutations are an important cause of the initiation and progression of many diseases, including atherosclerosis, heart diseases, and cancer. Phytochemicals that reduce mutagenesis may offer preventive potential. Naphthoquinones, such as ubiquinone, plastoquinone, and K vitamins, may have multiple health promoting effects [1]. However, further insight into their mechanisms of action is needed. In this study, three structurally similar naphthoquinones – plumbagin, menadione, and juglone (Fig. 1) – were examined. Plumbagin (5-hydroxyl-2-methyl-1,4-naphthoquinone), a yellow compound isolated from the genus *Plumbago* [2], has anti-inflammatory, anticarcinogenic, immunosuppressive, and anti-atherosclerotic activities [3–5]. Juglone (5-hydroxyl-1,4-naphthoquinone), known in the food industry as natural brown, is isolated from the black walnut (*Juglans nigra*) and other plants of the Juglandaceae family. It has been used as an herbicide, a dye for cloth and inks, and a colouring agent for foods and cosmetics [6]. Menadione (2-methyl-1,4-naphthoquinone), also known as vitamin K_3, is a synthetic compound. The antimutagenic properties of these naphthoquinones against ultraviolet (UV) and ethyl methanesulfonate (EMS) mutagenesis were evaluated by the Ames test and rifampicin resistance assay. The Ames test measures reversion of auxotrophic his^- mutants of *Salmonella typhimurium* and the rifampicin resistance assay measures acquisition of rifampicin resistance by *Escherichia coli* cells due to mutations in the *rpoB* gene, which encodes the β subunit of RNA polymerase [7,8].

Antioxidant activity was determined by quantifying the scavenging activities of free radicals such as DPPH, hydroxyl, and superoxide, and by measuring reducing power. Plasmid DNA protection from gamma radiation-induced oxidative damage was assayed using agarose gel electrophoresis, and its function by measuring transformation efficiency. Protection of *E. coli* cells against oxidative damage upon gamma radiation was also assayed by survival analysis using fluorescence activated cell sorting (FACS),

Abbreviations: Rif^R, rifampicin resistant; his, histidine; PL, plumbagin; JU, juglone; ME, menadione; AP, antimutagenic potential; SR, spontaneous reversion; SPC, standard plate count; aRNA, amplified RNA; cfu, colony-forming units; WT, wild type.

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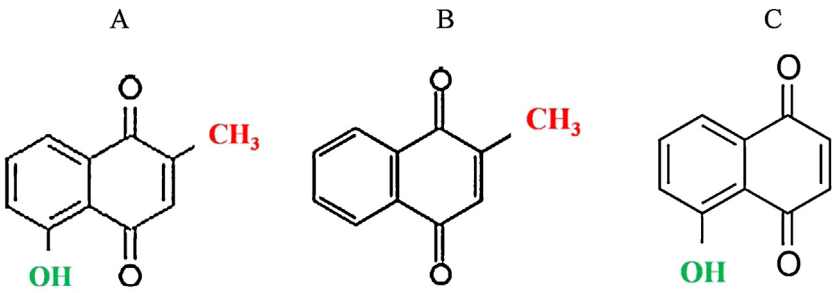

Fig. 1. Chemical structures of (A) plumbagin (PL), (B) menadione (ME), and (C) juglone (JU).

plotting the survival curve and calculating the decimal reduction dose (D_{10}). To examine the molecular mechanisms contributing to these activities, microarray transcriptome analyses were performed using *E. coli* cells grown in the presence of each naphthoquinone. The relevance of specific upregulated genes was also tested using *E. coli* knockout mutants.

This study demonstrates a strong structure–function relationship among the naphthoquinones and provides a mechanistic view of their differential activity.

2. Materials and methods

2.1. Bacterial strains

S. typhimurium and *E. coli* strains, their genotypes, and sources are listed in Table 1.

2.2. Genotype confirmation of Ames test strains

S. typhimurium strains TA102 and TA100 were confirmed for histidine requirement, *rfa* mutation, UV sensitivity and plasmid encoded antibiotic markers (pKM101-Amp^R, and pAQ1-Tet^R) [7,9].

2.3. Analysis of antimutagenicity of plumbagin and its analogues

Plumbagin (PL), juglone (JU), and menadione (ME) were obtained from Sigma–Aldrich (St. Louis, USA). Stock solutions (80 mM) were prepared in dimethyl sulfoxide (DMSO). The concentration of DMSO in the final reaction mixtures was below 0.75%. Antimutagenicity was determined at 25, 50 and 100 μM.

2.3.1. Ames test

The Ames test was performed using *S. typhimurium* TA102 and TA100 strains against UV- and EMS-induced mutagenesis as described earlier [7,10]. *S. typhimurium* strain TA102 culture was grown overnight in 50 ml nutrient broth (NB) on a rotary shaker (150 rpm) at 37 °C and centrifuged at 8000 × *g* for 10 min. For UV mutagenesis, the pellet was resuspended in the same volume of saline (0.85%) and placed on ice for 10 min; for EMS mutagenesis, it was resuspended in NB. Cell suspension (1 ml), was transferred to fresh microfuge tubes, mixed with test compounds, and incubated for 10 min. Aliquots (100 μl) were exposed to UV (254 nm; 8W; 220 μW/cm^2) for 10 or 15 s; or mixed with EMS (66.5 or 133 mM) and further incubated for 20 min. Treated cell suspensions were added to top agar (2 ml) containing histidine and biotin, vortexed, poured onto minimal glucose plates, incubated (37 °C, 48 h), and examined for revertant colonies. Additional antimutagenicity experiments were performed where the test compounds were added immediately after UV exposure of the cells. For EMS, the test compounds were also pre-incubated with the mutagen and analyzed for antimutagenicity as discussed above. The antimutagenic potential (AP) was determined by equation: AP = 100 − [(*T*/*M*) × 100], where *T* and *M* are the number of revertant colonies/plate in the presence of mutagen and test compound, and in presence of mutagen alone, respectively [11].

2.3.2. Rifampicin resistance assay

The rifampicin resistance assay was performed as described earlier [8,10,12]. *E. coli* cells were grown overnight in 50 ml LB on a rotary shaker (150 rpm) at 37 °C. Culture (1 ml) was inoculated in fresh LB (50 ml) and further grown for about 4 h to $OD_{600\,nm}$ = 0.5, centrifuged at 8000 × *g* for 10 min, and resuspended in 25 ml saline (0.85%). As described earlier for UV mutagenesis, the cell suspension (50 μl) was exposed for 10 or 15 s, and transferred to 1 ml LB and incubated overnight on a rotary shaker (150 rpm) at 37 °C. For EMS mutagenesis, an aliquot of the cell suspension (1 ml) was transferred to a fresh microfuge tube and the EMS as well as the test compound was added, incubated for 45 min on a rotary shaker (75 rpm) at 37 °C,

Table 1
List of *S. typhimurium* (TA) and *E. coli* strains used in the current study.

Strain designation	Genotype	Source
S. typhimurium		
TA102	*hisΔ(G)8476 galE503 rfa1027/pAQ1/pKM101*	Xenometrix Inc., USA
TA100	*hisG46 gal bio ch1005 rfa1004 uvrB pKM101*	Xenometrix Inc., USA
E. coli		
DH5α	*F⁻ endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG Φ80dlacZΔM15 Δ(lacZYA-argF)U169, hsdR17(r_K^- m_K^+), λ⁻*	Genei, Bangalore, India
MG1655	*F⁻ λ⁻ ilvG-rfb-50 rph-1*	M.Z. Humayun, UMDNJ, USA
JW2694	*F⁻, Δ(araD-araB)567, ΔlacZ4787(::rrnB-3), λ⁻, ΔhycB728::kan, rph-1, Δ(rhaD-rhaB)568, hsdR514*	Keio collection, Japan
JW1638	*F⁻, Δ(araD-araB)567, ΔlacZ4787(::rrnB-3), λ⁻, ΔsodC724::kan, rph-1, Δ(rhaD-rhaB)568, hsdR514*	Keio collection, Japan
JW1738	*F⁻, Δ(araD-araB)567, ΔlacZ4787(::rrnB-3), λ⁻, ΔxthA747::kan, rph-1, Δ(rhaD-rhaB)568, hsdR514*	Keio collection, Japan
JW5604	*F⁻, Δ(araD-araB)567, ΔlacZ4787(::rrnB-3), λ⁻, Δrep-729::kan, rph-1, Δ(rhaD-rhaB)568, hsdR514*	Keio collection, Japan
JW0470	*F⁻, Δ(araD-araB)567, ΔlacZ4787(::rrnB-3), ΔybaK764::kan, λ⁻, rph-1, Δ(rhaD-rhaB)568, hsdR514*	Keio collection, Japan
JW0117	*F⁻, Δ(araD-araB)567, ΔspeE739::kan, ΔlacZ4787(::rrnB-3), λ⁻, rph-1, Δ(rhaD-rhaB)568, hsdR514*	Keio collection, Japan
JW41441	*F⁻, Δ(araD-araB)567, ΔlacZ4787*(::rrnB-3), λ⁻, *rph-1, Δ(rhaD-rhaB)568, ΔyjfC736::kan, hsdR514*	Keio collection, Japan
JW11721	F-, *Δ(araD-araB)567, ΔlacZ4787(::rrnB-3), λ⁻, ΔumuD772::kan, rph-1, Δ(rhaD-rhaB)568, hsdR514*	Keio collection, Japan
JW11731	*F⁻, Δ(araD-araB)567, ΔlacZ4787(::rrnB-3), λ⁻, ΔumuC773::kan, rph-1, Δ(rhaD-rhaB)568, hsdR514*	Keio collection, Japan
JW26691	*F⁻, Δ(araD-araB)567, ΔlacZ4787*(::rrnB-3), λ⁻, *ΔrecA774::kan, rph-1, Δ(rhaD-rhaB)568, hsdR514*	Keio collection, Japan