SUMMARY OF DATA FOR CHEMICAL SELECTION

Juglone
481-39-0

BASIS OF NOMINATION TO THE CSWG

Juglone is brought to the attention of the CSWG as a potentially toxic natural product. Juglone, a brown dye, is found in several consumer products, including hair dye formulations and walnut oil stain. Juglone is an active ingredient in dietary supplements prepared from walnut hulls. Walnut hull extracts and poultices have been used for many years in folk remedies.

There is some evidence to suggest that juglone is a potential chemotherapeutic or chemopreventive agent. The Developmental Therapeutics Program, National Cancer Institute (NCI) evaluated juglone in its screening panel for HIV-1. However, German Commission E does not approve the use of walnut hull as an herbal medicine because of documented or suspected risk from juglone. This raises questions about the safety of products intended for human consumption that contain juglone as an active ingredient.

SELECTION STATUS

ACTION BY CSWG: 6/22/99

Studies requested:

Preliminary studies:

- Mechanistic studies predictive of carcinogenic/anticarcinogenic potential
- Metabolism studies
- Mouse lymphoma assay
- Mammalian mutagenicity assay

Follow-up:

Based on the results from the preliminary studies, select either juglone or plumbagin for carcinogenicity testing

Priority: High

Rationale/Remarks:

- Natural brown pigment; widespread human exposure through use of walnut-based stains, oils, and dyes

- Persistent environmental pollutant released from walnut and butternut trees

- Given the close relationship between juglone and plumbagin, chronic studies of only the more reactive compound are recommended.

- Existing information on the carcinogenic/anticarcinogenic potential of the two compounds is insufficient to determine which compound is more reactive.

- NCI will conduct mouse lymphoma assay.

CHEMICAL IDENTIFICATION

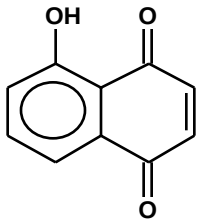
CAS Registry Number: 481-39-0

Chemical Abstracts Service Name: 1,4-Napthoquinone, 5-hydroxy-(8CI)

Synonyms and Trade Names: Akhnot; C.I. 75500; C.I. Natural Brown 7; 5-hydroxy-1,4-naphthalenedione;5-hydroxynaphthoquinone; juglone; regianin; walnut extract

Structural Class: Bicyclic; napthoquinone

Structure, Molecular Formula and Molecular Weight:



$C_{10}H_6O_3$ Mol. wt.: 174.16

Chemical and Physical Properties:

Description: Yellow needles from benzene plus petroleum ether; gives purplish-red solution in aqueous solutions of alkalis (Merck, 1997)

Melting Point: 155^{o}C (Merck, 1997)

Solubility: Slightly soluble in hot water; soluble in alcohol, acetone, chloroform, benzene, and acetic acid (Merck, 1997)

Technical Products and Impurities: Juglone is available at a purity of 97% from Fluka and Aldrich and 95% from Sigma (Sigma-Aldrich, 1999).

EXPOSURE INFORMATION

Production and Producers: Juglone is a napthoquinone pigment that occurs as a natural product in the roots, leaves, nut-hulls, bark and wood of black walnut (*Junglans nigra*), European walnut (*Junglans regia*) and butternut (*Junglans cinerea*) (Botanical Dermatology Database, 1999; Funt & Martin, 1999).

Twenty producers or distributors of juglone are listed by Chem Sources (Chemical Sources International, 1999). Some of these sources are Aldrich, Fluka, Sigma, and TCI America (TCI America, 1998; Sigma-Aldrich, 1999).

Juglone is not listed in the EPA's Toxic Substances Control Act (TSCA) Inventory.

Use Pattern: The primary uses of pure juglone are as a research chemical, a pH indicator, and a natural dye (Merck, 1997).

Crushed unripe walnut hulls have been used for generations in various types of folk medicine. The hulls are made into poultices and rubbed into the skin to treat fungal, bacterial, or viral infections such as herpes or warts. External applications of black walnut also kill ringworm, and Chinese herbalists use this substance to kill tapeworm. In certain regions of the United States fresh walnut hulls have been employed illegally to immobilize fish (Westfall *et al.*, 1961; Viable Herbal Solutions, 1997; Rye's Healthy Herbs, 1999).

Recently, herbal remedies containing black walnut have been marketed as dietary supplements. Viable Herbal Solutions (1997) sells an extract of black walnut that can be used in one of two ways: it can be taken orally by mixing 10-20 drops in water or juice daily, or it can be used externally by rubbing the extract directly on the skin twice daily. Chinese Tiao He Cleanse is an herbal cleansing program marketed through the Internet for acne, allergies, body odor, constipation, dry stools, fatigue, gastrointestinal disorders, halitosis, headaches, hemorrhoids, inflammatory skin conditions, intestinal parasites and worms, lymphatic inflammation, menstrual problems, obesity, and swollen abdomen. Chinese Tiao He Cleanse consists of 30 packets, one of which is a black walnut hull preparation (Rye's Healthy Herbs, 1999).

According to the German Commission E Report, walnut hull preparations containing juglone are used for catarrhs of the gastrointestinal tract, skin diseases, abscesses, inflammation of the eyes, in combinations for diabetes, gastritis, for "blood purification," blood poisoning, and anemia. According to Commission E, the effectiveness for the claimed applications is not documented and the risks of juglone are known, so that the application of walnut hull preparations cannot be justified (Blumenthal, 1998).

The dark color of walnut juice has been used for centuries to stain white skin, to darken the hair, and for an astringent effect. Oil of Walnut is used in varnish, paint, and perfumery. Attempts to use juglone as a topical sunscreen were abandoned because commercially available juglone was capable of causing allergic contact dermatitis (Botanical Dermatology Database, 1999).

The ground shells of black walnut have industrial applications including use as a nonslip agent in automobile tires, as an air pressure propellant in strip paints, and as a filtering agent for scrubbers in smoke stacks. The automobile industry uses the ground shell products to deburr precision gears, and the airline industry uses the ground shells to clean jet engines (Williams, 1990).

Between 1976 and 1999, 73 patents involving juglone were obtained in the United States (US Patents and Trademarks Office, 1999). These patents demonstrate a variety of potential uses for juglone, for example, to prepare antiviral naphthoquinone derivatives useful for AIDS treatment, in skin-coloring preparations, and in hair dyes (Kurz *et al.*, 1996; Boyd *et al.*, 1999; Schmitt *et al.*, 1999). According to Hocquaux and coworkers at L'Oreal (1990), juglone has a tinctorial strength in the right range of hues for hair dyes, but has the disadvantage that its resistance to oxidation is low.

Human Exposure: There is a potential for low-level dermal exposure to juglone because of its presence in hair dye formulations and other consumer items dyed with C.I. Natural Brown 7. Exposure to juglone also occurs through ingestion of the dietary supplements described above and through the handling of walnut or butternut hulls and leaves.

Environmental Occurrence: As noted above, juglone is present in the roots, leaves, nut-hulls, bark and wood of the black walnut, European walnut, and butternut. Therefore, humans may be exposed to juglone while raking the leaves from these trees or working in soils within their root zones.

Juglone has been identified in water run-off from black walnut, European walnut, and butternut trees (Botanical Dermatology Database, 1999).

Many plants such as tomato, potato, blackberry, blueberry, azalea, mountain laurel, rhododendron, red pine, and apple may be injured or killed within months when placed within the root zone of juglone-releasing trees. Plants adversely affected exhibit symptoms such as foliar yellowing, wilting, and eventual death caused by inhibition of respiration. Because decaying roots still release juglone, toxicity can persist for years after a tree is removed (Funt & Martin, 1999; WVU, 1999).

Juglone also occurs in the leaves, bark, and wood of walnut, but at lower concentrations than in the roots. Horses bedded on wood shavings containing more than about 20 percent black walnut may develop clinical signs of laminitis. Consumption of the shavings may also cause a mild colic. Juglone is poorly soluble in water and thus does not move very far in the soil; breakdown in soil may take up to two months (Funt & Martin, 1999; Purdue, 1999).

Regulatory Status:

No standards or guidelines have been set by NIOSH or OSHA foroccupational exposure to or workplace allowable levels of juglone. No listing was found for juglone in the National Occupational Exposure Survey (NOES), which was conducted by the National Institute for Occupational Safety and Health (NIOSH).

EVIDENCE OF POSSIBLE CARCINOGENIC ACTIVITY

Human Data: No epidemiological studies or case reports investigating the association of exposure to juglone and cancer risk in humans were identified in the available literature.

According to the German Commission E monograph on walnut hull, the topical, daily use of juglone-containing preparations of walnut bark is tied to an increased occurrence of cancer of the tongue and leukoplakia of the lips (Blumenthal, 1998).

Animal Data: No two year carcinogenicity studies of juglone were identified in the available literature. Information on the acute toxicity of juglone is presented in Table 1.

Table 1. Acute toxicity data for juglone

Route	Species	LD_{50} (mg/kg)	Reference
Oral	Mouse	2.5 mg/kg	Westfall *et al*., 1961
Oral	Rat	112 mg/kg	NLM, 1999a
Intraperitoneal (ip)	Mouse	25 mg/kg	NLM, 1999a

Dogs administered juglone intravenously (iv) at 5 mg/kg developed visible hemorrhages in the lungs, most likely related to increased capillary permeability. Since there were no significant changes in EKG, heart rate, or blood pressure, juglone did not appear to have a direct effect on the cardiovascular system (Boelkins *et al.*, 1968).

Short-Terms Tests: *Genotoxicity.* Juglone has been tested for mutagenicity, chromosomal damage, and DNA damage in several standard assays. Table 2 presents mutagenicity data using the *Salmonella* assay conducted with or without activation by the S-9 microsomal enzymes.

Table 2. Mutagenic activity of juglone in *Salmonella* assay

S. typhimuium strain	Results without S-9	Results with S-9	Comments	References
TA98	negative	negative	sensitive to frameshift mutations	Tikkanen *et al.*, 1983 Matsushima *et al.*, 1986 Edenharder & Tang, 1997
TA100	negative	negative	sensitive to base pair mutations	Tikkanen *et al.*, 1983 Matsushima *et al.*, 1986
TA2637	negative	positive	detects bulky DNA adducts	Tikkanen *et al.*, 1983 Matsushima *et al.*, 1986

A strong mutagenic response was obtained in the sex-linked recessive lethal test in adult male *Drosophila melanogaster.* Brood size was reduced significantly, suggesting some degree of toxicity. Because juglone is very toxic to *Drosophila* larvae, the larval stages were not suitable for testing mutagenic activity (Clark, 1982).

Gaivão and coworkers (1999) used the *w/w+* somatic mutation and recombination test (SMART) of *Drosophila melanogaster* to evaluate the genotoxicity of reactive oxygen species inducers. Juglone was positive in this assay, producing a high relative value of genotoxic activity as evidenced by the increased percentage of mosaic eye progeny produced.

Tumor Promoting Activity. Juglone promoted 7,12-dimethylbenz[a]anthracene (DMBA)- initiated skin carcinomas and papillomas in Sencar mice when applied dermally at 440, 880 or 1760 nmol/mouse once a week for 40 weeks. Tumor incidence and tumor multiplicity were both dose dependent. Several other structurally related quinones were also examined, and a good correlation between the ability to induce epidermal ornithine decarboxylase and the ability to behave as a tumor promoter was noted (Monks *et al.*, 1990).

Juglone promoted skin tumors in female ICR/Ha Swiss mice (30 per group)

pretreated with a subcarcinogenic dose of DMBA followed by dermal application of juglone at 62 ug 3x/week for 52 weeks. Skin tumors were not observed in mice treated with the same regimen of juglone but not pre-treated with the initiator (Van Duren *et al.*, 1978).

Metabolism: No information on the metabolism, distribution, or excretion of juglone was identified in the literature.

Other Biological Effects: *Anticarcinogenicity.* Okada and coworkers (1967) examined the cytological effects of juglone on Ehrlich ascites tumor cells transmitted in Swiss/HaICR mice. Mitotic abnormalities in the tumor cells were noted 6-12 hours after ip injections at doses as low as 0.25 mg. The most notable effects observed were a decrease in the percentage of mitotic figures with a concomitant accumulation of cells in metaphase indicating that juglone appeared to be preventing cells from entering mitosis. Chromosomes in prophase from juglone-treated tumors appeared diffuse and sticky and accumulation of abnormal metaphase figures occurred.

Bhargava and Westfall (1968) administered extracts of *Juglans nigra* leaves ip to Swiss mice for 9 days and observed a decrease of the growth rate of spontaneous mammary adenocarcinomas ($p<0.001$) . The authors considered the effectiveness of juglone to be questionable because the mice also had a significant decrease in body weight ($p = 0.003$).

Sugie and coworkers (1998) reported that male F344 rats administered 200 ppm of juglone in the diet for two weeks beginning one week before the injection of azoxymethane (AOM) had a lower incidence and multiplicity of tumors in the small intestine (7% and 0.07 Å 0.25) than those exposed to carcinogen alone (25% and 0.32 Å 0.6) and multiplicity of tumors of the entire intestine (0.60 Å 0.76) than those administered the carcinogen alone (1.04 Å 0.91) ($P<0.05$ in each). Based on these data, the authors suggested that juglone could be a promising chemopreventive agent for human intestinal neoplasia.

Juglone was inactive in the AIDs Screen conducted by the NCI's Developmental Therapeutics Program (NCI, 1999).

Antimutagenicity. Several napthoquinones (juglone, plumbagin, menadione, 5,8-hydroxy- 1,4-napthoquinone, chimaphilin) were tested in *Salmonella typhimurium* TA98 for their activities against mutagenicities induced by 2-nitrofluorene (2-NF), 3-nitrofluoranthene (3-NFA), and 1-nitropyrene (1-NP). All of the napthoquinones tested were potent antimutagens irrespective of the presence of methyl or hydroxyl functions (Edenharder & Tang, 1997).

Mechanistic studies. The mechanism of toxicity of juglone and related napthoquinones to eukaryotic cells *in vitro* has been investigated in various cell lines including rat hepatocytes, human HepG2 , and BALB/c 3T3 mouse fibroblasts. In general, juglone was more cytotoxic than the parent 1,4-napthoquinone (Doherty *et al.*, 1987; Öllinger & Brunmark, 1991; Babich & Stern, 1993).

Segura-Aguilar and coworkers (1992) compared the cytotoxicity of juglone to human leukemia (HL-60) cells and doxorubicin-resistant human leukemia (HL-60R) cells. The cell-killing effect after incubation of HL-60 cells with juglone was similar to the effect seen for doxorubicin. However, the HL-60R cells were equally sensitive to juglone. Thus, the multidrug resistance that develops in the doxorubicin-resistant HL-60R cell line did not prevent the cytotoxic effect of juglone.

Effects on other cellular macromolecules. Biologically active naphthoquinones readily pass through the cellular membranes where their electrophilicity enables them to conjugate with other compounds. This reaction has been implicated in the toxicity of quinones. Nucleophilic targets include thiol groups (Gant *et al*., 1986) which results in inhibition of enzymes such as parvulin-like peptidyl-prolyl *cis/trans* isomerases (Hennig *et al.*, 1998), glutathione-S-transferase (Vos *et al.*, 1989), and cardiac sarcoplasmic reticulum Ca2+ ATPase (Floreani *et al.*, 1994). Recent studies have shown that juglone can penetrate the plasma membrane and induce polarization by blocking K+ channels (Varga *et al.*, 1996). As part of a study to identify novel plant-derived inhibitors of signaling kinases, Frew and coworkers (1995) discovered that juglone and methyljuglone are potent inhibitors of protein kinase C (PKC).

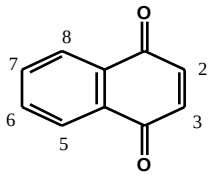
Juglone also showed potent inhibitory activity against aromatase cytochrome P450 in human placental microsomes in a dose dependent manner. The inhibitory effects of juglone were thought to be due to direct binding of the napthoquinone to cytochrome P450 rather than an interaction with the thiol groups or formation of superoxide radicals (Muto *et al.*, 1987).

Structure-Activity Analysis: The quinoid structure is ubiquitous in nature, encompassing over 1200 naturally occurring compounds, including juglone. The toxicity of quinone compounds has been extensively studied and is generally accepted to be a function of (a) the capacity of quinones to produce oxygen free radicals and (b) the electrophilicity of quinones, which enables them to form adducts to cellular macromolecules. The production of intracellular free radicals is thought to be a consequence of 0_2 reduction by semiquinone intermediates in a process known as redox cycling. This process generates hydrogen peroxide and oxygen radicals, which cause oxidative stress (Öllinger & Brunmark, 1991).

The capacity of quinone derivatives to produce free radicals is largely influenced by the substituents on the molecule which in turn determine the efficiency of one-electron reduction to semiquinone metabolites. *In vitro* experiments designed to examine the relative rates of enzymatic single-electron reduction demonstrated that napthoquinones, especially juglone, undergo rapid single-electron reduction (Lewis & Shibamoto, 1989).

Unsubstituted napthoquinones generally do not show mutagenicity in the *Salmonella* mutation assay in the presence or absence of S-9 metabolic activation. However, substituted napthoquinones containing one or more hydroxyl groups and/or methoxyl groups have been shown to be mutagenic in *S. typhimurium* in the presence of S-9 (Matshushima *et al.*, 1986). For the purposes of this report, three structurally related napthoquinones (1,4-napthoquinone, lawsone, and menadione) were screened for relevant information on mutagenicity and carcinogenicity. Table 3 presents the structures of the napthoquinones studied with their respective substituent groups.

Table 3. Structurally related naphthoquinones

Structure	Naphthoquinone	Substituent
	Juglone 1,4-Naphthoquinone Menadione Lawsone	5-OH None 2-CH_3 2-OH

No 2-year carcinogenicity studies of juglone, 1,4-naphthoquinone, lawsone, or menadione were identified in the available literature. RTECS describes 1,4-naphthoquinone as an equivocal tumorigenic agent (lungs, thorax and respiration, skin and appendages) on the basis of a 29 week skin painting study in mice (NLM, 1999a). RTECS describes menadione as an equivocal tumorigenic agent on the basis of tumors at the application site in two skin painting studies in mice, one at 1860 mg/kg/27 weeks and the other at 8400 mg/kg/21 weeks (NLM, 1999a).

Table 4 provides a summary of information found on the genotoxicity of juglone, 1,4-naphthoquinone, lawsone, and menadione.

Table 4. Information on genotoxicity of selected naphthoquiones

Chemical Name	Mutagenicity Data
Juglone [481-39-0]	*S. typhimurium* with S-9: positive in strain TA2637; negative in TA98 & TA100 (Tikkanen *et al.*, 1983; Matsushima *et al.*, 1986; Edenharder & Tang, 1997) *S. typhimurium* without S-9: negative in strains TA98, TA100 & TA2637 (Tikkanen *et al.*, 1983; Matsushima *et al.*, 1986; Edenharder & Tang, 1997) *Drosophila*: positive, specific locus (Clark, 1982); positive, somatic & recombination test (Gaiv_o *et al.*, 1999)

1,4-Naphthoquinone [130-1-4]	*S. typhimurium* with arochlor induced S-9: negative in strains TA97, TA98 & TA100 *S. typhimurium* with phenobarbital (PB)/5,6-benzoflavone (BF) induced S-9: positive in TA97, TA100, TA102, TA104 & TA2637; negative in TA98, TA1537 & TA1538 *S. typhimurium* without S-9: positive in TA104 and TA2637; negative in TA97, TA98, TA102, TA1535, TA1537 & TA1538; inconclusive in TA100 (NLM, 1999b) Human lymphocytes: induced micronuclei (NLM, 1999a)
Lawsone [83-72-7]	*S. typhimurium* with arochlor induced S-9: positive in strain TA1537; negative in TA98; TA100 & TA1535 *S. typhimurium* with PB/BF induced S-9: negative in TA98, TA100, TA102, TA104, TA1535, TA1537, TA1538 & TA2637 *S. typhimurium* without S-9: negative in TA98, TA100, TA102, TA104, TA1535, TA1537, TA1538 & TA2637 (NLM, 1999b) *In vivo* mouse (ip): induced micronuclei (NLM, 1999a) *Drosophila*: negative for sex-linked recessive lethal/reciprocal translocation (NTP, 1999)
Menadione [58-27-5]	*S. typhimurium* with arochlor induced S-9: negative in strains TA98, TA100, TA1535, TA1537 *S. typhimurium* with PB/BF induced S-9: positive in TA98 & TA2637; negative in TA97, TA100, TA102, TA104, TA1535, TA1537 & TA1538 *S. typhimurium* without S-9: positive in TA 97 & TA2637; negative in TA 97A, TA98, TA102, TA1535, TA1537 & TA1538; inconclusive in TA100 & TA104 (NLM, 1999b) *Drosophila*: positive, specific locus (NLM, 1999a); marginally positive in somatic and recombination test (Gaivão *et al.*, 1999) Human fibroblast: DNA damage (NLM, 1999a) Hamster cells: DNA damage (NLM, 1999a) Rat liver: DNA damage (NLM, 1999a) Mouse embryo: morphological transformation (NLM, 1999a) Hamster lung: positive cytogenetic analysis (NLM, 1999a) Human lymphocytes: induced sister chromatid exchanges (SCE) (NLM, 1999a) Hamster lung: induced SCE (NLM, 1999a)

Other information related to the potential toxicity of the napthoquinones listed in Table 3 includes:

- Juglone inhibited aromatase cytochrome P450; lawsone was negative (Muto *et al.*, 1987)

- Juglone and 1,4-napthoquinone exhibited potent activities reducing cytochrome c;
- other naphthoquinones tested showed only weak effects (Muto *et al*,. 1987)

- Hydroxyl derivatives of napthoquinone were mutagenic to *S. typhimurium* TA2637
- and TA98 but not to TA100; a methyl group at position 2 enhanced mutagenicity, while a
- methyl group at position 7 decreased mutagenicity (Matsushima *et al.*, 1986).

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