B. *ARISTOLOCHIA* SPECIES AND ARISTOLOCHIC ACIDS

1. Exposure Data

1.1 Origin, type and botanical data

Aristolochia species refers to several members of the genus (family *Aristolochiaceae*) (WHO, 1997) that are often found in traditional Chinese medicines, e.g., *Aristolochia debilis*, *A. contorta*, *A. manshuriensis* and *A. fangchi*, whose medicinal parts have distinct Chinese names. Details on these traditional drugs can be found in the *Pharmacopoeia of the People's Republic of China* (Commission of the Ministry of Public Health, 2000), except where noted. This Pharmacopoeia includes the following *Aristolochia* species:

Aristolochia species	Part used	Pin Yin Name
Aristolochia fangchi	Root	Guang Fang Ji
Aristolochia manshuriensis	Stem	Guan Mu Tong
Aristolochia contorta	Fruit	Ma Dou Ling
Aristolochia debilis	Fruit	Ma Dou Ling
Aristolochia contorta	Herb	Tian Xian Teng
Aristolochia debilis	Herb	Tian Xian Teng
Aristolochia debilis	Root	Qing Mu Xiang

In traditional Chinese medicine, *Aristolochia* species are also considered to be interchangeable with other commonly used herbal ingredients and substitution of one plant species for another is established practice. Herbal ingredients are traded using their common Chinese Pin Yin name and this can lead to confusion. For example, the name 'Fang Ji' can be used to describe the roots of *Aristolochia fangchi*, *Stephania tetrandra* or *Cocculus* species (EMEA, 2000).

Plant species supplied as 'Fang Ji'

Pin Yin name	Botanical name	Part used
Guang Fang Ji	*Aristolochia fangchi*	Root
Han Fang Ji	*Stephania tetrandra*	Root
Mu Fang Ji	*Cocculus trilobus*	Root
Mu Fang Ji	*Cocculus orbiculatus*	Root

Similarly, the name 'Mu Tong' is used to describe *Aristolochia manshuriensis*, and certain *Clematis* or *Akebia* species. There are some reports in Chinese literature where substitution can occur with 'Ma Dou Ling' (EMEA, 2000).

Plant species supplied as 'Mu Tong'

Pin Yin name	Botanical name	Part used
Guan Mu Tong	*Aristolochia manshuriensis*	Stem
Chuan Mu Tong	*Clematis armandii*	Stem
Chuan Mu Tong	*Clematis montana*	Stem
Bai Mu Tong	*Akebia quinata*	Stem
Bai Mu Tong	*Akebia trifoliata*	Stem

The Pin Yin name 'Mu Xiang' is applied to a number of species; there is no evidence of substitution between the species but the common names have potential for confusion in both Chinese and Japanese (EMEA, 2000).

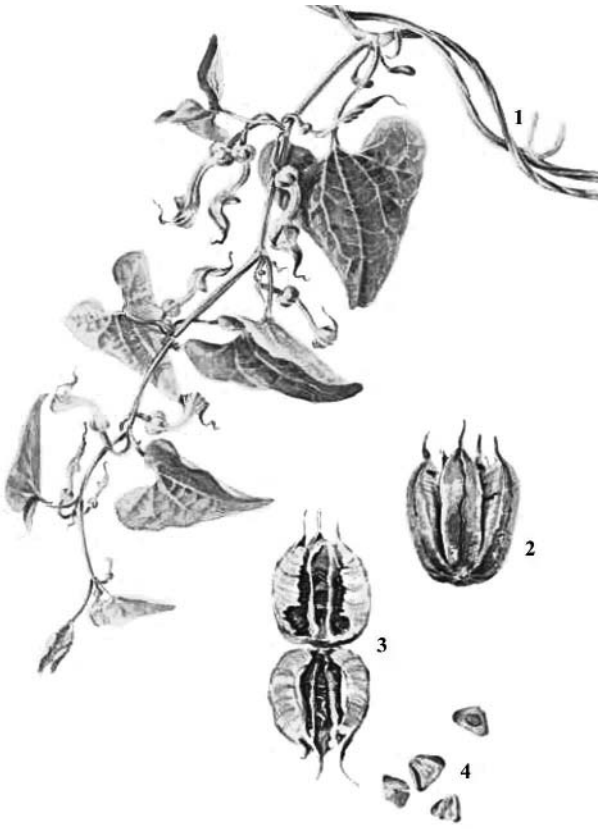
Plant species supplied as 'Mu Xiang'

Pin Yin name	Botanical name	Japanese name
Qing Mu Xiang	*Aristolochia debilis*	Sei-Mokkou
Mu Xiang	*Aucklandii lappa*	
Guang Mu Xiang	*Saussurea lappa*	Mokkou
Tu Mu Xiang	*Inula helenium*	
	Inula racemosa	
Chuan Mu Xiang	*Vladimiria souliei*	Sen-Mokkou
	Vladimiria souliei var. cinerea	

1.1.1 Aristolochia contorta *Bunge and* Aristolochia debilis *Sieb. et Zucc.*

Aristolochia contorta (see Figure 1) is a perennial climbing herb. The stem is convoluted, smoothish, more than 2 metres long. Leaves are alternated, petioled, entire, triangular cordate-shaped, 3–13 cm long and 3–10 cm wide, with a soft (effeminate) stem (petiole), 1–7 cm long. Axillary racemes have 3–10 flowers clustered with a dark purple perianth which is 2–3 cm long and zygomorphic. Peduncles are 2 cm long, with a small ovate bract, 1.5 cm long and 1 cm wide, near the base. Flowers have an obliquely trumpet-shaped upper part with acuminate apex and a tubular middle part. The lower part is enclosing the style and globular, six stamens and six stigmas. Capsules, broadly obovate-shaped, burst into six valves when ripe. The plant grows in valleys along streams and in thickets (see WHO, 1997, 1998).

Figure 1. *Aristolochia contorta* Bunge



From Qian (1996)
1, flower twig; 2, fruit; 3, longitudinal section of fruit; 4, seed

Aristolochia debilis (see Figure 2) differs from *A. contorta* chiefly in the following: Leaves are 3–8 cm long and 2–4 cm wide, cordate at the base, with round auricles on both sides and a petiole 12 cm long. Capsules are subglobose or oblong.

The dried fruits of both *A. debilis* and *A. contorta* (Ma Dou Ling), also known as *Fructus Aristolochiae* in Latin and Dutchman's pipe fruit in English, are ovoid and 3–7 cm long and 2–4 cm in diameter. The outer surface is yellowish green, greyish green or brown, with 12 longitudinal ribs, from which extend numerous horizontal parallel veinlets. The apex is flattened and obtuse and the base has a slender fruit stalk. The pericarp, which is light and fragile, is easily divided into six valves; the fruit stalk is also divided into six splittings. The inner surface of the pericarp is smooth and lustrous, with dense transverse veins. The fruit is six-locular, with each locule containing many seeds, which are overlapped and arranged regularly. The seeds are flat and thin, obtuse triangular or fan-shaped, 6–10 mm long, 8–12 mm wide and winged all around, and pale brown. The fruit has a characteristic odour and a slightly bitter taste.

Figure 2. *Aristolochia debilis* Siebold and Zuccarini

From Qian (1996)
1, flower twig; 2, root; 3, fruit

Tian Xian Teng (*Herba Aristolochiae* in Latin and Dutchman's pipe vine in English) consists of stems of *A. debilis* and *A. contorta* that are slenderly cylindrical, slightly twisted, 1–3 mm in diameter, yellowish green or pale yellowish brown in colour, with longitudinal ridges and nodes and internodes varying in length. The texture is fragile; the stems are easily broken and when fractured exhibit several vascular bundles of variable size. The leaves are mostly crumpled and broken, but, when whole, are deltoid narrow ovate or deltoid broad ovate and cordate at the base, dark green or pale yellowish brown and basal leaves are clearly veined and slenderly petioled. It has a delicately aromatic odour and is weak to the taste.

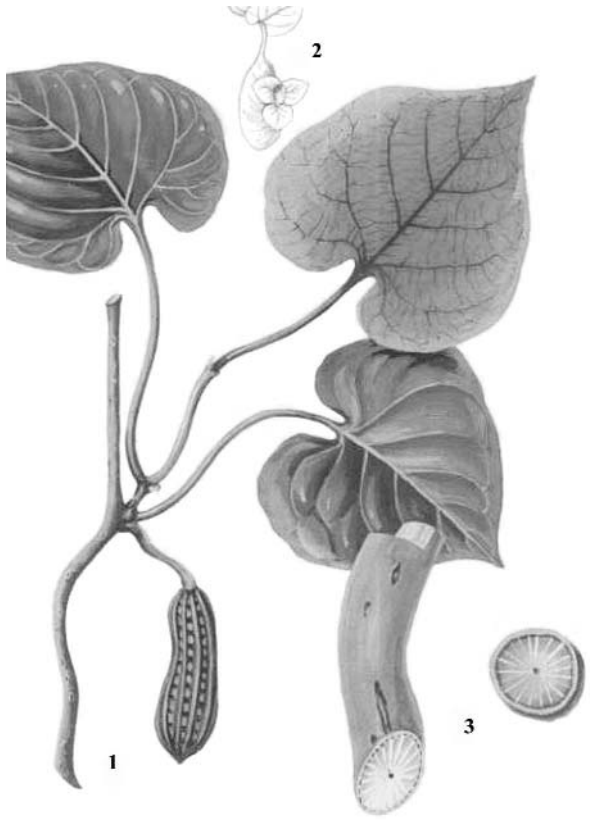
Qing Mu Xiang (*Radix Aristolochiae* in Latin and slender Dutchman's pipe root in English) is the root of *A. debilis* and is cylindrical or compressed cylindrical, slightly tortuous, 3–15 cm long and 0.5–1.5 cm in diameter. It is yellowish brown or greyish brown in colour, rough and uneven, and exhibits longitudinal wrinkles and rootlet scars. The texture is fragile; the root is easily broken and when fractured shows an uneven, pale

yellow bark and a wood with broad, whitish rays arranged radially and a distinct, yellowish brown cambium ring. Its odour is aromatic and characteristic and it has a bitter taste.

1.1.2 Aristolochia manshuriensis

The dried stem of *A. manshuriensis* (see Figure 3) is called Guan Mu Tong (*Caulis Aristolochiae manshuriensis* in Latin and commonly Manchurian Dutchman's pipe stem).

Figure 3. *Aristolochia manshuriensis*



From Qian (1996)
1, fruit twig; 2, flower; 3, transverse section

The stem is twined and woody, grey and striated, from 6 to 14 m long. Its branches are dark purple, with whitish villi. Leaves are either petioled, entire or ovate-cordate and are 15–29 cm long and 13–28 cm wide with a whitish down on each side. Petioles are 6–8 cm long. Flowers are axillary, single and peduncled; the apex is disc-shaped, with a

diameter of 4–6 cm or more, lobed, wide triangular and greenish outside; the lower part is tubular, 5–7 cm long, 1.5–2.5 cm in diameter and pink outside. Peduncles are drooping, with an ovate cordate, whitish, villous, sessile, greenish bract, 1 cm long. Six stamens adhibit to the outside of the stigma in pairs; they are inferior to the ovary, cylindrical, angular, whitish villi and 1–2 cm long. Capsules are cylindrical, dark brown, 9–10 cm long and 3–4 cm in diameter and burst into six valves when ripe. Seeds are numerous in each cell and cordate to triangular-shaped, with no winged margin.

The dried stem is long, cylindrical, slightly twisted, 1–2 m long and 1–6 cm in diameter. Externally, it is greyish yellow or brownish yellow, with shallow longitudinal grooves and has adhering remains of brown patches of coarse bark. Nodes are slightly swollen, with a branch scar. The stem is light and hard, not easily broken, but when fractured shows a narrow yellow or pale yellow bark, and broad wood having vessels arranged in many rings, with radial rays and indistinct pith. It has a slight odour and a bitter taste and gives off a smell like camphor when the remains of the coarse bark are rubbed.

1.1.3 Aristolochia fangchi

The dried root of *A. fangchi* (see Figure 4) is named *Guang Fang Ji* (*Radix Aristolochiae fangchi* in Latin and commonly southern fangchi root).

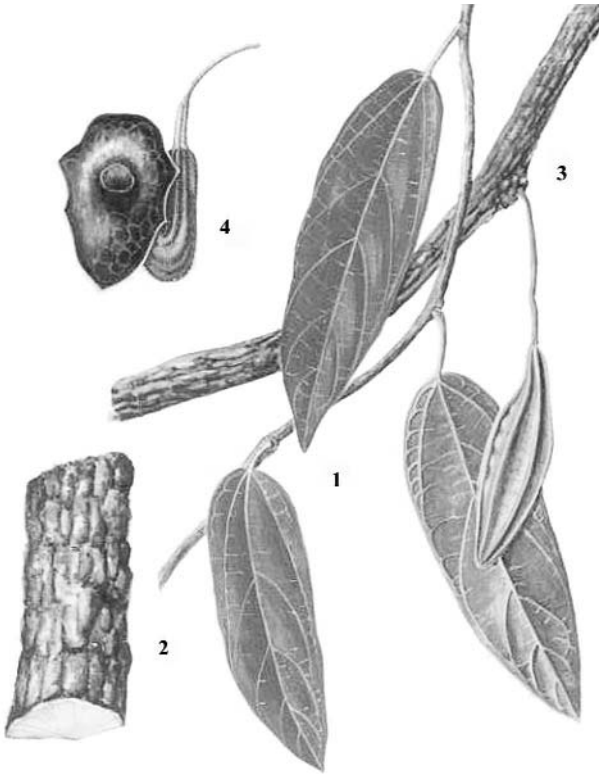
A. fangchi is a perennial climbing vine with crassitudinous roots. The stem is slender, greyish brown, with brownish villi and is 3–4 m long. The leaves are either petioled, entire, oblong or ovate-shaped, with grey whitish villi when young, and are 3–11 cm long and 2–6 cm wide. The petiole is 1–4 cm long. The perianth is canister-shaped, purple with yellowish spots and 2–3 cm long. Six stamens adhibit to the outside of the trilobed stigma. The capsules have numerous seeds. The plant grows in valleys along streams and in thickets and blooms from May to June.

The dried root is cylindrical or semi-cylindrical, slightly curved, 6–18 cm long and 1.5–4.5 cm in diameter. Externally it is greyish brown, rough and longitudinally wrinkled; the peeled stems are yellowish. The root is heavy and compact and is broken with difficulty; when fractured, the inside is starchy, exhibiting alternately greyish brown and whitish radial lines. It is odourless with a bitter taste.

1.1.4 Aristolochia clematitis

The medicinal parts of birthwort (*A. clematitis*) are the aerial portion (when in blossom) and the root. The plant has dirty yellow flowers, usually in axillary groups of seven. The perigone forms a straight tube, which is bulbous beneath and has a linguiform, oblong-ovate, obtuse border. There are six stamens, the style is upward-growing , and the stigma is six-lobed. The flower briefly traps insects that pollinate it. The fruit is a globose, pear-shaped capsule. The plant grows to a height of 30–100 cm. The stem is erect, simple, grooved and glabrous. The leaves are alternate, long-petioled, cordate-reniform, yellow-green, with prominent ribs. The plant has a fruit-like fragrance and is poiso-

Figure 4. ***Aristolochia fangchi***



From Qian (1996)
1, twig leaf; 2, longitudinal section; 3, fruit twig; 4, flower

nous. The plant is indigenous to Mediterranean regions, Asia Minor and the Caucasus, but is found in numerous other regions (Medical Economics Co., 2000).

1.1.5 Aristolochia indica

Aristolochia indica (Indian birthwort) is a perennial climber with greenish white woody stems found throughout India in the plains and low hills. The leaves are glabrous and very variable, usually obovate-oblong to sub-pandurate, entire with somewhat undulate margins, somewhat cordate, acuminate. Flowers are few, in axillary racemes with a perianth up to 4 cm long having a glabrous pale-green inflated and lobed base narrowed into a cylindrical tube terminating in a horizontal funnel-shaped purple mouth and a lip clothed with purple-tinged hair. Capsules are oblong or globose-oblong, 3–5 cm long and the seeds are flat, ovate and winged (Anonymous, 1985).

1.2 Use

1.2.1 Aristolochia contorta *and* Aristolochia debilis

Several parts of *A. debilis* and *A. contorta* have been used for various therapeutic purposes in Chinese traditional medicine. The dried ripe fruits of both plants are used as a remedy for haemorrhoids, coughs and asthma. The dried stems or leaves are used for treatment of epigastric pain, arthralgia and oedema. The dried roots from *A. debilis* are used for treatment of dizziness, headache, abdominal pain, carbuncles, boils and snake and insect bites.

1.2.2 Aristolochia manshuriensis

There are no clinical reports concerning the use of dried stems of *A. manshuriensis* alone. It is usually used in complex prescriptions as an anti-inflammatory and diuretic for acute infections of the urinary system, and as emmenagogue and galactagogue for amenorrhoea and scanty lactation in traditional Chinese medicine.

1.2.3 Aristolochia fangchi

There are no clinical reports concerning the use of the stems of *A. fangchi* alone. It is usually used in complex prescriptions as a diuretic for oedema and for antipyretic and analgesic remedies in traditional Chinese medicine.

1.2.4 Aristolochia clematitis

Birthwort (*A. clematitis*) has been used to stimulate the immune system and in the treatment of allergically caused gastrointestinal and gall-bladder colic. The plant is used in a wide variety of ways in the folk medicine of nearly all European countries. In homeopathy, the drug is used for gynaecological disorders and climacteric symptoms, in addition to the treatment of wounds and ulcers. It is also used as a treatment after major surgery and in ear–nose–throat treatments (Medical Economics Co., 1998, 2000).

1.2.5 Aristolochia indica

The roots of Indian birthwort (*Aristolochia indica* L.) have been used in Indian folk medicine as an emmenagogue and an abortifacient (Che *et al.*, 1984).

1.3 Chemical constituents

[As most of the literature is in Chinese, the Working Group had difficulty in identifying some of the chemicals cited.]

1.3.1 Aristolochia contorta *and* Aristolochia debilis

Dried ripe fruits

Alkaloids: *A. debilis* contains aristolochic acids[1] I, II, IIIa and IVa, debilic acid, 7-hydroxyaristolochic acid I and 7-methoxyaristolochic acid I. *A. contorta* contains aristolochic acids I, IIIa and E, 7-methoxy-8-hydroxyaristolochic acid, methyl aristolochate, aristolic acid, aristolic acid methyl ester, aristolamide, aristolochic acid III methyl ester, aristolochic acid IV methyl ester, 6-methoxyaristolochic acid [methyl] ester, aristolochic acid BII methyl ester (3,4-dimethoxy-10-nitrophenanthrenic-1-acid methyl ester) and aristolophenanlactone I. Aristolactam (also known as aristololactam), magnoflorine and cyclanoline have also been detected in these plants (Zheng *et al*., 1998; Commission of the Ministry of Public Health, 2000).

Terpenoids and steroids characterized in these fruits include aristolene, $\Delta^{1(10)}$-aristolene, $\Delta^{1(10)}$-aristolenone, debilone, $\Delta^{1(10),8}$-aristolodion-2-one, 9-aristolene, aristolone, Δ^{9}-aristolone, 3-oxoishwarane, β-sitosterol, stigmast-4-en-3-one, stigmast-4-en-3,6-dione and stigmastane-3,6-dione.

Other components include allantoin, flavones, coumarins, saccharides, gum, resinstanin, lignanoids and lipids (Zheng *et al*., 1999).

Dried roots

Essential oil constituents found in *A. debilis* roots include 2-furaldehyde, camphene, 2-pentylfuran, 1,8-cineole, camphor, borneol, bornyl formate, bornyl acetate, α-copaene, β-elemene, *cis*-caryophyllene, 1,2-aristolene, α-gurjunene, β-gurjunene, δ-cadinene, tetradehydroaristolane, aristolenone, isoaristolenone, $\Delta^{1(10),8}$-aristol-2-one, 9-aristolene, Δ^{9}-aristolone and 3-oxoishwarane.

Alkaloids found in this product include aristolochic acids I, II, IIIa, IV, 7-hydroxyaristolochic acid I, 7-methylaristolochic acid I, aristolochic acid III methyl ester, methyl aristolochate, debilic acid, magnoflorine, aristolactam and cyclanoline.

Other components include allantoin (Zheng *et al*., 1997, 1998).

1.3.2 Aristolochia manshuriensis

Components of this plant include aristolic acid II, aristolochic acids I, II, IV, IIIa and IVa, aristolactam IIIa, tannin, aristoloside, magnoflorine and β-sitosterol (Wang *et al*., 2000).

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[1] Aristolochic acids: I = A, II = B, IIIa = C, IVa = D

1.3.3 Aristolochia fangchi

Components of this plant include mufongchins A, B, C and D, *para*-coumaric acid, syringic acid, palmitic acid, aristolochic acids I, II and IIIa, allantoin, magnoflorine, aristolactam and β-sitosterol (Zheng *et al*., 1999; Commission of the Ministry of Public Health, 2000).

1.3.4 Aristolochia clematitis

Components of this plant include aristolochic acids I and II, as well as the alkaloids magnoflorine and corytuberine.

1.3.5 Aristolochia indica

The essential oil of the aerial parts of *A. indica* is dominated by sesquiterpenes and monoterpenes such as β-caryophyllene, α-humulene, ishwarone, caryophyllene oxide I, ishwarol, ishwarane, aristolochene, linalool and α-terpinolene (Jirovetz *et al*., 2000).

The roots of *A. indica* contain aristolindiquinone, aristololide, 2-hydroxy-1-methoxy-4*H*-dibenzo[de,g]quinoline-4,5-(6*H*)-dione, cepharadione, aristolactam IIa, β-sitosterol-β-D-glucoside, aristolactam glucoside I, stigmastenones II and III, methyl aristolate, ishwarol, ishwarane and aristolochene.

Other components found in *A. indica* include 12-nonacosenoic acid methyl ester, aristolic acid, (12*S*)-7,12-secoishwaran-12-ol, (+)-ledol, ishwarone, methyl aristolate, *para*-coumaric acid, 5βH,7β,10α-selina-4(14),11-diene, isoishwarane, aristolochic acids I, IVa, aristolochic acid IVa methyl ether lactam and aristolactam β-D-glucoside (Kupchan & Merianos, 1968; Ganguly *et al*., 1969; Fuhrer *et al*., 1970; Govindachari *et al*., 1970; Govindachari & Parthasarathy, 1971; Teng & DeBardeleben, 1971; Govindachari *et al*., 1973; Pakrashi *et al*., 1977; Pakrashi & Chakrabarty, 1978a,b; Pakrashi & Pakrasi, 1978; Pakrashi & Shaha, 1978; Cory *et al*., 1979; Pakrashi & Shaha, 1979a,b; Pakrashi *et al*., 1980; Achari *et al*., 1981, 1982, 1983; Che *et al*., 1983, 1984; Achari *et al*., 1985; Ganguly *et al*., 1986; Mahesh & Bhaumik, 1987).

1.4 Active components

1.4.1 Aristolochia contorta *and* Aristolochia debilis

Hypotensive activity: magnoflorine, some aristolochic acid derivatives (Xu, 1957).
Analgesic effect: aristolochic acid

1.4.2 Aristolochia manshuriensis

Cardiotonic action: Calcium, tannin, dopamine (Zhou & Lue, 1958; Bulgakov *et al*., 1996).

Antitumour action: Aristoloside, aristolic acid II, aristolochic acids I, II, IV, IIIa, IVa and aristolactam IIIa (Nagasawa *et al*., 1997; Wang *et al*., 2000).

1.4.3 Aristolochia fangchi

Hypotensive activity: magnoflorine (Zheng *et al*., 1999).

1.5 Sales and consumption

About 320 tonnes of dried stems of *A. manshuriensis* were consumed in China in 1983 (Chinese Materia Medica, 1995).

1.6 Components with potential cancer hazard: aristolochic acids

For the purpose of this monograph, unless otherwise specified, the term 'aristolochic acids' refers to an extract of *Aristolochia* species comprising a mixture of aristolochic acid I and its demethoxylated derivative, aristolochic acid II. *Aristolochia* species also contain the related aristolactams, which are phenanthrene cyclic amides (EMEA, 2000). In some of the older literature, it is unclear whether individual compounds or mixtures are being discussed when referring to 'aristolochic acid'.

1.6.1 *Nomenclature*

Aristolochic acid I

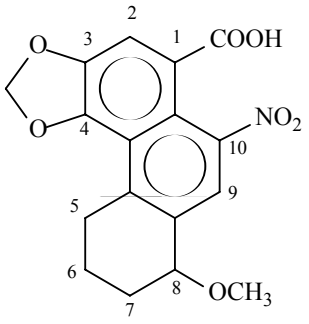
Chem. Abstr. Serv. Reg. No.: 313-67-7
Deleted CAS Nos.: 12770-90-0; 61117-05-3
Chem. Abstr. Serv. Name: 8-Methoxy-6-nitrophenanthro[3,4-d]-1,3-dioxole-5-carboxylic acid
Synonyms and trade names: Aristinic acid; aristolochia yellow; aristolochic acid A; aristolochin; aristolochine; Descresept; isoaristolochic acid; 8-methoxy-3,4-methylenedioxy-10-nitrophenanthrene-1-carboxylic acid; 3,4-methylenedioxy-8-methoxy-10-nitro-1-phenanthrenecarboxylic acid; Tardolyt; TR 1736

Aristolochic acid II

Chem. Abstr. Serv. Reg. No.: 475-80-9
Deleted CAS No.: 79468-63-6
Chem. Abstr. Serv. Name: 6-Nitrophenanthro[3,4-d]-1,3-dioxole-5-carboxylic acid
Synonyms: Aristolochic acid B; 3,4-methylenedioxy-10-nitrophenanthrene-1-carboxylic acid

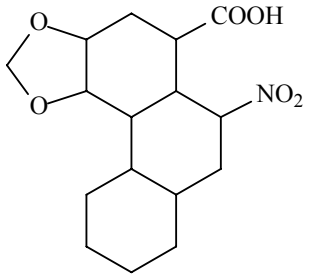
1.6.2 *Structural and molecular formulae and relative molecular mass*

Aristolochic acid I



$C_{17}H_{11}NO_7$ Rel. mol. mass: 341.27

Aristolochic acid II



$C_{16}H_9NO_6$ Rel. mol. mass: 311.25

1.6.3 *Chemical and physical properties of the pure substance*

Aristolochic acids

(*a*) *Description*: Crystalline solid [aristolochic acid I] (Buckingham, 2001)
(*b*) *Melting-point*: 281–286 °C, decomposes [aristolochic acid I] (Buckingham, 2001)
(*c*) *Solubility*: Slightly soluble in water; soluble in acetic acid, acetone, aniline, alkalis, chloroform, diethyl ether and ethanol; practically insoluble in benzene and carbon disulfide (O'Neil, 2001)
(*d*) *Octanol/water partition coefficient (P)*: log P, 3.48 (Buckingham, 2001)

1.6.4 *Analysis*

A procedure based on an extraction method used in Germany for the determination of aristolochic acids in botanical products has been developed and applied to a variety of botanicals and botanical-containing dietary supplements. Aristolochic acids are extracted from the sample matrix with aqueous methanol/formic acid. The concentration of aristolochic acids in the extract is determined by gradient high-performance liquid chromatography (HPLC) with UV absorption detection at 390 nm and their identity is confirmed by liquid chromatography/mass spectrometry using either an ion-trap mass spectrometer or a triple quadrupole mass spectrometer. The quantitation limit is equivalent to 1.7 μg/g in solid samples and 0.14 μg/mL in liquid samples (Flurer *et al*., 2000).

Lee *et al*. (2001) developed an HPLC procedure with a silica gel RP-18 reversed-phase column to determine aristolochic acids I and II in medicinal plants and slimming products. The recovery of these two compounds in medicinal plants and slimming products by extracting with methanol and purifying through a PHP-LH-20 (piperidino-hydroxypropyl Sephadex LH-20) column was better than 90%.

Targeted liquid chromatography/serial mass spectrometry (LC/MS/MS) analysis, using a quadrupole ion-trap mass spectrometer, permitted the detection of aristolochic acids I and II in crude 70% methanol extracts of multi-component herbal remedies without any clean-up or concentration stages. The best ionization characteristics were obtained using atmospheric pressure chemical ionization (APCI) and by including ammonium ions in the mobile phase. Limits of detection for aristolochic acids were influenced by the level of interference due to other components in the sample matrix. They were determined to be between 250 pg and 2.5 ng on-column within a matrix containing compounds extracted from 2 mg of herbal remedy (Kite *et al*., 2002).

Ong and Woo (2001) developed a method for the analysis of aristolochic acids in medicinal plants or Chinese prepared medicines using capillary zone electrophoresis (CZE). The limits of detection for aristolochic acids I and II were 30 and 22.5 mg/kg, respectively. The proposed method using pressurized liquid extraction with CZE was used to determine the amount of aristolochic acids in medicinal plants or samples of Chinese prepared medicines with complex matrix and the results were compared with those from HPLC. Results obtained for aristolochic acids I and II in medicinal plants by CZE and HPLC are presented in Table 1.

Ong *et al*. (2000) compared extraction and analysis of aristolochic acids I and II in medicinal plants (*Radix aristolochiae*) using a pressurized liquid extraction method in a dynamic mode with ultrasonic and Soxhlet extraction. The effects of temperature, volume of solvent required and particle size were investigated. The pressurized liquid extraction method showed some advantages over ultrasonic and Soxhlet extraction methods.

Singh *et al*. (2001a,b) developed a reversed-phase HPLC method with photodiode array detection for quantitative detection of aristolochic acids in *Aristolochia* plant samples. The procedure involves extraction of aristolochic acids with methanol and

Table 1. Comparison of results obtained for aristolochic acids I and II in medicinal plants by capillary zone electrophoresis (CZE) and high-performance liquid chromatography (HPLC)[a]

	CZE (mg/kg)	HPLC (mg/kg)
Aristolochic acid I in *Radix aristolochiae fangchi*	541.3	479.8
Aristolochic acid II in *Radix aristolochiae fangchi*	314.6	57.0
Aristolochic acid I in *Radix aristolochiae fangchi* [b]	612.0	564.1
Aristolochic acid I in *Radix aristolochiae (qing mu xiang)*	654.9	567.6
Aristolochic acid II in *Radix aristolochiae (qing mu xiang)*	190.0	208.4

[a] From Ong & Woo (2001)
[b] Analysis of a different batch of *Radix aristolochiae fangchi*

chromatographic separation with a mobile phase of acetonitrile–water–trifluoroacetic acid–tetrahydrofuran (50:50:1:1). The average recovery of aristolochic acids was 97.8%; the minimum quantity detectable was 0.10 µg per injection with a 5 µL injection volume.

1.6.5 *Production*

Aristolochic acids are produced commercially only as a reference standard and as research chemicals (Sigma-Aldrich, 2002).

1.6.6 *Use*

The aristolochic acid occurring in *Aristolochia* species used in traditional herbal medicines has been reported to function as a phospholipase A_2 inhibitor and as an anti-neoplastic, antiseptic, anti-inflammatory and bactericidal agent (Buckingham, 2001).

1.6.7 *Occurrence*

Aristolochic acids are alkaloid components of a wide range of species of the family Aristolochiaceae (e.g., *Aristolochia*, *Asarum*). They are also found in several species of butterflies (e.g., *Atrophaneura*, *Battus*, *Pachliopta*, *Troides*) which feed on the *Aristolochia* plants (von Euw *et al.*, 1968; Urzúa & Priestap, 1985; Urzúa *et al.*, 1983, 1987;

Nishida & Fukami, 1989a,b; Nishida *et al*., 1993; Sachdev-Gupta *et al*., 1993; Fordyce, 2000; Klitzke & Brown, 2000; Sime *et al*., 2000; Wu *et al*., 2000).

Aristolochic acids I and II have been determined in several samples of medicinal plants and slimming products using HPLC. The major component was aristolochic acid I in *Aristolochia fangchi* and the level ranged from 437 to 668 ppm (mg/kg). Aristolochic acid II was the major component in *Aristolochia contorta*, at levels ranging from less than 1 to 115 ppm (mg/kg). Twelve out of 16 samples of slimming pills and powders contained aristolochic acids I and/or II. The major component in most slimming products was aristolochic acid II and the level ranged from less than 1 to 148 ppm (Lee *et al*., 2001).

The amounts of aristolochic acids I and II in four groups of medicinal plants from the family Aristolochiaceae and some related plants were determined by HPLC. Aristolochic acids I and II were detected in all the plants from the genus *Aristolochia* (Aristolochiaceae) and at trace levels in some from the genus *Asarum* (Aristolochiaceae) (Hashimoto *et al*., 1999). The levels of these compounds in several medicinal plants are presented in Table 2.

Table 2. Aristolochic acid content in individual samples of medicinal plants of the Aristolochiaceae family[a]

Botanical name	AA-I (mg/kg)	AA-II (mg/kg)
Aristolochia debilis	1010	180
	1080	120
	790	80
Aristolochia manshuriensis	3010	210
	1690	140
	8820	1000
Aristolochia fangchi	2220	220
	1430	60
	1030	40

[a] From Hashimoto *et al*. (1999)
AA-I, aristolochic acid I
AA-II, aristolochic acid II

1.6.8 *Regulations*

The Therapeutic Goods Administration (TGA) (2001a) of Australia has issued a Fact Sheet stating that all species of *Aristolochia* are prohibited for supply, sale or use in therapeutic goods in Australia. The Therapeutic Goods Administration (2001b) also issued a Practitioner Alert to communicate its concern about traditional Chinese medicine herbal products that are known to contain, or suspected to contain, *Aristolochia* species, which

may contain aristolochic acids. The TGA has published three lists of botanicals or products at risk of containing aristolochic acids: botanicals known or suspected to contain aristolochic acid (Group A); botanicals which may be adulterated with aristolochic acid (Group B); and products which have Mu Tong and Fang Ji as declared ingredients (Group C).

The use of *Aristolochia* in unlicensed medicines was prohibited by the Medicines Control Agency (2001) in the UK in July 1999, and a further temporary prohibition covering certain herbal ingredients at risk of confusion with *Aristolochia* came into force in June 2000. The UK Committee on Safety of Medicines (2001) made these prohibitions permanent by issuing a Statutory Instrument to prohibit the sale, supply and importation of any medicinal product consisting of or containing certain plants belonging to a species of the genus *Aristolochia* or consisting of or containing Guan Mu Tong; or belonging to any of eight specifically listed plants (*Akebia quinata, Akebia trifoliata, Clematis armandii, Clematis montana, Cocculus laurifolius, Cocculus orbiculatus, Cocculus trilobus, Stephania tetrandra*); or consisting of or containing an extract from such a plant.

In addition, the European Agency for the Evaluation of Medicinal Products issued a position paper in October 2000 (EMEA, 2000), warning European Union Member States to 'take steps to ensure that the public is protected from exposure to aristolochic acids arising from the deliberate use of *Aristolochia* species or as a result of confusion with other botanical ingredients'.

The European Commission (EC) (2000) has prohibited 'aristolochic acid and its salts, as well as *Aristolochia* species', and their preparations in cosmetic products.

In 1999, Health Canada issued a warning not to use products containing *Aristolochia* due to potential risk of cancer, cell changes and kidney failure. Health Canada issued four additional warnings and advisories in 2001 advising not to use products labelled to contain *Aristolochia* (Health Canada, 1999, 2001a,b,c,d).

The Food and Drug Administration (2001a) of the USA has issued a Consumer Advisory to communicate its concern about the use and marketing of dietary supplements or other botanical-containing products that may contain aristolochic acids. It has also posted a listing of botanical ingredients of concern, including: botanicals known or suspected to contain aristolochic acids; botanicals which may be adulterated with aristolochic acids; products in which 'Mu Tong' and 'Fang Ji' are declared ingredients; and botanical products determined by FDA to contain aristolochic acids (Food and Drug Administration, 2001b).

2. Studies of Cancer in Humans

Aristolochia spp.

The possibility of adulteration of herbal products with *Aristolochia* species exists as a result of similarities in their common names, e.g. substitution of *Stephania tetrandra* (Han Fang Ji) with *Aristolochia* species (e.g. Guang Fang Ji).

In 1992, a cluster of patients with interstitial renal fibrosis rapidly progressing to end-stage renal disease after having followed a slimming regimen containing powdered extracts of Chinese herbs was recorded in Brussels, Belgium (Vanherweghem *et al.*, 1993; Cosyns *et al.*, 1994a; Depierreux *et al.*, 1994; Vanherweghem, 1998), followed by some reports from several other countries (see Section 4.2.1 for details). The herbal product was labelled as including *Stephania tetrandra*, but was later found to contain *Aristolochia fangchi*, which had been erroneously substituted for *Stephania tetrandra.*

Subsequent reports have shown the etiology of this disease to be related to exposure to aristolochic acids which are components of *Aristolochia* species (see Section 1.4.1(*c*)) (Vanhaelen *et al.*, 1994; Schmeiser *et al.*, 1996; Nortier *et al.*, 2000; Muniz Martinez *et al.*, 2002).

The renal disease associated with prolonged intake of some Chinese medicinal herbs is called by various names. The term 'Chinese herb nephropathy' has been widely used in scientific nephrology publications. However, this could be considered misleading in relation to the hundreds of Chinese medicinal herbs that are safely used throughout the world, including for renal diseases. Thus alternatives such as 'aristolochic acid-associated nephropathy' and 'Aristolochia nephropathy' are also used. However, throughout this monograph, the term 'Chinese herb nephropathy' is consistently used to refer specifically to the Aristolochia-associated disease.

2.1 Case reports

Mild-to-moderate atypia and atypical hyperplasia of the urothelium were detected in three women in Brussels, Belgium, who had undergone nephroureterectomies as part of a transplantation programme (Cosyns *et al.*, 1994a). All three cases of end-stage renal disease were attributed to the use of Chinese herbs containing aristolochic acids (Schmeiser *et al.*, 1996). One of these three women developed, 12 months after transplantation, at the age of 25 years, two papillary tumours of the posterior bladder wall, histologically classified as low-grade transitional-cell carcinomas, without invasion. Microscopic transitional-cell carcinomas of low-to-intermediate grade were also detected in the two ureters (right and remnant distal part of left ureter) and in the right pelvis (Cosyns *et al.*, 1994b).

A second report from Belgium described a 42-year-old woman presenting with end-stage renal disease and haematuria. Apart from the Chinese herbal product labelled as *Stephania tetrandra*, she had regularly taken paracetamol (1.2–2.4 g per day) for 27 years. In medical surveillance, no side-effects on renal function were recorded previous to the consumption of the herbal product. The haematuria was secondary to a transitional-cell carcinoma with moderate atypia of the right renal pelvis (Vanherweghem *et al*., 1995).

In a report from Taiwan, China, of 12 cases with rapidly progressive interstitial fibrosis associated with Chinese herbal drugs, one case of a bladder carcinoma was observed (Yang *et al*., 2000).

A bilateral multifocal transitional-cell urothelial carcinoma occurring six years after the onset of end-stage renal disease was described (Lord *et al.*, 1999, 2001) in one of two cases of Chinese herbal nephropathy in the United Kingdom related to aristolochic acid from *Aristolochia manshuriensis* contained in Mu Tong.

2.2 Prevalence of urothelial cancers among patients with Chinese herb nephropathy

Following the previously described case reports of rare urothelial tumours among some patients who had suffered end-stage renal disease after consumption of Chinese herbs, other patients were offered a bilateral removal of their native kidneys and ureters. High prevalence of urothelial cancers was documented in two series.

In the first series (Cosyns *et al.*, 1999), nephroureterectomies were performed in 10 renal-grafted Chinese herb nephropathy patients. The patients were all women and had a mean age of 40 years (range 27–59 years). In the pelviureteric urothelium, moderate atypia was observed in all samples. Multifocal high-grade carcinoma *in situ* was observed in four patients, in the renal pelvis (three patients), upper ureter (four patients), mid ureter (one patient) and lower ureter (three patients).

In the second series of 39 Chinese herb nephropathy patients with end-stage renal disease in Brussels (31 transplanted patients and eight dialysis patients), bilateral nephroureterectomy of the native kidneys and ureters was performed. Except for a 60-year-old man, all the patients were women (aged 54 ± 7 years). Aristolochic acid-specific DNA adducts were detected in tissue samples from kidneys. Among the 39 patients, 18 cases of urothelial carcinomas were found (prevalence, 46%; 95% confidence interval [CI], 29–62%). Except for one case of bladder cancer, all the carcinomas were located in the upper urinary tract and were almost equally distributed between the pelvis and the ureter. Mild to moderate dysplasia of the urothelium was found in 19 of the 21 patients without urothelial carcinoma. Cumulative doses of herbs labelled as *Stephania tetrandra* (which on analysis proved to contain various levels of *Aristolochia fangchi*) taken by the patients were significantly higher in the group of 18 Chinese herb nephropathy patients with urothelial cancer than in the group of 21 Chinese herb nephropathy patients without cancer (226 ± 23 g versus 167 ± 17 g; $p = 0.035$). Among the 24 patients with a cumulative dose of 200 g or less, eight cases of urothelial cancer were recorded and among the 15 patients who had ingested more than 200 g, 10 cases of urothelial cancer were observed ($p = 0.05$) (Nortier *et al.*, 2000).

3. Studies of Cancer in Experimental Animals

Aristolochic acids

3.1 Oral administration

Mouse: A group of 39 female NMRI mice [age not specified] was given daily doses of 5.0 mg/kg bw aristolochic acids (77.2% aristolochic acid I and 21.2% aristolochic acid II) by gavage for three weeks. A group of 11 vehicle controls was given solvent [unspecified]. The mice were kept for up to 56 weeks with interim sacrifice at 3, 9, 18, 26, 37 and 48 weeks. The remaining eight animals were killed at 56 weeks. At 18 and 26 weeks stages, low- to middle-grade papillomatosis was observed in the forestomach of all mice. Of the mice sacrificed at 37 and 48 weeks, 1/5 mice at each time point had squamous-cell carcinoma. Forestomach carcinoma was diagnosed in all of the eight mice killed at 56 weeks. Adenocarcinoma of the glandular stomach was observed in one mouse at 37 weeks. In addition, cystic papillary adenomas in the renal cortex (6/8 mice), malignant lymphomas (4/8 mice), alveologenic carcinomas (8/8 mice) and haemangiomas in the uterus (3/8 mice) were found at 56 weeks. No tumours were detected in 11 control animals at 56 weeks (Mengs, 1988).

Rat: Groups of 30 male and 30 female Wistar rats, 10 weeks of age, were given aristolochic acids (77.2% aristolochic acid I and 21.2% aristolochic acid II) as their sodium salts in distilled water at 10.0 or 1.0 mg/kg bw for three months and held for up to an additional six months or at 0.1 or 0 mg/kg bw for 3, 6 or 12 months and held for up to an additional four months. Forestomach carcinomas were observed in 13/18 males and 8/13 females given 10 mg/kg bw for three months and killed at six months. In addition, renal pelvis carcinomas (8/18) and urinary bladder carcinomas (3/18) were observed in the males given 10 mg/kg bw. Forestomach carcinomas were observed in 4/4 female rats treated with 10 mg/kg bw for three months and killed at nine months. In the groups dosed with 1.0 mg/kg bw for three months, forestomach carcinomas were observed in 3/11 males and 0/10 females after six months and 6/9 males and 2/11 females after nine months. In rats treated with 0.1 mg/kg bw for three months and killed at 12 months, 2/7 males and 0/6 females had forestomach carcinomas. In rats treated with 0.1 mg/kg bw for 12 months and killed at 16 months, 4/4 males and 1/5 females had forestomach tumours. Only one tumour (a spontaneous endometrial polyp) was observed in the controls (0/30 in males and 1/31 in females) (Mengs *et al.*, 1982).

Two groups of male Wistar rats, eight weeks old, were examined for the histogenesis of forestomach carcinoma caused by aristolochic acids [77.2% aristolochic acid I and 21.2% aristolochic acid II]. A group of 108 rats was given aristolochic acids daily by gavage at a dose of 10 mg/kg in distilled water for up to 90 days. A group of 37 controls

received distilled water. The animals were sacrificed sequentially. Administration of the aristolochic acids caused extensive necrosis of the squamous epithelium in the forestomach, followed by regeneration and hyperplasia, papilloma formation and ultimately invasive squamous-cell carcinoma. No pathological changes were seen in the controls (Mengs, 1983).

Three groups of 20 male BD-6 rats weighing 140 g received twice-weekly doses of 10 mg/kg bw aristolochic acid [components not otherwise specified] by gavage for 12 weeks. One of these groups also received 150 mg/kg bw diallyl sulfide by gavage 4 h before aristolochic acid treatment, and another group received diallyl sulfide 24 h and 4 h before the treatment. A fourth group received diallyl sulfide only, four times per week, for 12 weeks. Treatment with diallyl sulfide 4 h before aristolochic acid treatment decreased the development of forestomach tumours that appeared within 6–9 months after the start of the experiment. The incidence of aristolochic acid-induced forestomach tumours (60%; 12/20 rats) was reduced to 10% (2/20 rats) by the prior 4 h-treatment with diallyl sulfide. The prior 4 and 24-h treatment with diallyl sulfide prevented the induction of squamous-cell carcinomas in the forestomach (aristolochic acid alone, 9/20; aristolochic acid with diallyl sulfide, 0/20), but did not prevent the induction of forestomach and urinary bladder papillomatosis [number of control animals and incidences of tumours not reported] (Hadjiolov *et al*., 1993).

3.2 Intraperitoneal administration

Rabbit: Twelve female New Zealand white rabbits, 15 weeks of age, were given daily intraperitoneal injections of 0.1 mg/kg bw aristolochic acid [components not otherwise specified] in 25 mM NaOH on five days per week for 17–21 months. All 11 surviving rabbits developed fibrotic changes in the kidneys resembling Chinese herb nephropathy and two developed kidney tumours (renal-cell carcinoma or tubulopapillary adenoma). One rabbit had a transitional-cell carcinoma of the ureter as well as a peritoneal mesothelioma. Mild to moderate atypia of the epithelium of the collecting ducts and of the pelvis was present in 5/11 and 11/11 rabbits, respectively. These changes were not detected in 10 female control rabbits (Cosyns *et al.*, 2001).

3.3 Subcutaneous administration

Rat: In a study to model the renal fibrosis seen in Chinese herb nephropathy, 66 male Wistar rats, four weeks of age, were given a single intraperitoneal injection of furosemide and fed a low-salt normal-protein diet. This group was divided into three groups which received daily subcutaneous injections of either 1 mg/kg bw aristolochic acid [components not otherwise specified] (low-dose group; $n = 24$), 10 mg/kg bw aristolochic acid (high-dose group; $n = 24$) or vehicle (control group; $n = 18$) for 35 days. On days 10 and 35, six rats from each group were sacrificed for assessment of kidney function. Urothelial dysplasia was detected in two rats on day 10, one rat on day 35 and three of the remaining

11 rats on day 105 in the high-dose group. Urothelial dysplasia was also seen in a few rats of the low-dose group on day 10 or 105. Three of the high-dose group developed papillary urothelial carcinomas by day 105. Malignant fibrohistiocytic sarcomas were found on day 105 around the sites of subcutaneous injection in two of the six rats of the low-dose group and in seven of the 11 of the high-dose group. Marked interstitial fibrosis of the kidney was noted in the high-dose group on day 35. None was seen in the low-dose or controls groups (Debelle *et al.*, 2002).

4. Other Data Relevant to an Evaluation of Carcinogenicity and its Mechanisms

4.1 Absorption, distribution, metabolism and excretion

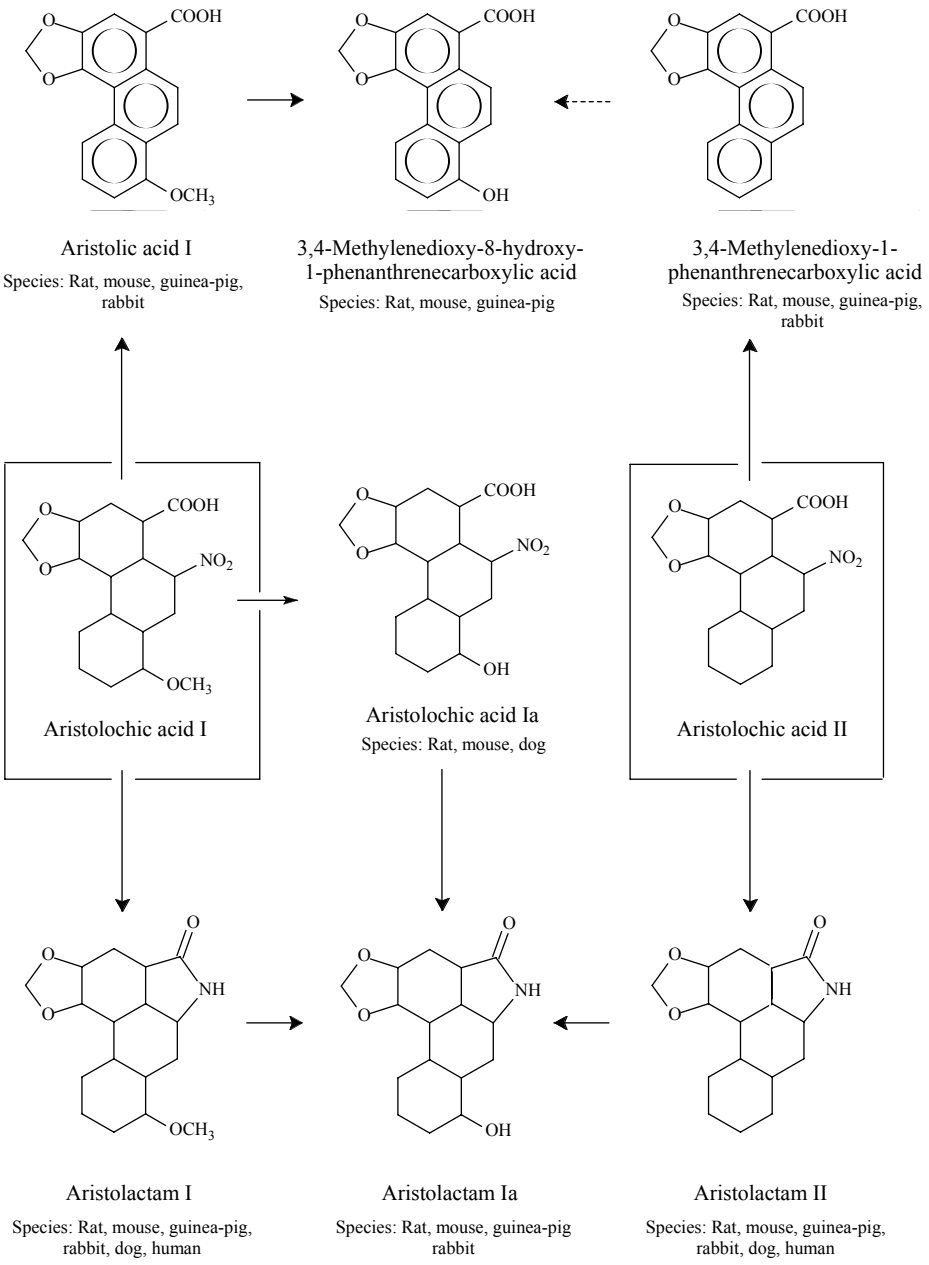
4.1.1 *Humans*

As part of a clinical study designed to investigate the effects of aristolochic acids on the phagocytic activity of granulocytes, six healthy volunteers were given a daily dose [presumably oral but not explicitly stated] of 0.9 mg of a mixture of aristolochic acids I and II [ratio not specified] for several days, and 24-h urine samples from day 3 of this trial were analysed for metabolites. The only metabolites detected were aristolactam I (metabolite of aristolochic acid I) and aristolactam II (metabolite of aristolochic acid II) (Figure 5). The percentage conversions to these two metabolites were not reported (Krumbiegel *et al.*, 1987). This contradicts an earlier report of oral absorption of aristolochic acid in humans resulting in the compound(s) being excreted unchanged in urine, bile, breast milk and cerebrospinal fluid (Schulz *et al.*, 1971).

4.1.2 *Experimental systems*

In an extensive study following oral administration of aristolochic acids I and II to male Wistar rats (pure compounds given; 3 mg), the following metabolites were detected in urine and faeces: from aristolochic acid I — aristolactam I, aristolactam Ia, aristolochic acid Ia, aristolic acid I and 3,4-methylenedioxy-8-hydroxy-1-phenanthrenecarboxylic acid; from aristolochic acid II — aristolactam II, aristolactam Ia and 3,4-methylenedioxy-1-phenanthrenecarboxylic acid (Krumbiegel *et al.*, 1987). The structures of these compounds (Figure 5) were determined by spectroscopic methods including low-resolution electron-impact mass spectrometry and low-field ^{1}H-NMR spectrometry (100 MHz) (Krumbiegel & Roth, 1987). A further metabolite of aristolochic acid II with a lactam moiety was not fully characterized. The principal metabolite of aristolochic acid I in rats was aristolactam Ia (46% of the dose converted to this was found in the urine and 37% in the faeces). In the urine, most of the aristolactam Ia was present as a conjugated form, which required alkaline treatment (3 M NaOH) for hydrolysis. The

Figure 5. Proposed metabolic transformations of aristolochic acids I and II in various species



From Krumbiegel *et al.* (1987)
The metabolites were detected in the urine of the species indicated.

metabolites determined for aristolochic acid II in rats were all minor compounds, with the largest proportion accounted for by aristolactam II (4.6% in the urine and 8.9% in the faeces) (Krumbiegel *et al.*, 1987).

This study also examined the metabolism of aristolochic acids I and II by other laboratory animals. The amounts of aristolochic acids I and II administered were similar in proportion to the weight of each test animal. The mouse (NMRI, female) was the only species to exhibit the same metabolic profiles for these compounds as the rat. In general, dogs (beagle, male), rabbits (White Vienna, male) and guinea-pigs (Pirbright White, male) showed smaller numbers of metabolites than mice and rats (summarized in Figure 5) (Krumbiegel *et al.*, 1987).

4.2 Toxic effects

4.2.1 *Humans*

Aristolochic acid I [purity not specified] was given by infusion to 20 patients having various malignant tumours, at different dose schedules ranging from 0.1 mg/kg bw per day for five days to a single dose of 2 mg/kg bw. The compound was too toxic to the kidneys for further trial (Jackson *et al*., 1964).

The so-called *Stephania tetrandra* powder, first introduced in a slimming regimen in a Belgian clinic in early 1990, was withdrawn from the Belgian market at the end of 1992. Altogether, 1500–2000 persons are thought to have followed the same regimen during the period 1990–92. Among them, about 100 patients with renal disease (70% of them being in end-stage renal disease that had to be treated by dialysis or kidney transplantation) had been recorded by 1998, or about 5% of the exposed population (Vanherweghem, 1998).

The pathology of the renal disease was characterized by extensive interstitial fibrosis with atrophy and loss of the tubules, while the glomeruli were relatively untouched (Vanherweghem *et al*., 1993; Cosyns *et al*., 1994a; Depierreux *et al*., 1994). The typical slimming treatment followed by the patients included intradermal injection of artichoke extract (Chophytol S) and euphyllin as well as oral intake of a mixture of fenfluramine, diethylpropion, meprobamate, cascara powder, acetazolamide, belladonna extract, *Magnolia officinalis* and *Stephania tetrandra*. Replacement of *Stephania tetrandra* by the nephrotoxic *Aristolochia fangchi* was suspected because *Stephania tetrandra* (Han Fang Ji) belongs to the same family and the Chinese characters are identical to those for *Aristolochia fangchi* (Guang Fang Ji) (Vanherweghem *et al*., 1993).

The inadvertent substitution of *Stephania* by *Aristolochia* was confirmed by phytochemical analysis of 12 different batches of herb powders delivered in Belgium under the name of *Stephania tetrandra*: only one batch contained tetrandrine and not aristolochic acids I and II, one contained both tetrandrine and aristolochic acids and 10 contained aristolochic acids only. The amount of aristolochic acids in the 12 batches

varied from undetectable (< 0.02 mg/g) to 1.56 mg/g (mean ± SD, 0.66 ± 0.56 mg/g) (Vanhaelen *et al*., 1994).

Further evidence supports the involvement of aristolochic acids I and II in the kidney disease: 7-(deoxyadenosin-N^6-yl)aristolactam I–DNA adduct (dA-AAI) was detected in renal tissue obtained from five patients with Chinese herb nephropathy, while none was found in renal tissue from six patients with other renal diseases (Schmeiser *et al*., 1996; see Section 4.4.1). A larger series of kidney samples from 38 patients with Chinese herb nephropathy confirmed the presence of DNA adducts formed by aristolochic acid six years after their exposure to the so-called *Stephania tetrandra* powder (actually, *Aristolochia fangchi*). Such adducts were absent in kidney tissues obtained from eight patients with renal disease of other origin (Nortier *et al*., 2000; see Section 4.4.1).

After the description of the initial cases (Vanherweghem *et al*., 1993), similar cases of Chinese herb nephropathy were reported in many other countries: four cases in France secondary to the intake of slimming pills containing *Stephania tetrandra* which was, in fact, *Aristolochia fangchi* (Pourrat *et al*., 1994; Stengel & Jones, 1998); one case in Spain after chronic intake of a tea made with a mixture of herbs containing *Aristolochia pistolochia*, a herb that was grown in the Catalonia region (Peña *et al*., 1996); two cases in the United Kingdom after treatment of eczema with Mu Tong containing aristolochic acid (Lord *et al*., 1999); 12 cases in Taiwan related to the use of various unidentified herbal medications for different purposes (Yang *et al*., 2000); one case in the USA after intake of herbal medicine containing aristolochic acid for low back pain (Meyer *et al.*, 2000); and 12 cases in Japan, in five of which the presence of aristolochic acid was demonstrated in the herbal medicine; in the other cases, confusion of Mokutsu (*Akebia quinata*) with Kan-Mokutsu (*Aristolochia manshuriensis*) and Boui (*Sinomenium acutum*) with Kou-Boui (*Aristolochia fangchi*) or Kanchu-Boui (*Aristolochia heterophylla*) was suspected (Tanaka *et al*., 2001). In Japan, the cases of Chinese herb nephropathy often presented with adult-onset Fanconi syndrome (Tanaka *et al*., 2000a,b). A similar case was reported in Germany after intake of a purported *Akebia* preparation containing aristolochic acid (Krumme *et al*., 2001).

4.2.2 *Experimental systems*

In a study of acute effects of a mixture of aristolochic acids I (77.2%) and II (21.2%), intragastric or intravenous administration at high doses to male and female mice (NMRI) and rats (Wistar) resulted in death from acute renal failure within 15 days. The oral LD_{50} ranged from 56 to 203 mg/kg bw and the intravenous LD_{50} from 38 to 83 mg/kg bw, depending on the species and sex. The predominant histological features were severe necrosis of the renal tubules, atrophy of the spleen and thymus, superficial ulceration of the forestomach by both routes, followed by hyperplasia and hyperkeratosis of the squamous epithelium (Mengs, 1987).

In a follow-up study, a no-effect level of 0.2 mg/kg bw — given daily by gavage for four weeks — was observed for aristolochic acids in male Wistar rats. Mild changes only

were observed at 1.0 mg/kg bw, with clear toxic effects first seen at 5.0 mg/kg bw given daily for four weeks. Degenerative lesions in the kidneys, forestomach, urinary bladder and testes were observed at a dose of 25 mg/kg bw. Two of the rats in the 25-mg/kg bw group died due to tubular necrosis following renal failure. Renal lesions were also found within three days in female Wistar rats given single doses of 10, 50 or 100 mg/kg bw intragastrically (Mengs & Stotzem, 1992). There was a dose-dependent decrease in body weight, which was significant at doses above 10 mg/kg bw. At necropsy, a grey-brown discoloration of the kidneys was seen in the 100-mg/kg bw group and the relative kidney weights of this group were significantly greater than those of the controls. There was evidence of dose-dependent tubular epithelial necrosis of the renal tubules, with (in the 100-mg/kg bw group) degenerative changes predominantly localized in the *pars recta* of the proximal tubules and widespread necrosis affecting all of the nephrons. In the 50 mg/kg bw group, single cell necrosis predominated, while no necrotic lesions were observed in the 10-mg/kg bw group. There were specific increases in the urinary enzymes malate dehydrogenase, γ-glutamyltranspeptidase and *N*-acetyl-β-glucosaminidase in the 50- and 100-mg/kg bw groups. In these same two groups, urinary electrolyte determinations showed decreases in calcium and magnesium concentrations, although the levels of sodium, potassium and chloride remained unaffected. Urine testing showed significantly increased protein concentrations and some increases in haemoglobin in the 50- and 100-mg/kg bw groups, with glucose concentrations elevated significantly in all three dose groups. Urinary volume, specific gravity and pH were unaffected in all dosed animals (Mengs & Stotzem, 1993).

In a recent study on the effects of chronic administration of a commercially available mixture (97% purity) of aristolochic acids I (44%) and II (56%), female New Zealand white rabbits were given intraperitoneal injections of either the test mixture (0.1 mg/kg bw) or saline (control) for five days per week for 17–21 months. All dosed animals developed renal hypocellular interstitial fibrosis and urothelial atypia, whereas no significant pathological changes were seen in the control animals. Three animals developed tumours of the urinary tract. The treated group also showed impaired growth, increased serum creatinine, glucosuria, tubular proteinuria and anaemia (Cosyns *et al.*, 2001).

Groups of 5–6 male Fischer 344 rats, weighing 140–150 g, were given a single intraperitoneal injection of 10 mg/kg bw aristolochic acids (a commercially available mixture of aristolochic acids I and II) 18 h after a two-thirds partial hepatectomy. After a one-week recovery period, one group was kept on the basal diet while the second group was given a diet with 1% orotic acid to stimulate cell proliferation. In the second group, the percentage of rats with nodules and the number of nodules per rat were increased compared with the group given aristolochic acids alone, suggesting that aristolochic acids act as initiating agents (Rossiello *et al.*, 1993).

The aristolochic acids have a phagocytosis- and metabolism-activating effect. They are also thought to enhance the production of lymphokines. Activation of phagocytes has been demonstrated in tests in rabbits and guinea-pigs. In addition, in animal tests, aristo-

lochic acids enhanced immune resistance to herpes simplex viruses in the eye (Medical Economics Co., 2000).

4.3 Reproductive and developmental effects

4.3.1 *Humans*

The roots of *Aristolochia indica L.* are reputedly used in Indian folk medicine as an abortifacient (references cited in Che *et al*., 1984). However, no data concerning reproductive effects in humans were available to the Working Group.

4.3.2 *Experimental systems*

With a view to developing fertility-regulating agents from plants, various extracts of *A. indica* have been investigated for effects during pregnancy in mice, rats, hamsters and rabbits.

An ethanolic extract of *A. indica* roots decreased the number of pregnancies in both rats and hamsters when administered daily by gavage on days 1–10 and 1–6 post-coitum, respectively. The extract was fractionated and the various materials were tested. Aristolochic acid I was lethal to rats at a daily dose of 40 mg/kg bw and to hamsters at a daily dose of 25 mg/kg bw. A dose of 10 mg/kg bw per day was not lethal to rats and had no effect on the number of pregnancies, while a daily dose of 12.5 mg/kg bw caused some lethality in hamsters (4/10 died), and tended to decrease the number of pregnancies in survivors. However, this may have been a result of non-specific toxic effects of the test substance (Che *et al*., 1984).

Aristolochic acid I from *A. mollisima* H. given orally to mice on gestation days 1–6 or 7–10 caused a marked decrease in the number of pregnancies at a daily dose of ≥ 3.7 mg/kg bw. In similarly dosed rats, no effect was found. Single injections given intra-amniotically to rats on one of gestation days 14–16 and to dogs on one of gestation days 30–45 led to fetal death and termination of pregnancy at doses per fetus of 50 or 100 μg (for rats) and 1–18 mg (dogs) (Wang & Zheng, 1984).

In female Swiss albino mice given a single oral dose of 100 mg/kg bw of a crude extract of *A. indica* on day 6 or 7 of gestation, the number of pregnancies was markedly reduced. Dosing with 50 mg/kg bw of various purified fractions also led to a marked effect. No toxic effects were reported at the doses used; however, no other data were given on, for example, body weight of the animals (Pakrashi *et al*., 1976).

Aristolochic acid I metabolites

A number of studies have investigated *aristolic acid*, a metabolite of aristolochic acid I detected in rats, mice, guinea-pigs and rabbits, but not in humans (see Figure 5).

Oral administration of 90 mg/kg bw aristolic acid to Swiss albino mice on gestation day 6 resulted in termination of pregnancy and in-utero death (Pal *et al*., 1982).

A single oral dose of 120 or 90 mg/kg bw aristolic acid given by gavage to Swiss albino mice on gestation day 1 or 6 caused marked reduction in uterine weight and increased concentrations of acid phosphatase and a decrease in alkaline phosphatase in the uterus. A subcutaneous dose of progesterone (1 mg per mouse) on gestation days 5–8 with aristolic acid on gestation day 6 did not prevent the effects. The authors concluded that aristolic acid did not appear to block hormone synthesis in the ovary (Pakrashi & Ganguly, 1982).

Aristolic acid disrupted nidation in Swiss albino mice when given at a dose of 150 mg/kg bw on gestation day 1. The treatment did not affect tubal transport of eggs, but affected implantation. In addition, it inhibited the increase in specific uterine alkaline phosphatase activity of the uterus, which in control mice was about three-fold through gestation days 4–6. Based on these results, it was inferred that aristolic acid interferes with estrogenic conditioning of the uterus (Ganguly *et al*., 1986).

Studies of endocrine properties of aristolic acid revealed anti-estrogenic effects, as the compound inhibited estrogen-induced weight increase and epithelial growth of the uterus in immature female Swiss albino mice. Aristolic acid given on gestation day 1 caused total inhibition of implantation at 60 mg/kg bw and decreased implantation at dose levels down to 15 mg/kg bw (43% reduction) (Pakrashi & Chakrabarty, 1978a).

The methyl ester of aristolic acid caused a 100% abortifacient effect when a single oral dose of 60 mg/kg bw was administered to Swiss albino mice on gestation day 6 or 7. At a dose of 30 mg/kg bw, the effect was 40%. The 60-mg/kg bw dose caused 25% and 20% reduction of fertility when given on gestation day 10 or 12, respectively. No toxic effects were reported in the dams and no malformations were found in the offspring (Pakrashi & Shaha, 1978).

Aristolic acid administered orally to rabbits on gestation day 9 caused 65% fetal loss at a dose of 60 mg/kg bw and 80% fetal loss at 90 mg/kg bw (Pakrashi & Chakrabarty, 1978b).

In male mice, oral feeding of the water-soluble part of the chloroform extract of *A. indica* at a dose of 75 mg/kg bw caused a marked decrease in the weight of the testes (55%) and accessory genital organs. There were varying degrees of arrest of spermatogenesis and nuclear degeneration in various germinal cell types. The treatment also caused a decrease of approximately 30% in body weight. The effects on the male sex organs may have been a result of non-specific toxicity (Pakrashi & Pakrasi, 1977).

4.4 Genetic and related effects

4.4.1 *Humans*

(*a*) *DNA-adduct formation in patients with Chinese herb nephropathy* (see Table 3 for details of studies and references)

Aristolochic acid-specific DNA adducts have been detected by the ^{32}P-postlabelling method in the kidneys and ureters of patients with Chinese herb nephropathy (a total of 47 women and one man). The major DNA adduct, which co-chromatographed with 7-(deoxyadenosin-N^6-yl)aristolactam I (dA-AAI), was detected in all urothelial tissues analysed, whereas the two minor ones, chromatographically indistinguishable from 7-(deoxyguanosin-N^2-yl)aristolactam I (dG-AAI) and 7-(deoxyadenosin-N^6-yl)aristolactam II (dA-AAII), were found in most cases (see Figure 6). Total aristolochic acid-specific adduct levels in DNA obtained from whole organs or biopsies from Chinese herb nephropathy patients were in the range of 1.7–530 adducts per 10^9 normal nucleotides. All studies presented evidence that these patients had taken herbal preparations containing a natural mixture of aristolochic acids.

(*b*) *p53 overexpression in patients with Chinese herb nephropathy*

Overexpression of the p53 protein, a common finding in human tumours, was observed in carcinoma *in situ*, papillary transitional-cell carcinoma and urothelial atypia found in 10 Belgian patients with Chinese herb nephropathy (Cosyns *et al*., 1999).

4.4.2 *Experimental systems*

(*a*) *DNA adduct formation by aristolochic acids in rats* in vivo (see Table 4 for details of studies and references)

Aristolochic acid–DNA adducts were formed *in vivo* in many organs of male rats given oral doses of aristolochic acid (natural mixture) or the pure major components aristolochic acid I or aristolochic acid II. Aristolochic acid–DNA adducts were also formed *in vivo* in the kidney (the only organ examined) of male and female rats given multiple oral doses of a slimming regimen of plant material that contained aristolochic acids. The results confirm that the three major DNA adducts formed *in vivo* co-chromatograph with 7-(deoxyadenosin-N^6-yl)aristolactam I (dA-AAI), 7-(deoxyguanosin-N^2-yl)-aristolactam I (dG-AAI) and 7-(deoxyadenosin-N^6-yl)aristolactam II (dA-AAII). Oral administration of a single dose of aristolochic acid I to rats led to formation of the dA-AAI adduct that persisted in DNA of several organs, consistent with the results obtained from studies in patients with Chinese herb nephropathy.

Table 3. DNA adduct formation in Chinese herb nephropathy (CHN) patients

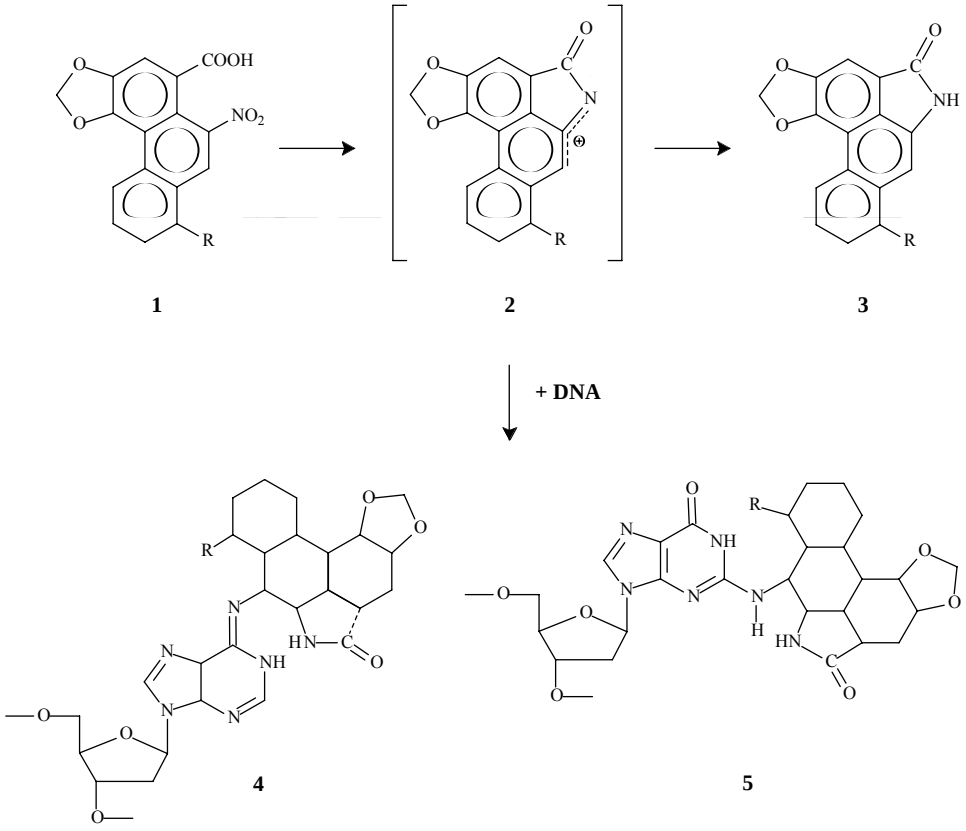
Details of study	DNA binding	Reference
Detection of AA-specific DNA adducts in renal tissue from 5 female patients with CHN from Belgium by ^{32}P-postlabelling. DNA was obtained from cortical or cortico-medullary tissue. The patients had taken pills containing Chinese herbs for 13–23 months. The adduct chromatographically indistinguishable from dA-AAI was detectable up to 27 months after termination of the regimen.	dA-AAI: 0.7–5.3/10^7 nucleotides	Schmeiser *et al*. (1996)
Detection of AA-specific DNA adducts in the kidneys and one ureter from 6 female patients with CHN from Belgium by ^{32}P-postlabelling (five cases were from Schmeiser *et al*., 1996). Three AA-specific adducts chromatographically indistinguishable from dA-AAI, dG-AAI and dA-AAII were detectable up to 44 months after termination of the regimen.	dA-AAI: 0.7–5.3/10^7 nucleotides dG-AAI: 0.02–0.12/10^7 nucleotides dA-AAII: 0.06–0.24/10^7 nucleotides	Bieler *et al*. (1997)
Detection of AA-specific DNA adducts in the kidneys of CHN patients (37 female and one male), and in 17 ureters from 11 of these patients from Belgium by ^{32}P-postlabelling. Among these patients were 18 cases of urothelial carcinoma. Data were related to cumulative doses of compounds in the weight-reducing pills on the basis of all prescriptions made during the period of exposure (1990–92). The cumulative dose of aristolochia was a significant risk factor for developing urothelial carcinoma. The kidneys and ureters from the CHN patients had the same pattern of adducts consisting of dA-AAI, dG-AAI and dA-AAII. The major adduct dA-AAI was detectable up to 89 months after discontinuation of use of the weight-reducing pills. No statistically significant difference was observed between mean levels of dA-AAI DNA adducts determined in renal tissue samples from patients who had developed urothelial carcinoma and those from tumour-free patients.	In the kidneys: dA-AAI: 1.2–165/10^9 nucleotides dG-AAI: 0.4–8.2/10^9 nucleotides dA-AAII: 0.6–6.8/10^9 nucleotides In the ureters: dA-AAI: 2.2–34/10^9 nucleotides	Nortier *et al*. (2000)
Detection of AA-specific DNA adducts in the kidneys from 2 new female CHN patients from the Belgian cohort by ^{32}P-postlabelling.	2.9–5.0/10^8 nucleotides	Arlt *et al*. (2001a)

Table 3 (contd)

Details of study	DNA binding	Reference
Detection of the dA-AAI DNA adduct in a renal biopsy from a female CHN patient outside the Belgian cohort by ^{32}P-postlabelling. The patient ingested a Chinese herbal preparation bought in Shanghai for the preceding 6 months (2 pills/day). The presence of AA in the pills was determined by HPLC analysis (0.3 mg AA/pill).	dA-AAI: $1.8/10^8$ nucleotides	Gillerot *et al*. (2001)
Detection of the dA-AAI DNA adduct in the kidney and ureter from a female CHN patient outside the Belgian cohort by ^{32}P-postlabelling. The patient took a herbal preparation containing aristolochic acid for 2 years prescribed for eczema. She developed invasive transitional cell carcinoma of the urinary tract.	In the kidney: dA-AAI: $3.8/10^9$ nucleotides In the ureter: dA-AAI: $40/10^9$ nucleotides	Lord *et al*. (2001)

AA, aristolochic acid; CHN, Chinese herb nephropathy; dA-AAI, 7-(deoxyadenosin-N^6-yl)aristolactam I; dA-AAII, 7-(deoxyadenosin-N^6-yl)aristolactam II; dG-AAI, 7-(deoxyguanosin-N^2-yl)aristolactam I; HPLC, high-performance liquid chromatography

Figure 6. Metabolic activation and DNA adduct formation of aristolochic acids I (R = OCH_3) and II (R–H)



From Schmeiser *et al.* (1997)
(1) Aristolochic acid; (2) cyclic nitrenium ion of aristolochic acids I or II; (3) aristolactams; (4) 7-(deoxyadenosin-N^6-yl)aristolactam I or II (dA-AAI or dA-AAII); (5) 7-(deoxyguanosin-N^2-yl)aristolactam I or II (dG-AAI or dG-AAII)

(*b*) *Mutations in proto-oncogenes in tumours induced by aristolochic acids in rodents* in vivo (see Table 5 for details of studies and references)

Activated c-Ha-*ras* proto-oncogenes were found in 7/7 ear-duct tumours (squamous-cell carcinoma), in 13/14 forestomach tumours (squamous-cell carcinoma) and in the transformant of the 14th forestomach tumour, and in one metastasis in the lung induced in rats by aristolochic acid I. The mutations were all A → T transversions at the second base of codon 61. In the same animal study, activated c-Ki-*ras* proto-oncogenes were found in 1/7 ear duct tumours and 1/8 tumours of the small intestine. In the one metas-

Table 4. DNA adduct formation with aristolochic acid (AA) in rats *in vivo*

Details of study	Compound, route and dose	DNA binding	Reference
Adduct formation in DNA of several organs of male Wistar rats *in vivo* given multiple doses of AAI or AAII (equimolar doses as sodium salts). Adduct formation by both compounds was detected by the standard ^{32}P-postlabelling method in forestomach, stomach, liver, kidney and lung. In addition, adduct formation by AAII was detected in bladder and brain.	AAI and AAII, gavage AAI: 11 mg/kg bw daily for 5 days AAII: 10 mg/kg bw daily for 5 days	 + [not quantified] + [not quantified]	Schmeiser *et al.* (1988)
Detection and quantitation of adducts in DNA of several organs of male Wistar rats *in vivo* given multiple doses of AAI or AAII (equimolar doses as sodium salts). AAI induced higher adduct levels than AAII in fore-stomach, glandular stomach, liver, kidney and urinary bladder analysed by the nuclease P1 enhancement of the ^{32}P-postlabelling method. Assignment of characterized *in vitro* nucleoside adducts of AAI to the biphosphate derivatives obtained by the ^{32}P-postlabelling procedure.	As above (Schmeiser *et al.*, 1988)	AAI: 17–330/10^8 nucleotides AAII: 24–80/10^8 nucleotides	Pfau *et al*. (1990a)
Adduct formation in DNA of forestomach and liver of male Wistar rats (APf SD strain) given multiple doses of AA. Adduct detection was by the nuclease P1 enhancement of the ^{32}P-postlabelling method. Butylated hydroxyanisole pretreatment enhanced adduct formation by AA.	AA mixture, gavage, 1 mg/kg bw daily for 5 days	Liver: 63/10^9 nucleotides forestomach: 77/10^9 nucleotides	Routledge *et al*. (1990)
Adduct formation in DNA of whole urinary bladder, urothelial cells and exfoliated cells in urine of male Wistar rats given daily doses of AAI. Animals were killed 3 months after the last dose. Adduct detection was by the nuclease P1 and the butanol extraction enhancement of the ^{32}P-postlabelling method. AA-specific adducts were detectable in whole urinary bladder, urothelial cells and exfoliated cells in urine pooled from several rats over a period of 2 weeks after the first dose. The major adduct spots were identified by co-chromatography.	AAI, gavage, 10 mg/kg bw daily, five times a week for 3 months	Total adduct levels (dG-AAI, dA-AAI) determined by the nuclease P1 version exfoliated cells: 5.8/10^9 nucleotides urinary bladder: 37/10^9 nucleotides urothelial cells: 126/10^9 nucleotides	Fernando *et al*. (1992)

Table 4 (contd)

Details of study	Compound, route and dose	DNA binding	Reference
Adduct formation in DNA of several organs of male Wistar rats given a single dose of AAI and sacrificed 1 day and 1, 2, 4, 16 and 36 weeks later. The nuclease P1 enhancement of the ^{32}P-postlabelling method was used to analyse adducts in forestomach, glandular stomach, liver, lung and urinary bladder. In the target organ (forestomach), dA-AAI and dG-AAI adducts were removed rapidly within the first 2 weeks; thereafter, extensive removal of the dG-AAI continued, whereas dA-AAI remained at constant levels from 4 to 36 weeks.	AAI, gavage, 5 mg/kg bw	Initial level in forestomach: dA-AAI: $30/10^8$ nucleotides, dG-AAI: $21/10^8$ nucleotides Level after 36 weeks in forestomach: dA-AAI: $2/10^8$ nucleotides, dG-AAI: $0.4/10^8$ nucleotides	Fernando *et al.* (1993)
Adduct formation in DNA of the forestomach of male BD-6 rats given multiple doses of AA mixture. The nuclease P1 enhancement of the ^{32}P-postlabelling method was used to analyse adducts in forestomach. Chronic diallyl sulfide co-administration decreased adduct levels in forestomach DNA.	AA mixture, gavage, 10 mg/kg bw twice a week for 12 weeks	Total level in forestomach: $87/10^8$ nucleotides	Hadjiolov *et al*. (1993)
Adduct formation in DNA of the forestomach of male Sprague Dawley rats given multiple doses of AAI or AAII or AA mixture. The AA mixture consisted of 65% AAI and 34% AAII. The nuclease P1 enhancement of the ^{32}P-postlabelling method was used to analyse adducts in forestomach. Adduct spots were identified by co-chromatography with in-vitro prepared standard compounds. Adduct formation with AAI was more efficient than with AAII.	AAI or AAII or AA mixture, gavage, 10 mg/kg bw twice a week for 2 weeks	Total level in forestomach (dA and dG adducts): AAI: $62/10^7$ nucleotides AAII: $2.5/10^7$ nucleotides AA: $3.2/10^7$ nucleotides	Stiborová *et al.* (1994)
Adduct formation in DNA of the kidney of male Wistar rats given a single dose of AAI and sacrificed 1 day and 1, 2, 4, 16 and 36 weeks later. The nuclease P1 enhancement of the ^{32}P-postlabelling method was used to analyse adducts. The dA-AAI adduct showed lifelong persistence.	AAI, gavage, 5 mg/kg bw	Initial level in kidney: dA-AAI: $6.5/10^8$ nucleotides, dG-AAI: $3.8/10^8$ nucleotides Level after 36 weeks in kidney: dA-AAI: $1.6/10^8$ nucleotides, dG-AAI: $0.5/10^8$ nucleotides	Bieler *et al*. (1997)

Table 4 (contd)

Details of study	Compound, route and dose	DNA binding	Reference
Adduct formation in DNA of the kidney of male and female Wistar rats given multiple doses of AA as plant material of the slimming regimen. Animals were sacrificed 11 months after last treatment. The nuclease P1 enhancement of the ^{32}P-postlabelling method was used to analyse adducts. AA-specific DNA adduct levels were higher in female than in male rats.	Slimming regimen containing AA mixture as plant material, gavage, 0.15 mg/kg bw per day, 5 times a week for 3 months	Total level in kidneys: 51–83/10^9 nucleotides	Arlt *et al*. (2001a)

AA, aristolochic acids (mixed); AAI and AAII, aristolochic acids I and II; dA-AAI, 7-(deoxyadenosin-N^6-yl)aristolactam I; dA-AAII, 7-(deoxyadenosin-N^6-yl)aristolactam II; dG-AAI, 7-(deoxyguanosin-N^2-yl)aristolactam I

Table 5. Mutations in oncogenes found in rodents treated *in vivo* with aristolochic acids (AA)

Species	Treatment	Incidence and type of tumours	Method of analysis	No. of mutated genes/no. of tumours analysed	Details of mutations		
					Gene, codon	Base change	Reference
Male Wistar rats	10 mg/kg bw AAI given daily by gavage to 8-week-old rats 5 times a week for 3 months. Rats were killed over a 15-week period after treatment	15/40 forestomach tumours (SCC), 7/40 ear duct tumours (SCC), 23/40 adeno-carcinomas or sarcomas of the small intestine, 2/40 metastases of SCC in lung and pancreas	DNA isolated from 5 excised forestomach tumours was transfected into NIH 3T3 cells which induced tumours in nude mice. c-Ha-*ras* fragments were amplified by PCR of DNA from nude mouse tumours and analysed by sequencing. DNA extracted from rat tumours was amplified by PCR for regions of c-Ha-*ra*s, c-Ki-*ra*s and c-N-*ra*s gene and analysed by selective oligonucleotide hybridization with probes carrying different *ra*s base-pair substitutions.	7/7 ear duct tumours 14/14 forestomach tumours 1/8 tumours of the small intestine 1/1 metastasis in the pancreas 1/1 metastasis in the lung	c-Ha-*ras*, 61 c-Ki-*ras*, 61 c-Ha-*ras*, 61 c-Ki-*ras*, 61 c-N-*ras*, 61 c-Ha-*ras*, 61	CAA → CTA (7/7) CAA → CAT (1/7) CAA → CTA (14/14) CAA → CTA (1/8) CAA → CTA CAA → CTA	Schmeiser *et al*. (1990)
Female NMRI mice	5 mg/kg bw AA mixture, by gavage, daily for 3 weeks (80% AAI, 20% AAII). Animals killed after 56 weeks	SCC of the forestomach, adenocarcinoma of the lung	DNA extracted from histologically normal and neoplastic tissue in paraffin sections, c-Ha-*ra*s fragments around codon 61 amplified by PCR and analysed by oligo-nucleotide hybridization with probes carrying different c-Ha-*ra*s base-pair substitutions	1/1 fore-stomach tumour 1/3 lung tumours	c-Ha-*ras*, 61	CAA → CTA (2/4)	Schmeiser *et al*. (1991)

Table 5 (contd)

Species	Treatment	Incidence and ype of tumours	Method of analysis	No. of mutated genes/no. of tumours analysed	Details of mutations		
					Gene, codon	Base change	Reference
Male Wistar rats	10 mg/kg bw AAI given daily by gavage to 8-week-old rats, 5 times a week for 3 months. Rats were killed over a 15-week period after treatment	SCC of the fore-stomach and pancreas	DNA extracted from histologically normal and neoplastic tissue in paraffin sections, c-Ha-*ra*s fragments around codon 61 amplified by PCR and analysed by oligonucleotide hybridization with probes carrying different c-Ha-*ra*s base-pair substitutions	2/2 fore-stomach tumours 0/1 pancreas tumour	c-Ha-*ra*s, 61	CAA → CTA (2/3)	Schmeiser *et al*. (1991)

SCC, squamous-cell carcinoma; PCR, polymerase chain reaction; AA, aristolochic acid; AAI, aristolochic acid I; AAII, aristolochic acid II

tasis in the pancreas, an activated c-N-*ras* proto-oncogene was detected. All mutations were A → T transversions at either the second or the third base of codon 61.

In mice, a mixture of aristolochic acids induced squamous-cell carcinoma in the forestomach and adenocarcinoma in the lung. In the tumours analysed, one forestomach squamous-cell carcinoma and 1/3 lung adenocarcinomas contained activated c-Ha-*ras* proto-oncogenes both mutated by A → T transversions at the second base of codon 61.

(*c*) *In-vitro studies* (see Tables 6–8 for details of studies and references)

After metabolic activation, aristolochic acid I and aristolochic acid II form adducts *in vitro* with calf thymus DNA, MCF-7 DNA, plasmids, polydeoxyribonucleotides, oligodeoxyribonucleotides, deoxyribonucleotide-3′-monophosphates (purines), deoxyadenosine and deoxyguanosine. In-vitro systems capable of activating aristolochic acids I and II to reactive species that may form adducts are S9 mix from Aroclor 1254- or β-naphthoflavone-pretreated rats, xanthine oxidase, peroxidases (horseradish peroxidase, lactoperoxidase, prostaglandin H synthase), zinc at pH 5.8 and microsomal preparations from various species other than the rat. Aristolochic acid-specific adducts were formed in calf thymus DNA after activation of aristolochic acids I and II with hepatic microsomes from humans, mini-pigs and rats, as well as with microsomes containing recombinant human CYP1A1 and CYP1A2. From studies with specific inducers and selective inhibitors, it can be concluded that most of the microsomal activation of aristolochic acids is due to CYP1A1 and CYP1A2.

Activated aristolochic acids I and II react with DNA to form three and two major adducts, respectively. These major adducts co-chromatograph with 7-(deoxyadenosin-N^6-yl)aristolactam I (dA-AAI), 7-(deoxyguanosin-N^2-yl)aristolactam I (dG-AAI), 7-(deoxyadenosin-N^6-yl)aristolactam II (dA-AAII) and 7-(deoxyguanosin-N^2-yl)aristolactam II (dG-AAII) (see Figure 6), indicating that aristolochic acid reacts preferentially with the exocyclic amino group of purine bases. On the basis of the adduct structures, it can be concluded that reduction of the nitro group is the main metabolic pathway for the activation of aristolochic acid.

The major metabolites, the aristolactams, form DNA adducts *in vitro* after activation by hepatic microsomes or horseradish peroxidase. Adducts with calf thymus DNA are also formed by aristolochic acids I and II *in vitro* in the presence of rat faecal bacteria. In explants of rat stomach tissue, both acids formed adducts in the DNA of the epithelial layer. DNA adducts have been detected in MCF-7 cells after exposure to aristolochic acid I and in opossum kidney cells after exposure to an aristolochic acid mixture.

After reaction of aristolochic acids with DNA, DNA synthesis by T7 DNA polymerase and human DNA polymerase α is mainly blocked at the nucleotide 3′ to the aristolochic acid-induced DNA adducts. This property has allowed the use of polymerase arrest assays that revealed binding of aristolochic acids I and II *in vitro* to the c-Ha-*ras* gene and the *TP53* gene.

Aristolochic acids I and II and the aristolochic acid mixture induced SOS repair and mutations in bacteria. In nitroreductase-deficient strains of *Salmonella typhimurium*,

Table 6. DNA adduct formation by aristolochic acids (AAs) *in vitro*

Test system	Assay	Dose	Result	Reference
Adduct formation by AAI in DNA (calf thymus) *in vitro* with S9 mix[a], aerobic	Standard ^{32}P-postlabelling	0.4 mM	4 adducts	Schmeiser *et al.* (1988)
Adduct formation by AAI in DNA (calf thymus) *in vitro* with S9 mix [a], anaerobic[b]			2 adducts	
Adduct formation by AAII in DNA (calf thymus) *in vitro* with S9 mix [a], aerobic			No adducts	
Adduct formation by AAII in DNA (calf thymus) *in vitro* with S9 mix [a], anaerobic[b]			2 adducts	
Adduct formation by AAI in DNA (calf thymus) *in vitro* with xanthine oxidase (0.1 µg/mL), anaerobic[b]			4 adducts	
Adduct formation by AAII in DNA (calf thymus) *in vitro* with xanthine oxidase (0.1 µg/mL), anaerobic[b]			2 adducts	
Adduct formation by AAI in polydG *in vitro* with S9 mix aerobic or anaerobic			2 adducts	
Adduct formation by AAI in DNA (calf thymus) *in vitro* with rat faecal bacteria, anaerobic[b]	Nuclease P1-enhanced ^{32}P-postlabelling	1 mmol/2 mL	Same pattern as *in vivo* (cf Table 4)	Pfau *et al.* (1990a)
Adduct formation by AAII in DNA (calf thymus) *in vitro* with rat faecal bacteria, anaerobic[b]			Same pattern as *in vivo* (cf Table 4)	
Adduct formation by AAI in DNA of forestomach and glandular stomach in explanted stomach of rats		30 µmol/kg bw daily for	4 adducts	
Adduct formation by AAII in DNA of forestomach and glandular stomach in explanted stomach of rats		5 days (oral)	2 adducts	
Adduct formation by AAI with deoxyguanosine and deoxyadenosine *in vitro* in the presence of xanthine oxidase, anaerobic	UV/vis and fluorescence spectroscopy. Adducts were spectroscopically identified as dG-AAI and dA-AAI.		Same adducts are formed in DNA	Pfau *et al.* (1990b)

Table 6 (contd)

Test system	Assay	Dose	Result	Reference
Adduct formation by AAII with deoxyadenosine *in vitro* in the presence of xanthine oxidase, anaerobic	UV/vis, fluorescence and NMR spectroscopy. Adduct was spectroscopically identified as dA-AAII.		Same adduct is formed in DNA *in vitro* and *in vivo*	Pfau *et al.* (1991)
Adduct formation by AAI with deoxyadenosine 3′-monophosphate or deoxyguanosine 3′-monophosphate *in vitro* in the presence of xanthine oxidase, anaerobic	Butanol extraction-enhanced ^{32}P-postlabelling	100 µM (with 0.3 µM substrate)	dA-AAI and dG-AAI	Stiborová *et al.* (1994)
Adduct formation by AAII with deoxyadenosine 3′-monophosphate or deoxyguanosine 3′-monophosphate *in vitro* in the presence of xanthine oxidase, anaerobic			dA-AAII and dG-AAII	
Adduct formation by AAI in 18-mer oligonucleotides containing either a single guanosine or adenosine in the presence of zinc at pH 5.8	Standard ^{32}P-postlabelling	2 mM (with 100 µM substrate)	dA-AAI and dG-AAI	Broschard *et al.* (1994)
Adduct formation by AAII in 18-mer oligonucleotides containing either a single guanosine or adenosine in the presence of zinc at pH 5.8			dA-AAII and dG-AAII Adenine adducts have greater miscoding potential than guanine adducts (see Table 7).	
Adduct formation by AAI in 30-mer oligonucleotides containing either a single guanosine or adenosine in the presence of zinc at pH 5.8	Standard ^{32}P-postlabelling	2 mM (with 100 µM substrate)	dA-AAI and dG-AAI	Broschard *et al.* (1995)
Adduct formation by AAII in 30-mer oligonucleotides containing either a single guanosine or adenosine in the presence of zinc at pH 5.8			dA-AAII and dG-AAII Adenine adducts formed by AAI and AAII and guanine adducts formed by AAI block DNA replication more efficiently than dG-AAII (see Table 7).	

Table 6 (contd)

Test system	Assay	Dose	Result	Reference
Adduct formation by aristolactam I in DNA (calf thymus) *in vitro* with microsomes[c]	Nuclease P1-enhanced ^{32}P-postlabelling	0.3 mM (with 1.3 mg DNA/mL)	Total adduct level: $1.3/10^7$ nucleotides	Stiborová *et al.* (1995)
Adduct formation by aristolactam II in DNA (calf thymus) *in vitro* with microsomes[c]			Total adduct level: $0.5/10^7$ nucleotides	
Adduct formation by aristolactam I in DNA (calf thymus) *in vitro* with horseradish peroxidase			Total adduct level: $93/10^7$ nucleotides	
Adduct formation by aristolactam II in DNA (calf thymus) *in vitro* with horseradish peroxidase			Total adduct level: $19/10^7$ nucleotides	
Adduct formation by AAI in DNA (calf thymus) *in vitro* with hepatic microsomes[c], aerobic	Nuclease P1-enhanced ^{32}P-postlabelling (adducts dG-AAI, dG-AAII, dA-AAI and dA-AAII identified by chromatography)	0.3 mM (with 1.3 mg DNA/mL)	Total adduct level: $14/10^7$ nucleotides	Schmeiser *et al*. (1997)
Adduct formation by AAI in DNA (calf thymus) *in vitro* with hepatic microsomes [c], anaerobic[b]			Total adduct level: $34/10^7$ nucleotides	
Adduct formation by AAII in DNA (calf thymus) *in vitro* with hepatic microsomes [c], aerobic			Total adduct level: $2.3/10^7$ nucleotides	
Adduct formation by AAII in DNA (calf thymus) *in vitro* with hepatic microsomes [c], anaerobic[b]			Total adduct level: $7.1/10^7$ nucleotides	
Adduct formation by AAI in DNA (calf thymus) *in vitro* with xanthine oxidase, aerobic			Total adduct level: $76/10^7$ nucleotides	
Adduct formation by AAI in DNA (calf thymus) *in vitro* with xanthine oxidase, anaerobic[b]			Total adduct level: $145/10^7$ nucleotides	
Adduct formation by AAII in DNA (calf thymus) *in vitro* with xanthine oxidase, aerobic			Total adduct level: $4.4/10^7$ nucleotides	
Adduct formation by AAII in DNA (calf thymus) *in vitro* with xanthine oxidase, anaerobic[b]			Total adduct level: $6.1/10^7$ nucleotides	
Adduct formation by AAI in DNA (calf thymus) *in vitro* with zinc pH 5.8, aerobic			Total adduct level: $22420/10^7$ nucleotides	
Adduct formation by AAII in DNA (calf thymus) *in vitro* with zinc pH 5.8, aerobic			Total adduct level: $52700/10^7$ nucleotides	
Adduct formation by AAI in DNA (calf thymus) *in vitro* with horseradish peroxidase, aerobic			Total adduct level: $74/10^7$ nucleotides	
Adduct formation by AAI in DNA (calf thymus) *in vitro* with horseradish peroxidase, anaerobic[b]			Total adduct level: $100/10^7$ nucleotides	

Table 6 (contd)

Test system	Assay	Dose	Result	Reference
Adduct formation by AAII in DNA (calf thymus) *in vitro* with horseradish peroxidase, aerobic		0.3 mM	Total adduct level: $4.1/10^7$ nucleotides	Schmeiser *et al*. (1997) (contd)
Adduct formation by AAII in DNA (calf thymus) *in vitro* with horseradish peroxidase, anaerobic[b]			Total adduct level: $19/10^7$ nucleotides	
Adduct formation by AAI in DNA (calf thymus) *in vitro* with lactoperoxidase, aerobic			Total adduct level: $10.2/10^7$ nucleotides	
Adduct formation by AAI in DNA (calf thymus) *in vitro* with lactoperoxidase, anaerobic			Total adduct level: $13.8/10^7$ nucleotides	
Adduct formation by AAII in DNA (calf thymus) *in vitro* with lactoperoxidase, aerobic			Total adduct level: $0.6/10^7$nucleotides	
Adduct formation by AAII in DNA (calf thymus) *in vitro* with lactoperoxidase, anaerobic			Total adduct level: $3.6/10^7$nucleotides	
Adduct formation by aristolactam I in DNA (calf thymus) *in vitro* with rat liver microsomes[c], aerobic	Nuclease P1-enhanced ^{32}P-postlabelling	0.3 mM (1.3 mg DNA/mL)	Total adduct level: $1.3/10^7$ nucleotides[d]	Stiborová *et al.* (1999)
Adduct formation by aristolactam I in DNA (calf thymus) *in vitro* with horseradish peroxidase, aerobic			Total adduct level: $93/10^7$ nucleotides	
Adduct formation by AAI activated by zinc in a plasmid containing exon 2 of the mouse c-Ha-*ras* gene	Standard ^{32}P-postlabelling	1.2 mM	Total adduct level: $5.2/10^3$ nucleotides	Arlt *et al*. (2000)
		0.12 mM	Total adduct level: $0.6/10^3$ nucleotides	
Adduct formation by AAII activated by zinc in a plasmid containing exon 2 of the mouse c-Ha-*ras* gene		1.2 mM	Total adduct level: $9.9/10^3$ nucleotides	
		0.12 mM	Total adduct level: $6.1/10^3$ nucleotides	
Adduct formation by AAI activated by zinc in polydeoxynucleotides poly(dA), poly(dG)-poly(dC) and poly (dC)		1.2 mM	dA-AAI, dG-AAI, dC-AAI	
Adduct formation by AAII activated by zinc in polydeoxynucleotides poly(dA), poly(dG)-poly(dC) and poly (dC)		1.2 mM	dA-AAI, dG-AAII, dC-AAII	

Table 6 (contd)

Test system	Assay	Dose	Result	Reference
Adduct formation of AAI in MCF-7 cells with different doses for 24 h	Nuclease P1-enhanced ^{32}P-postlabelling	10 μM 100 μM 200 μM	Total adduct level: $1.2/10^7$ nucleotides $33/10^7$ nucleotides $52/10^7$ nucleotides	Arlt *et al.* (2001b)
Adduct formation of AAI in MCF-7 DNA *in vitro*, activated by zinc	Standard ^{32}P-postlabelling	0.24 mM	Total adduct level: $7.1/10^4$ nucleotides	
Adduct formation of AAII in MCF-7 DNA *in vitro*, activated by zinc		0.24 mM	Total adduct level: $55/10^4$ nucleotides	
Adduct formation of AAI in MCF-7 DNA *in vitro*, activated by xanthine oxidase		2.4 mM	Total adduct level: $1.3/10^4$ nucleotides	
Adduct formation of AAII in MCF-7 DNA *in vitro*, activated by xanthine oxidase		2.4 mM	Total adduct level: $6.3/10^4$ nucleotides	
Adduct formation of AAI in DNA (calf thymus) *in vitro*, activated by ram seminal vesicle microsomes, anaerobic	Nuclease P1-enhanced ^{32}P-postlabelling	0.5 mM	Total adduct level: $0.54/10^7$ nucleotides[e]	Stiborová *et al.* (2001a)
Adduct formation of AAII in DNA (calf thymus) *in vitro*, activated by ram seminal vesicle microsomes, anaerobic			Total adduct level: $0.3/10^7$ nucleotides[e]	
Adduct formation of AAI in DNA (calf thymus) *in vitro*, activated by pure prostaglandin H synthase (PHS-1), anaerobic			Total adduct level: $4.6/10^7$ nucleotides[e]	
Adduct formation of AAII in DNA (calf thymus) *in vitro*, activated by pure prostaglandin H synthase (PHS-1), anaerobic			Total adduct level: $1.9/10^7$ nucleotides[e]	
Adduct formation of AAI in DNA (calf thymus) *in vitro*, activated by human hepatic microsomes, anaerobic	Nuclease P1-enhanced ^{32}P-postlabelling (adducts identified by co-chromatography)	0.5 mM (4 mM DNA)	Total adduct level: $6.4/10^7$ nucleotides	Stiborová *et al.* (2001b)
Adduct formation of AAII in DNA (calf thymus) *in vitro*, activated by human microsomes, anaerobic			Total adduct level: $0.8/10^7$ nucleotides	
Adduct formation of AAI in DNA (calf thymus) *in vitro*, activated by hepatic microsomes from minipig, anaerobic			Total adduct level: $11/10^7$ nucleotides	
Adduct formation of AAII in DNA (calf thymus) *in vitro*, activated by hepatic microsomes from minipig, anaerobic			Total adduct level: $2.1/10^7$ nucleotides	
Adduct formation of AAI in DNA (calf thymus) *in vitro*, activated by hepatic microsomes from rats, anaerobic			Total adduct level: $5.7/10^7$ nucleotides	

Table 6 (contd)

Test system	Assay	Dose	Result	Reference
Adduct formation of AAII in DNA (calf thymus) *in vitro*, activated by hepatic microsomes from rats, anaerobic			Total adduct level: $0.7/10^7$ nucleotides	Stiborová *et al*. (2001b) (contd)
Adduct formation of AAI in DNA (calf thymus) *in vitro* activated by recombinant human CYP1A1 in microsomes from a baculovirus insect cell expression system, anaerobic			Total adduct level: $22/10^7$ nucleotides	
Adduct formation of AAII in DNA (calf thymus) *in vitro*, activated by recombinant human CYP1A1 in microsomes from a baculovirus insect cell expression system, anaerobic			Total adduct level: $2.5/10^7$ nucleotides	
Adduct formation of AAI in DNA (calf thymus) *in vitro*, activated by recombinant human CYP1A2 in microsomes from a baculovirus insect cell expression system, anaerobic			Total adduct level: $19/10^7$ nucleotides	
Adduct formation of AAII in DNA (calf thymus) *in vitro*, activated by recombinant human CYP1A2 in microsomes from a baculovirus insect cell expression system, anaerobic			Total adduct level: $2/10^7$ nucleotides	
Adduct formation of AA mixture in DNA of opossum kidney cells after 24 h incubation	Nuclease P1-enhanced ^{32}P-postlabelling	10 µM 20 µM	Total adduct level: $47/10^7$nucleotides $87/10^7$nucleotides $15/10^7$ nucleotides after 6 days of recovery	Lebeau *et al*. (2001)

AA, aristolochic acids (mixed); AAI and AAII, aristolochic acids I and II; dA-AAI, 7-(deoxyadenosin-N^6-yl)aristolactam I; dA-AAII, 7-(deoxyadenosin-N^6-yl)-aristolactam II; dG-AAI, 7-(deoxyguanosin-N^2-yl)aristolactam I; dG-AAII, 7-(deoxyguanosin-N^2-yl)aristolactam II; vis, visible

[a] S9 mix from Aroclor 1254-pretreated rats

[b] Anaerobic: reaction mixture purged with argon for 15 min before addition of substrates

[c] Microsomes from β-naphthoflavone-pretreated rats

[d] Adduct formed in incubations of aristolactam I with DNA and microsomes is found in the ureter of a Chinese herb nephropathy patient, shown by co-chromatography.

[e] Same adducts as found *in vivo* in the kidney of Chinese herb nephropathy patients, as shown by co-chromatography

Table 7. Polymerase action on aristolochic acid-induced adducts *in vitro*

Test system	Result	Reference
Site-specifically adducted oligonucleotides containing AA-DNA adducts were used as templates for T7 DNA polymerase in primer extension reactions. DNA sythesis products were analysed on polyacrylamide gels using 5′-^{32}P-labelled primers	Mainly block of DNA synthesis at the nucleotide 3′ to each adduct, but translesional synthesis was also observed. dA-AAI and dA-AAII allowed incorporation of dAMP and dTMP directly across equally well, deoxyguanosine adducts allowed preferential incorporation of dCMP	Broschard *et al.* (1994)
Site-specifically adducted oligonucleotides containing AA-DNA adducts were used as templates for human DNA polymerase α in primer extension reactions. DNA synthesis products were analysed on polyacrylamide gels using 5′-^{32}P-labelled primers	Mainly block of DNA synthesis at the nucleotide 3′ to adducts dA-AAI, dG-AAI and dA-AAII. Only dG-AAII allowed substantial translesional synthesis	Broschard *et al.* (1995)
Polymerase arrest assay was used to determine the distribution of DNA adducts formed by AAI and AAII *in vitro* in the mouse c-Ha-*ras* gene. Arrest spectra were obtained on sequencing gels using 5′-^{32}P-labelled primers and polymerase Sequenase	AAI-induced adducts showed preference for adenine residues. AAII-induced damage was found at guanine, adenine and cytosine residues. Adduct distribution was not random.	Arlt *et al*. (2000)
Polymerase arrest assay combined with a terminal transferase-dependent PCR (TD-PCR) was used to map the distribution of DNA adducts formed by AAI and AAII *in vitro* in the human *TP53* gene of MCF-7 DNA. Adducted DNA was used as template for TD-PCR.	AA-DNA binding spectrum in the *TP53* gene: CpG sites are not preferential targets for AAI or AAII.	Arlt *et al*. (2001b)

AA, aristolochic acids (mixed); AAI and AAII, aristolochic acids I and II; dA-AAI, 7-(deoxyadenosin-N^6-yl)aristolactam I; dA-AAII, 7-(deoxyadenosin-N^6-yl)aristolactam II; dG-AAI, 7-(deoxyguanosin-N^2-yl)aristolactam I; dG-AAII, 7-(deoxyguanosin-N^2-yl)aristolactam II; PCR, polymerase chain reaction; dAMP, deoxyadenosine 3′-monophosphate; dTMP, deoxythymidine 3′-monophosphate; dCMP, deoxycytidine 3′-monophosphate

Table 8. Genetic and related effects of aristolochic acid (AA)

Test system	Result[a]		Compound, dose (LED or HID)[b]	Reference
	Without exogenous metabolic system	With exogenous metabolic system		
Escherichia coli PQ37 SOS repair induction	+	+[c]	AA plant extract, 0.38 µg/assay	Kevekordes *et al*. (1999)
Escherichia coli PQ37 SOS repair induction	+	+[c]	AAI, 0.17 µg/assay	Kevekordes *et al*. (1999)
Escherichia coli PQ37 SOS repair induction	+	(+)[c]	AAII, 0.16 µg/assay	Kevekordes *et al*. (1999)
Salmonella typhimurium TM677, forward mutation, *hprt* locus *in vitro*	+	NT	AAI, 8.5	Pezzuto *et al*. (1988)
Salmonella typhimurium TA100, TA1537, reverse mutation	+	+	AA mixture, 50 µg/plate	Robisch *et al*. (1982)
Salmonella typhimurium TA1535, TA1538, TA98, reverse mutation	–	–	AA mixture, 200 µg/plate	Robisch *et al.* (1982)
Salmonella typhimurium TA100, TA1537, reverse mutation	+	+	AAI, 100 µg/plate	Schmeiser *et al*. (1984)
Salmonella typhimurium TA100, TA1537, reverse mutation	–	+	Aristolactam I and aristolactam II, 50 µg/plate	Schmeiser *et al*. (1986)
Salmonella typhimurium TA100, TA102, TA1537, reverse mutation	+	NT	AAI, 100 µg/plate	Pezzuto *et al*. (1988)
Salmonella typhimurium TA98, YG1020, YG1021, reverse mutation	(+)	NT	AAI, 170 µg/plate	Götzl & Schimmer (1993)
Salmonella typhimurium TA98, YG1020, YG1021, reverse mutation	(+)	NT	AAII, 78 µg/plate	Götzl & Schimmer (1993)
Salmonella typhimurium YG1024, reverse mutation	+	NT	AAI, 34 µg/plate	Götzl & Schimmer (1993)
Salmonella typhimurium YG1024, reverse mutation	+	NT	AAII, 31 µg/plate	Götzl & Schimmer (1993)
Salmonella typhimurium TA100NR, reverse mutation	–	–	AAI, 200 µg/plate	Schmeiser *et al*. (1984)
Salmonella typhimurium TA100NR, TA98NR, reverse mutation	–	NT	AAI, 200 µg/plate	Pezzuto *et al*. (1988)
Salmonella typhimurium TA100, YG1025, YG 1026, YG 1029, reverse mutation	+	NT	AAI and AAII, 34 µg/plate	Götzl & Schimmer (1993)

Table 8 (contd)

Test system	Result[a]		Compound, dose (LED or HID)[b]	Reference
	Without exogenous metabolic system	With exogenous metabolic system		
Drosophila melanogaster, somatic mutations, recombination, sex-linked recessive mutation	+	NT	AA mixture (65% AAI, 35% AAII), 17 µg/mL in feed	Frei *et al*. (1985)
DNA strand breaks, rat hepatocytes, *in vitro* (alkaline elution assay)	–	NT	AAI and AAII [dose not reported]	Pool *et al*. (1986)
Gene mutation, rat subcutaneous granuloma tissue, *Hprt* locus *in vitro*	+	NT	AAI and AAII, 20	Maier *et al*. (1987)
Gene mutation, Chinese hamster ovary cells, *Hprt* locus *in vitro*	+	NT	AAI, 18.2	Pezzuto *et al*. (1988)
Sister chromatid exchange, human lymphocytes *in vitro*	+	NT	AA mixture, 1.0	Abel & Schimmer (1983)
Micronucleus induction, human lymphocytes and hepatoma cells (Hep-G2) *in vitro*	+	+	AA mixture, 17	Kevekordes *et al*. (2001)
Chromosomal aberrations, human lymphocytes *in vitro*	+	NT	AA mixture, 1.0	Abel & Schimmer (1983)
Unscheduled DNA synthesis, male F344/Ducrj rat glandular stomach mucosa *in vitro*, after in-vivo treatment	–		AA mixture, 400 × 1 po	Furihata *et al*. (1984)
Unscheduled DNA synthesis, male PV6 rat glandular stomach mucosa *in vivo*	–		AA mixture, 300 × 1 po	Burlinson (1989)
Gene mutation, male SD rat subcutaneous granuloma tissue, *Hprt* locus *in vivo*	+		AA mixture, 40 µg in pouch	Maier *et al*. (1985)
Gene mutation, male SD rat subcutaneous granuloma tissue, *Hprt* locus *in vivo*	+		AAI, 80 µg; AAII, 32 µg in pouch	Maier *et al*. (1987)
Micronucleus induction, male and female NMRI mouse bone-marrow cells *in vivo*	+		AA mixture, 20 × 1 iv	Mengs & Klein (1988)

AA, aristolochic acids (mixed); AAI and AAII, aristolochic acids I and II

[a] +, positive; (+), weak positive; –, negative; NT, not tested

[b] LED, lowest effective dose; HID, highest ineffective dose; in-vitro tests, µg/mL; in-vivo tests, mg/kg bw/day; iv, intravenous; po, oral

[c] Activity is much less in the presence of metabolic activation.

aristolochic acids I and II were not mutagenic. Both aristolactams were mutagenic in *S. typhimurium* only with S9 mix. Aristolochic acids I and II did not induce DNA strand breaks in hepatocytes. In cultured human lymphocytes, the aristolochic acid mixture induced chromosomal aberrations, sister chromatid exchange and micronuclei and it induced somatic and sex-linked recessive lethal mutations in *Drosophila melanogaster*. It also induced micronuclei in human hepatoma cells *in vitro* and in mouse bone-marrow cells after administration *in vivo*, but it did not induce unscheduled DNA synthesis in rat stomach after in-vivo treatment. The aristolochic acid mixture as well as aristolochic acids I and II induced mutations at the *hprt* locus in *Salmonella typhimurium*, in the granuloma pouch assay, and in Chinese hamster cells.

4.5 Mechanistic considerations

Aristolochic acid must be activated in order to form DNA adducts *in vitro* and *in vivo*. From the structures of the major aristolochic acid–DNA adducts identified in various in-vitro systems and in animals and humans *in vivo*, it can be concluded that the major pathway of activation of aristolochic acid is nitroreduction (Figure 6): a cyclic *N*-acylnitrenium ion with a delocalized positive charge is the ultimate DNA-reactive species that binds preferentially to the exocyclic amino groups of purine nucleotides in DNA or is hydrolysed to the corresponding 7-hydroxyaristolactam. Therefore, the activation of aristolochic acid is a unique example of an intra-molecular acylation which leads to the DNA-reactive species. This view is supported by the results of the *Salmonella* mutagenicity assays showing that only the nitro group is important for the mutagenic activity of aristolochic acid and by the demonstration that the enzymatic activation of both aristolochic acids by xanthine oxidase, a mammalian nitroreductase, produced an adduct pattern identical to that seen after metabolism mediated by rat liver S9 mix (Schmeiser *et al*., 1988). It was also demonstrated that both aristolochic acids could be activated by rat liver microsomes through simple nitroreduction (Schmeiser *et al*., 1997). In a single study, this anaerobic hepatic microsomal activation of aristolochic acid could be attributed to CYP1A1 and CYP1A2 and — to a lesser extent — to NADPH:CYP reductase (CYPOR) using specific CYP/CYPOR inhibitors (Stiborová *et al*., 2001a).

The adduct patterns in DNA from forestomach and kidney — target tissues of aristolochic acid-mediated carcinogenesis — and from non-target tissues, such as glandular stomach, liver and lung, were similar. This indicates that adduct formation is not sufficient to result in neoplasia.

In rodents, many chemical carcinogens activate the *ras* proto-oncogene by inducing a single point mutation, resulting in alteration of amino acid residue 12, 13 or 61. Similarly, aristolochic acid-initiated carcinogenesis in rodents is associated with a distinct molecular characteristic, the activation of *ras* by a specific AT → TA transversion mutation in codon 61 (CAA). This mutation occurs exclusively at the first adenine of codon 61 of Ha-*ras* (CAA → CTA) in all forestomach and ear-duct tumours of rats treated with aristolochic acid I (Schmeiser *et al*., 1990) and was also found in tumours

of the forestomach and lung of mice treated with a plant extract containing both aristolochic acids I and II (Schmeiser *et al*., 1991). The selectivity of aristolochic acid I for mutations at adenine residues is consistent with the extensive formation of dA-AAI adducts in the target organ (Pfau *et al*., 1990a; Stiborová *et al*., 1994). Moreover, an apparently lifelong persistence of the dA-AAI adduct in rat forestomach DNA was observed, whereas the less abundant dG-AAI adduct was removed continuously from the same DNA over a 36-week period after treatment with a single dose of aristolochic acid I (Fernando *et al*., 1993). Therefore, both the higher initial levels and the longer persistence of the dA-AAI adduct in urothelial tissue of patients with Chinese herb nephropathy probably contribute to the relative abundance of this adduct.

Investigations on the conversion of individual aristolochic acid–DNA adducts into mutations have shown that during in-vitro DNA synthesis, dAMP and dTMP were incorporated opposite the adenine adducts (dA-AAI, dA-AAII) equally well, whereas the guanine adducts (dG-AAI, dG-AAII) led to preferential incorporation of dCMP. The translesional by-pass of adenine adducts of aristolochic acid indicates a mutagenic potential resulting from dAMP incorporation by DNA polymerase, consistent with an AT → TA transversion as the mutagenic consequence. Therefore, the adenine adducts have a higher mutagenic potential than the guanine adducts, which may explain the apparent selectivity for mutations found at adenine residues in codon 61 of the *ras* genes in aristolochic acid-induced rodent tumours (Schmeiser *et al*., 1990, 1991).

An adduct-specific polymerase arrest assay with a plasmid containing exon 2 of the mouse c-H-*ras* gene demonstrated that both adenines in codon 61 of this gene are aristolochic acid–DNA binding sites (Arlt *et al*., 2000). Since *ras* genes are activated with high frequency by an AT → TA transversion mutation in codon 61 of *ras* DNA from aristolochic acid-induced tumours in animals (Schmeiser *et al*., 1990), this suggests an important role of the dA-AAI adduct in Chinese herb nephropathy-related urothelial cancer in humans. Polymerase arrest spectra showed a preference for reaction with purine bases in human *TP53* for both aristolochic acids. The aristolochic acid–DNA binding spectrum in the *TP53* gene did not suggest the existence of any mutational hotspot in urothelial tumours of the current *TP53* mutation database. Thus, aristolochic acid is not a likely cause of urothelial tumours not associated with Chinese herb nephropathy (Arlt *et al.*, 2001b).

5. Summary of Data Reported and Evaluation

5.1 Exposure data

Several *Aristolochia* species (notably *A. contorta*, *A. debilis*, *A. fangchi* and *A. manshuriensis*) have been used in traditional Chinese medicine as anti-rheumatics, as diuretics and in the treatment of oedema. Aristolochic acids are nitrophenanthrene carboxylic acid derivatives that are constituents of these plant species.

5.2 Human carcinogenicity data

An outbreak of rapidly progressive renal fibrosis in Belgium involved at least 100 patients, mostly middle-aged women undergoing a weight-loss regimen that included use of a mixture of Chinese herbs containing *Aristolochia* species incorrectly labelled as *Stephania tetrandra*. Additional cases of rapidly progressive renal disease involving Chinese herbs have been reported from at least five other countries in Europe and Asia. This syndrome has been called 'Chinese herb nephropathy'.

Because of a few early cases of urothelial cancer among Belgian patients suffering from Chinese herb nephropathy, individuals with end-stage renal disease were offered prophylactic nephroureterectomy. This surgical procedure led to the identification of a high prevalence of pre-invasive and invasive neoplastic lesions of the renal pelvis, the ureter and the urinary bladder in patients with Chinese herb nephropathy. The number of malignancies detected (18 cancers in 39 women undergoing prophylactic nephroureterectomy) greatly exceeded the expected number of these uncommon tumours. There was a positive dose–response relationship between the consumption of the herbal mixture and the prevalence of the tumours. Some cases of clinically invasive disease have been described in the follow-up of end-stage Chinese herb nephropathy patients not undergoing prophylactic nephroureterectomy.

Subsequent phytochemical investigation led to the identification of aristolochic acids in the herbal mixture consumed by these patients. While there was batch-to-batch variation in the chemical composition of such mixtures employed in the weight-loss clinics in Belgium, specific aristolochic acid–DNA adducts were found in urothelial tissue specimens from all the urothelial cancer patients, providing conclusive evidence of exposure to plants of the genus *Aristolochia*.

One additional case of urothelial cancer following treatment for eczema with another herbal mixture that contained aristolochic acid has been reported.

5.3 Animal carcinogenicity data

Aristolochic acids, when tested for carcinogenicity by oral administration in mice and rats and by intraperitoneal injection in rabbits, induced forestomach carcinomas in mice and rats, and fibrotic changes in the kidney together with a low incidence of kidney tumours in rabbits.

Subcutaneous injection of aristolochic acids into rats induced a low incidence of urothelial carcinomas in the kidney and malignant fibrohistiocytic sarcomas at the injection site.

5.4 Other relevant data

Several structurally defined metabolites (mainly nitroreduction products) have been reported following oral administration of aristolochic acid I (five metabolites) and aristo-

lochic acid II (three metabolites) to rats and mice. Fewer metabolites were observed in beagle dogs, rabbits, guinea-pigs and humans than in rats and mice.

The toxic effects of aristolochic acids I and II have been inferred from effects seen in patients suffering from kidney nephropathy as a result of consuming herbal mixtures containing *Aristolochia* species, which leads to rapidly progressive fibrosing interstitial nephritis. In experimental animals, high doses of aristolochic acids administered either orally or intravenously caused severe necrosis of the renal tubules, atrophy of the spleen and thymus, and ulceration of the forestomach, followed by hyperplasia and hyperkeratosis of the squamous epithelium.

Various constituents of *Aristolochia indica* including aristolochic acids and aristolic acid (a metabolite) caused termination of pregnancy in female mice, hamsters and rabbits, but not rats. The dose levels used, however, may also lead to general toxicity.

Aristolochic acids, when metabolically activated by nitroreduction, are consistently active in genotoxicity tests *in vivo* and *in vitro*. They form DNA adducts in rodent tissues and activate *ras* oncogenes through a specific transversion mutation in codon 61. Aristolochic acid-specific DNA adducts were identified in urothelial tissues of all patients with Chinese herb nephropathy.

5.5 Evaluation

There is *sufficient evidence* in humans for the carcinogenicity of herbal remedies containing plant species of the genus *Aristolochia*.

There are no data in experimental animals on the carcinogenicity of herbal remedies containing plant species of the genus *Aristolochia*.

There is *limited evidence* in humans for the carcinogenicity of naturally occurring mixtures of aristolochic acids.

There is *sufficient evidence* in experimental animals for the carcinogenicity of aristolochic acids.

Overall evaluation

Herbal remedies containing plant species of the genus *Aristolochia* are *carcinogenic to humans (Group 1)*.

Naturally occurring mixtures of aristolochic acids are *probably carcinogenic to humans (Group 2A)*.

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