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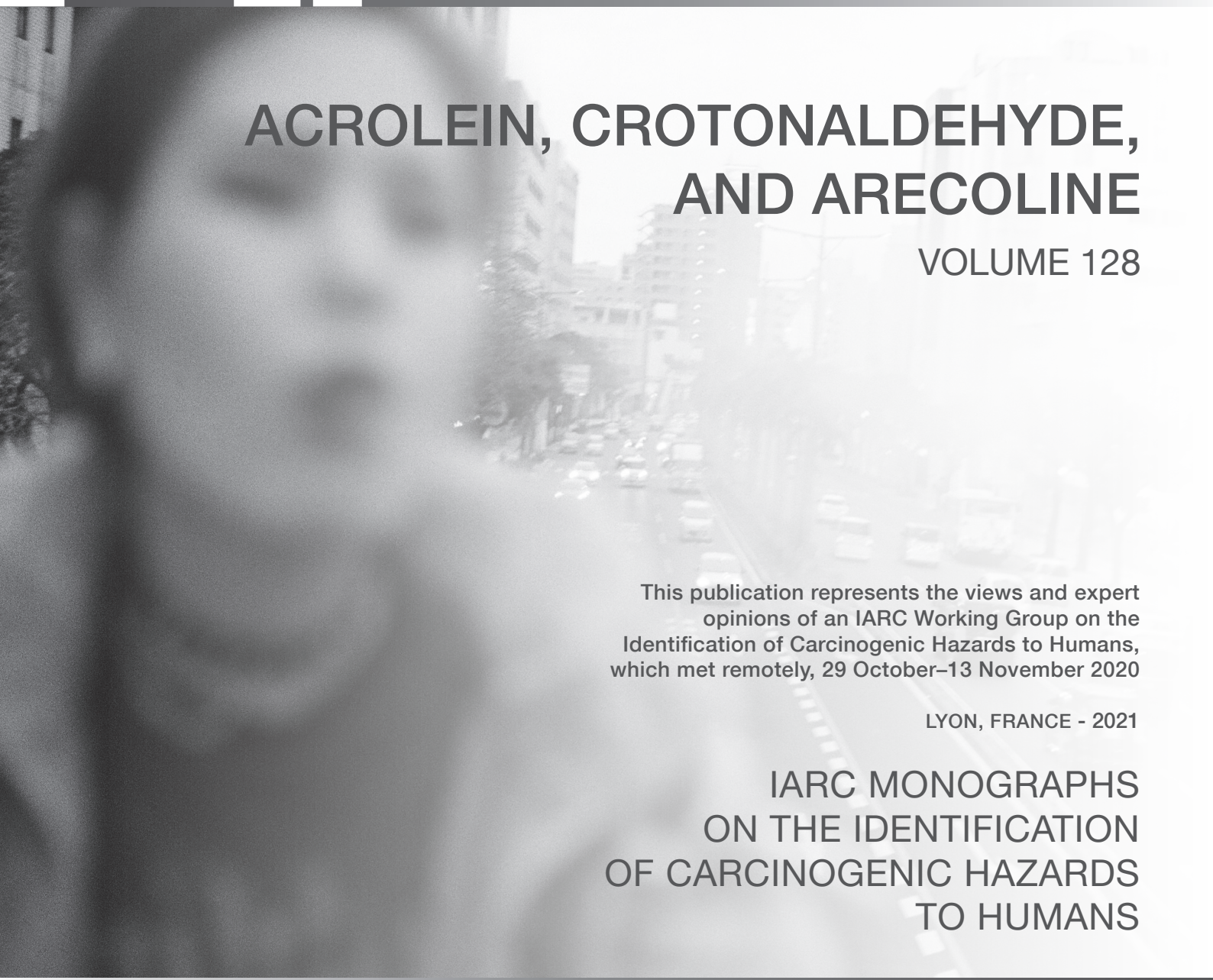


ACROLEIN, CROTONALDEHYDE, AND ARECOLINE

VOLUME 128

This publication represents the views and expert opinions of an IARC Working Group on the Identification of Carcinogenic Hazards to Humans, which met remotely, 29 October–13 November 2020

LYON, FRANCE - 2021

IARC MONOGRAPHS ON THE IDENTIFICATION OF CARCINOGENIC HAZARDS TO HUMANS

International Agency for Research on Cancer

World Health Organization

ARECOLINE

1. Exposure Characterization

1.1 Identification of the agent

1.1.1 Nomenclature

Chem. Abstr. Serv. Reg. No.: 63-75-2 (free base); 300-08-3 (hydrobromide); 61-94-9 (hydrochloride)

EC/List No.: 200-565-5 (free base); 206-087-3 (hydrobromide); 200-523-6 (hydrochloride)

Deleted CAS Reg. Nos: 1398-01-2 (free base)

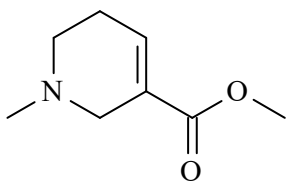
Chem. Abstr. Serv. name: 3-pyridinecarboxylic acid, 1,2,5,6-tetrahydro-1-methyl-, methyl ester (free base); 3-pyridinecarboxylic acid, 1,2,5,6-tetrahydro-1-methyl-, methyl ester, hydrobromide (1:1); 3-pyridinecarboxylic acid, 1,2,5,6-tetrahydro-1-methyl-, methyl ester, hydrochloride (1:1)

IUPAC systematic name: methyl 1-methyl-3,6-dihydro-2*H*-pyridine-5-carboxylate (free base); methyl 1-methyl-3,6-dihydro-2*H*-pyridinium-5-carboxylate hydrobromide; methyl 1-methyl-3,6-dihydro-2*H*-pyridinium-5-carboxylate hydrochloride

Synonyms: arecoline (6CI); nicotinic acid, 1,2,5,6-tetrahydro-1-methyl-, methyl ester (8CI); 1-methyl-1,2,5,6-tetrahydro-pydine-3-carboxylic acid methyl ester; 3-methoxycarbonyl-1-methyl-1,2,5,6-tetrahydropyridine; arecaidine methyl ester; arecolin; arecoline base; methyl 1,2,5,6-tetrahydro-1-methyl-nicotinate; methyl *N*-methyl-1,2,5,6-tetrahydronicotinate; methyl arecaidine; methylarecaidine; NSC 56321 (free base); arecoline hydrobromide; methyl 1,2,5,6-tetrahydro-1-methylnicotinate hydrobromide; methyl *N*-methyl-1,2,5,6-tetrahydronicotinate hydrobromide; nicotinic acid, 1,2,5,6-tetrahydro-1-methyl-, methyl ester, hydrobromide; taeniolin (hydrobromide); nicotinic acid, 1,2,5,6-tetrahydro-1-meth-yl, methyl ester, hydrochloride (O'Neil, 2013; ECHA, 2020a, b, c; NCBI, 2020a, b, c; SciFinder, 2020a, b, c).

1.1.2 Structural and molecular formulae, and relative molecular mass

Structural formula of the free base:



Molecular formula: $C_8H_{13}NO_2$ (free base); $C_8H_{13}NO_2$·HBr (hydrobromide); $C_8H_{13}NO_2$·HCl (hydrochloride)

Relative molecular mass: 155.20 (free base); 236.11 (hydrobromide); 191.65 (hydro-

chloride) (O'Neil, 2013; NCBI, 2020a, b, c; SciFinder, 2020a, b, c).

1.1.3 Chemical and physical properties

(a) Free base

Description: oily liquid (O'Neil, 2013)

Boiling point: 209 °C (O'Neil, 2013)

Melting point: < 25 °C (SciFinder, 2020a)

Density: 1.0495 g/cm^3 at 20 °C (SciFinder, 2020a)

Solubility: soluble in chloroform; miscible with water, alcohol, ether (O'Neil, 2013)

Acid dissociation constant: pK_a 6.84 (O'Neil, 2013)

Reactivity: arecoline is a strong base that forms salts with acids, e.g. it may be crystallized as hydrobromide (CAS No. 300-08-3) or hydrochloride (CAS No. 61-94-9)

(b) Hydrobromide

Melting point: 169–171 °C (SciFinder, 2020b)

(c) Hydrochloride

Melting point: 157–158 °C (SciFinder, 2020c).

1.1.4 Technical products and impurities

Commercial qualities with purities in the range of 90–98% for the free base and hydrochloride are available, while the hydrobromide is also available in purities > 99% (SciFinder, 2020a, b, c). No information about impurities of technical products was available.

1.2 Production and use

1.2.1 Production process

The isolation of arecoline from nuts of the areca palm *Areca catechu* was first described by Jahns (1888). According to a historical 1911 version of the German Pharmacopoeia, arecoline (as its hydrobromide) was produced from areca nuts using extraction with acidified water followed by several clean-up steps (Anselmino & Gilg, 1911). The first industrial-scale extraction, reported in 1927, was based on extraction of arecoline with diethyl ether (Chemnitius, 1927). There are various approaches for the synthetic production of arecoline starting from nicotinic acid and iodomethane; methylamine hydrochloride, formaldehyde and acetaldehyde; or ethyl acrylate and methylamine. The most modern approach involves nicotinic acid methyl ester and methyl iodide (Volgin et al., 2019). [The Working Group was unable to obtain information regarding which process is currently preferred in industrial practice.]

Arecoline salts such as arecoline hydrochloride or arecoline hydrobromide may be obtained by dissolving arecoline in an alcohol of low relative molecular mass (such as methanol, ethanol, isopropanol, butanol, or amyl alcohol) and adding sufficient amounts of acid (hydrochloric or bromic acid) to give a weakly acidic solution. The crystallized salts may be separated from the alcohol by filtration (Howland-Knox, 1950).

1.2.2 Production volume

The international database Chem Sources lists 5 companies worldwide that manufacture arecoline (freebase) (USA, Canada, China, France, and the UK), 3 companies that manufacture arecoline hydrochloride (USA, Germany, and the UK), and 26 companies that manufacture arecoline hydrobromide (USA, Germany, China, Switzerland, UK, France, Japan, and Ukraine) (Chem Sources, 2020). The Scifinder database

lists 37 companies worldwide that manufacture arecoline (free base) (USA, UK, Canada, China, France, and Hong Kong Special Administrative Region (SAR) China), 18 companies that manufacture arecoline hydrochloride (USA, China, France, Germany, and the UK), and 94 companies that manufacture arecoline hydrobromide (USA, Belgium, Singapore, India, Republic of Korea, Canada, China, France, Germany, Japan, UK, and Hong Kong SAR China) (SciFinder, 2020a, b, c).

[No data on production volume were available to the Working Group. Arecoline is not included on national or international lists of High Production Volume chemicals. Extrapolating from the number of manufacturing companies, the Working Group noted that arecoline appears to be most commonly traded and used in the form of its hydrobromide.]

1.2.3 Uses

Historically, arecoline was used as an antiparasitic drug and included in several pharmacopoeias, but it has been replaced by other drugs and is rarely administered directly at the present time (Ahuja et al., 2016; Zhao et al., 2018). Arecoline is, however, still applied indirectly in the form of patent medicines or crude preparations of the areca nut (seed) in traditional Chinese medicine (see Section 1.4.1; Yi et al., 2012; He et al., 2018; Zhao et al., 2018). Three types of crude herbal preparation of areca are used: raw Arecae semen, Arecae semen tostum and Arecae semen carbonisata. Raw Arecae semen is prepared by collecting the mature seeds of *Areca catechu* and following a series of processing steps involving purifying, soaking, nourishing, slicing, and drying. Arecae semen tostum and Arecae semen carbonisata are generally prepared by a stir-fry method at high temperature until the seed surface turns light brown to black-brown (Sun et al., 2017). Areca nut is also an integral part of traditional Indian Ayurveda medicine. Global consumption and production of areca nut are shown in Fig. 1.1 (Volgin et al., 2019).

Arecoline stimulates both muscarinic and nicotinic acetylcholine receptors (Brown et al., 2018). It has been studied in model systems as a treatment for Alzheimer disease (Asthana et al., 1995, 1996; Liu et al., 2016).

In veterinary medicine, arecoline has been used as an anthelmintic (for cestodes, a type of parasitic flatworm that includes tapeworm), cathartic, and cholinergic agent (Mascavage et al., 2010; O'Neil, 2013). The anthelmintic action is assumed to cause the cestode muscles to relax and the host to purge so that the detached worms are removed. Oral administration of 40 mg of arecoline hydrobromide for 5 days in dogs completely controlled tapeworm, but had a low efficiency against ascaris (a common parasitic roundworm) (Tang & Eisenbrand, 1992).

1.3 Methods of detection and quantification

[The Working Group noted that analytical methods specified for arecoline are applicable to arecoline hydrochloride and arecoline hydrobromide dissolved in suitable solvents.]

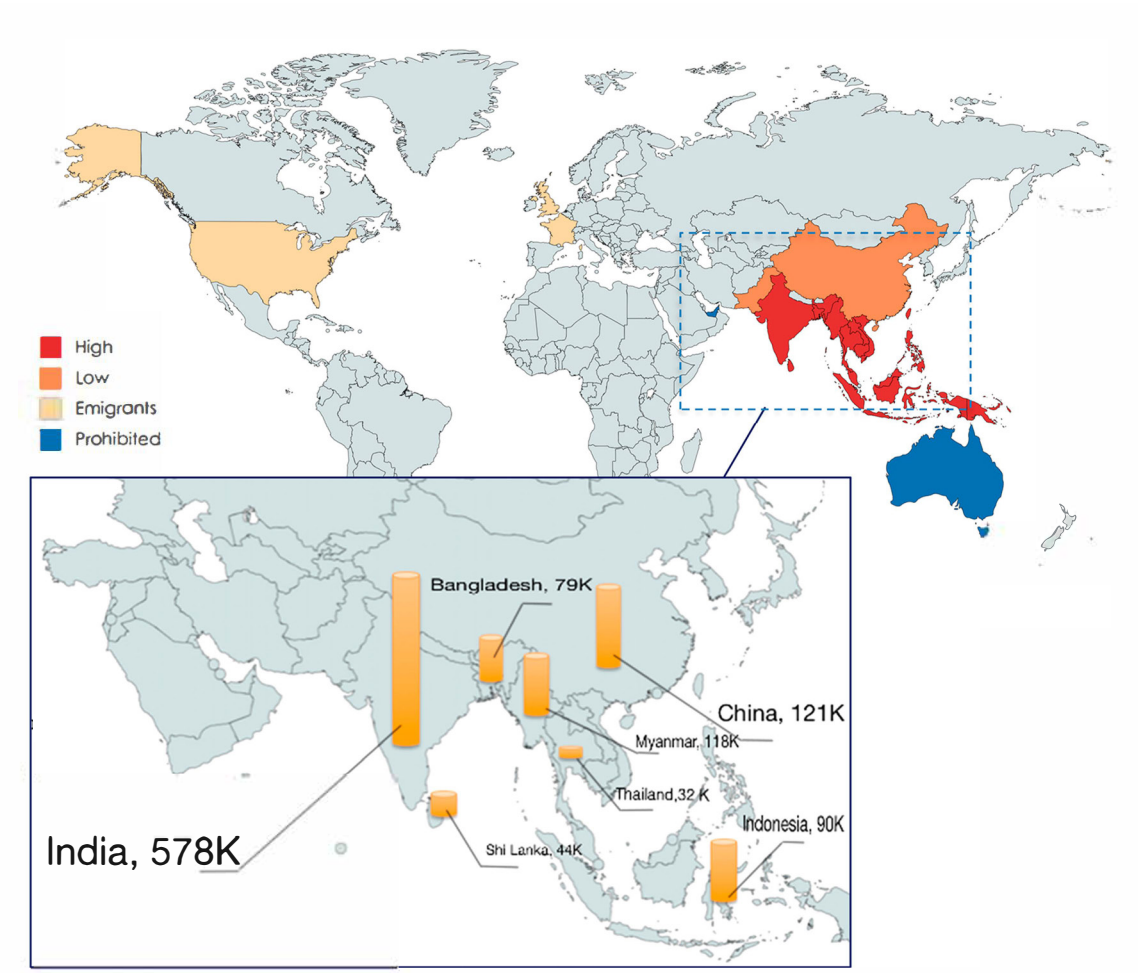
No methods for analysing arecoline in air, water, or soil were available to the Working Group.

1.3.1 Food, beverages, and consumer products

Historical methods for the identification of arecoline using various colour reactions, as well as quantitative determinations using paper chromatography, titrimetric methods, or colorimetric assays were reviewed by Arjungi (1976).

Recent analytical methods for arecoline have primarily focused on its detection in areca nuts as well as in areca nut-containing products such as *pan masala* (a powdered chewing mixture of areca nut with slaked lime, catechu, and other

Fig. 1.1 Global consumption and production of areca nuts



Global consumption (top panel) and production (inset) of areca nuts (red, high; orange, low; yellow, immigrants only). Blue denotes countries (Australia and United Arab Emirates) that currently ban areca-nut consumption. As shown in the inset, in 2015, India produced the majority of areca-nut products (578 000 tonnes/year), followed by China (121 000 tonnes/year), Indonesia (90 000 tonnes/year), and other countries in the region. For references to colour in this figure, please see the online version of this paper.
Reprinted with permission from Volgin et al. (2019). DARK Classics in Chemical Neuroscience: Arecoline. *ACS Chem Neurosci.* 10: 2176-85.

flavouring agents) (Table 1.1). After thin-layer or liquid chromatographic separation, arecoline can be detected using ultraviolet detection with absorption in the range of 200–250 nm owing to its chromophoric conjugated system of two double bonds (α,β-unsaturated carbonyl system) (Huang & McLeish, 1989; Lin et al., 1992; Sun et al., 2017). Mass spectrometry has been applied as a more specific and sensitive detection methodology allowing accurate detection down to the picogram range (Jain et al., 2017). Near-infrared spectroscopy has allowed rapid analysis of arecoline and process control during parching (Xue et al., 2011).

Table 1.1 Representative methods for the detection and quantification of arecoline in areca nut and products derived from areca nut

Sample matrix	Assay procedure	Limit of detection	Reference
Areca nut decoction pieces	MCE-CCD	5 μM	Cai et al. (2012)
Areca nut	HPLC	12.2 ng/mL	Huang & McLeish (1989)
Areca nut	MALDI-TOF-MS	0.2 μM	Feng & Lu (2009)
Areca nut	CE-ECL	5×10^{-9}mol/L	Xiang et al. (2013)
Areca nut (dried seed powder)	HPLC	2.86 μg/mL	Jantarat et al. (2013)
Areca nut	CZE	NR	Lord et al. (2002)
Areca nut and Indian nontobacco pan masala	HPTLC	3.25 ng	Adhikari et al. (2015)
Areca nut	PEC	30 pM	Dai et al. (2014)
Areca nut (raw, roasted, and boiled) and pan masala	HPTLC	35 ng/spot	Dutta et al. (2017)
Areca nut	HPLC-ES-MS	0.4 μg/mL	Ding & Shulian (2008)
Fruits of 11 *Arecaceae* species	UPLC-MS	NR	Wu et al. (2019)
Raw Arecae semen and its processed drugs	HPLC	0.89 ng	Sun et al. (2017)
Areca nut and different manufactured areca nut-containing products	LC-MS/MS	0.1 pg (on column) LOQ 0.5 pg (on column)	Jain et al. (2017)
Areca nut	Capillary electrophoresis	0.25 mg/L	Zhao et al. (2009)
Areca nut	NIR technology, HPLC	NR	Xue et al. (2011)
Areca nut	TLC-densitometric method	NR	Lin et al. (1992)
Areca nut	RP-HPLC	NR	He et al. (2011)

CE-ECL, capillary electrophoresis-electrochemiluminescence; CZE, capillary zone electrophoresis; HPLC, high-performance liquid chromatography; HPLC-ES-MS, high-performance liquid chromatography-electrospray mass spectrometry; HPTLC, high-performance thin-layer liquid chromatography; LC-MS/MS, liquid chromatography-tandem mass spectrometry; LOQ, limit of quantification; MALDI-TOF-MS, matrix-assisted laser desorption ionization mass spectrometry with a time-of-flight analyser; MCE-CCD, microchip capillary electrophoresis with contactless conductivity detection; NIR, near infrared; NR, not reported; PEC, photoelectrochemical detection; RP, reversed phase; TLC, thin-layer chromatography; UPLC, ultra-performance liquid chromatography.

1.3.2 Biological specimens

Several methods were available for the direct analysis of arecoline in various biological matrices, including saliva, urine, and serum, and typically combining liquid chromatography with mass spectrometry (Table 1.2). Chou et al. (2012) suggested a method for the determination of arecoline–protein adducts in human plasma.

[The Working Group was unable to identify an internationally accepted and validated biomarker for arecoline exposure.]

1.4 Occurrence and exposure

1.4.1 Occurrence

Arecoline occurs naturally in areca nut, the seed of the fruit of the areca palm (*Areca catechu* L.), which is widespread in south and south-east Asia (Volgin et al., 2019). For further information on the areca nut, see *IARC Monographs* Volumes 85 and 100E (IARC, 2004; 2012). Arecoline concentrations in areca nuts and various products derived from areca nut are summarized in Table 1.3.

Cai et al. (2012) reported the arecoline concentration in "semen Arecae" samples collected from Sri Lanka, and in Hainan and Guangzhou,

Table 1.2 Representative methods for the detection and quantification of arecoline in biological matrices

Sample matrix	Assay procedure	Limit of detection	Limit of quantification	Reference
Humans				
Plasma	GC-MS	0.5 ng/mL	1 ng/mL	Hayes et al. (1989)
Cord serum	LC-ESI-MS	0.001 µg/g	0.004 µg/g	Pichini et al. (2003)
Blood	LC-MS/MS	0.02 ng/mL	0.5 ng/mL	Wu et al. (2010)
Hair	LC-ESI-MS	0.09 ng/mg	0.30 ng/mL	Marchei et al. (2005)
Saliva	HPLC	50 pg	NR	Cox et al. (2004)
Saliva	HPLC-MS	NR	NR	Cox et al. (2010)
Saliva	LC-MS/MS	0.156 ng/mL	1.25 ng/mL	Lee et al. (2015)
Saliva	HPLC-MS/MS	NR	NR	Venkatesh et al. (2018)
Urine, newborn	LC-ESI-MS	0.0004 µg/g	0.001 µg/g	Pichini et al. (2003)
Urine	Online SPE LC-MS/MS	0.016 ng/mL	0.05 ng/mL	Hu et al. (2010)
Breast milk	LC-MS/MS	16 µg/L	50 µg/L (using 1 mL of human milk per assay)	Pellegrini et al. (2007)
Meconium	LC-ESI-MS	0.001 µg/g	0.005 µg/g	Pichini et al. (2003)
Typical stained tooth from an Iron Age skeleton	LC-MS/MS and LC-HR-ToF-MS	NR	NR	Krais et al. (2017)
Arecoline–protein adducts	Nanoscale LC-MS	NR	NR	Chou et al. (2012)
Experimental systems				
Rat urine	LC-MS and LC-MS/MS	< 8 ng/mL	NR	Zhu et al. (2006)
Rat plasma	LC-MS/MS	1 ng/mL (LLQ)	0.5 ng/mL	Pan et al. (2018)

GC-MS, gas chromatography-mass spectrometry; HPLC, high-performance liquid chromatography; HPLC-MS/MS, high-performance liquid chromatography-tandem mass spectrometry; LC-ESI-MS, liquid chromatography-electrospray quadrupole mass spectrometry; LC-HR-ToF-MS, liquid chromatography-high-resolution time-of-flight mass spectrometry; LC-MS/MS, liquid chromatography-tandem mass spectrometry; LLQ, lower limit of quantification; NR, not reported; SPE LC-MS/MS, solid-phase extraction liquid chromatography-tandem mass spectrometry.

China. The maximum average arecoline concentration was measured in semen Arecae from Sri Lanka (0.43%), followed by Guangzhou (0.41%) and Hainan (0.24%). Concentrations of arecoline were higher in unripe than in ripe areca nuts from Thailand (0.14% versus 0.09% w/w of dried seed powder, respectively) (Jantarat et al., 2013). Adhikari et al. (2015) analysed arecoline concentrations in various areca-nut products available on the Indian market, including pan masala, mouth freshener, scented *supari* (the colloquial name for areca nut in Hindi and other Indian languages), and areca nut. The average concentration of arecoline in areca-nut products was reported to be in the range of 132.7 to 415.6 ng/mg, while areca nut itself had a concentration of 434.4 ng/mg.

Wu et al. (2019) reported average concentrations of arecoline in the range of 0.06 to 0.77 mg/g dry weight in unripe (stages 1–3) and ripe fruits, spadices, female flowers, male flowers, tender leaves, and ripe leaves of *Areca triandra* from Hainan province in China. In another study from China by Sun et al. (2017), average concentrations of arecoline measured in raw, tostum, and carbonisata Arecae semen were 3.38, 2.23, and 1.36 mg/g, respectively. Similarly, concentrations of arecoline were measured at 0.64–2.22 mg/g in bulk areca nut, pan masala, and *gutkha* from India, China, and Minneapolis, USA, by Jain et al. (2017). *Gutkha*, or betel quid

Table 1.3 Concentrations of arecoline in products derived from areca nut

Product	Average concentration	Standard deviation or range	Unit	Country or region	Reference
Areca nut	0.2191	± 0.0070	%	Import (NR)	Lin et al. (1992)
Areca nut	0.1896	± 0.0072		Hainan, China	
Areca nut	0.5607	± 0.0125		Guangzhou, China	
Areca nut	0.4310	± 0.0067	%	Sri Lanka	Cai et al. (2012)
Areca nut	0.2396	± 0.0042		Hainan, China	
Areca nut	0.4078	± 0.0085		Guangzhou, China	
Areca nut	6.6	NR	mg/g	NR	Dai et al. (2014)
Areca nut	434.4	± 4.42	ng/mg sample	India	Adhikari et al. (2015)
Sundried areca nut	0.5	0–1.4	%	Mumbai, India	Awang (1986)
Areca nuts from boiled varieties	0.8	0.4–1.3			
Roasted areca nuts	0.9	0.4–1.3			
Unripe areca nut Ripe areca nut	0.1434 0.0944	± 0.0016 ± 0.0002	%w/w of dried seed powder	Thailand	Jantarat et al. (2013)
Raw areca nut	1.15	± 0.008	%	NR	Dutta et al. (2017)
Pan masala areca nut	0.94	± 0.006			
Boiled areca nut	0.79	± 0.009			
Roasted areca nut	0.85	± 0.007			
Chinese areca nut	[1.27]	1.07–1.44	mg/g product dry weight	Changsha City, Hunan Province, China	Jain et al. (2017)
Bulk areca nuts	[1.27]	0.91–2.22		Mumbai, India	
Raw Arecae semen	3.38	± 0.157	mg/g	China	Sun et al. (2017)
Arecae semen tostum	2.23	± 0.223			
Arecae semen carbonisata	1.36	± 0.219			
Rajnigandha flavoured pan masala	376.9	± 7.71	ng/mg sample	India	Adhikari et al. (2015)
Parag pan masala	362.5	± 4.63			
Paras premium pan masala	349.2	± 6.36			
Dilruba sahi pan masala	255.2	± 4.01			
Pan parag pan masala	386.1	± 2.90			
Shikhar pan masala	415.6	± 3.83			
Chutki mouth freshener	132.7	± 5.92			
Tiranga pan masala	384.7	± 2.65			
Sir g finest pan masala	294.9	± 4.06			
Bahar heritage pan masala	322.7	± 5.94			.
Sweety *supari* kesar scented	167.4	± 3.55			
Pan masala *Gutkha*	[0.93] [0.95]	0.64–1.25 0.74–1.16	mg/g product dry weight	Indore, Madhya Pradesh, India, and Minneapolis	Jain et al. (2017)
Areca triandra (unripe fruits stage 1)	0.271	± 0.00773	mg/g dry weight	Hainan Province, China	Wu et al. (2019)
Areca triandra (pericarp of unripe fruits stage 2)	0.402	± 0.00698			
Areca triandra (endosperm of unripe fruits stage 2)	0.772	± 0.0419			

Table 1.3 (continued)

Product	Average concentration	Standard deviation or range	Unit	Country or region	Reference
Areca triandra (pericarp of unripe fruits stage 3)	0.180	± 0.00202	mg/g dry weight	Hainan Province, China	Wu et al. (2019) (cont.)
Areca triandra (endosperm of unripe fruits stage 3)	0.332	± 0.00570			
Areca triandra (pericarp of ripe fruits)	0.0621	± 0.00388			
Areca triandra (endosperm of ripe fruits)	0.191	± 0.00307			
Areca triandra (spadices)	0.164	± 0.00130			
Areca triandra (female flowers)	0.186	± 0.00650			
Areca triandra (male flowers)	0.190	± 0.00720			
Areca triandra (tender leaves)	0.660	± 0.00497			
Areca triandra (ripe leaves)	0.177	± 0.00362			

NR, not reported; w/w, weight per weight.

with tobacco, is a form of chewing tobacco containing a mixture of tobacco, crushed areca nut (also called betel nut), spices, and other ingredients (IARC, 2004). The average concentration of arecoline in sundried (0.5%), boiled (0.8%), and roasted areca nuts (0.9%) from Mumbai, India, was measured by Awang (1986). Lin et al. (1992) recorded average arecoline concentrations of 0.19% and 0.56% in areca nuts from Hainan and Guangzhou, respectively, in China.

Several researchers estimated arecoline concentrations in areca nut and in products containing areca nut. Recently, Dutta et al. (2017) found that raw areca nut and pan masala contained the highest concentrations of arecoline at 1.15% and 0.94%, respectively. Boiled areca nut contained the lowest concentration (0.79%) and roasted areca nut showed an intermediate concentration of arecoline (0.85%).

Adhikari et al. estimated the concentrations of arecoline in 11 brands of pan masala from Kolkata, India. Arecoline concentrations ranging from 130 to 415 µg/g of dried pan masala were detected (Adhikari et al., 2015).

Areca nut is an integral part of traditional Indian Ayurveda and Chinese medicines. Arecoline, an alkaloid, is mainly responsible for the areca nut's pharmaceutical properties (Arjungi, 1976). Yi et al. (2012) determined the arecoline content of the Chinese patent medicine Si-Mo-Tang, a liquid preparation that is taken orally and that is used in the treatment of gastrointestinal dyspeptic disease. The observed mean arecoline concentration was 29 ± 7 µg/mL, and concentrations ranged from 19 to 43 µg/mL. Another Chinese traditional medicine, Simo decoction, which is widely used to treat gastrointestinal dysmotility, contains arecoline as one of 94 ingredients (He et al., 2018).

1.4.2 Exposure in the general population

No information on direct exposure to arecoline as an isolated chemical was available to the Working Group. Exposure of the general population to arecoline is generally indirect via the use of areca nut and areca nut-derived

products. For further information on areca nut see IARC (2004, 2012).

Areca nut, sometimes called "betel nut" (although the latter is not botanically correct), is reportedly consumed by ~10–20% of the global population, making arecoline the fourth most frequently consumed psychoactive substance in the world after alcohol, nicotine, and caffeine (Volgin et al., 2019). The use of areca nut has been an integral part of various social customs and ceremonies in many Asian countries for thousands of years (Volgin et al., 2019). Its consumption is socially accepted in many Asian countries due to its pharmacological properties, which have been described historically (Volgin et al., 2019). Areca nut is mainly consumed in Asian countries (e.g. south China, Malaysia, tropical India, Sri Lanka, Pakistan, Myanmar, Indonesia, the Philippines), but also in countries of Oceania (Micronesia, Polynesia, and South Pacific islands), as well as parts of east Africa. The nuts are used either fresh or processed by sun drying, baking, boiling, or roasting to alter their flavour (Sinor et al., 1990; Leghari et al., 2016). A variety of products containing areca nut are available in other parts of the world where there are Asian immigrants or habitual users, but with limited availability due to local regulations (see Section 1.5; Warnakulasuriya, 2002; Blank et al., 2008). For example, betel products were collected from Richmond, Virginia, USA, between March and May 2006 and included pure areca nut, areca nut with tobacco, and areca nut with additives. Most packaging labels did not contain health warnings specific to arecoline.

The manner in which areca nut is consumed varies around the world. Most commonly it is placed in the mouth as small pieces wrapped in betel leaf and slaked lime. Sometimes additives, spices, sweeteners, and tobacco are added to this preparation (Blank et al., 2008).

Betel quid is a combination of areca nut, betel leaf, slaked lime, and flavouring ingredients (varying according to region) and is the common form in which areca nut is consumed in Asia. An interviewer-administered survey followed by an examination for oral mucosal disorders was conducted by the Asian Betel-Quid Consortium to investigate the population burden of betel-quid use and its effect on oral premalignant disorders in south, south-eastern, and east Asia. A total of 8922 participants from Taiwan and mainland China, Malaysia, Indonesia, Nepal, and Sri Lanka, were recruited. The prevalence of betel-quid use varied from 0.8% to 46.3% across the six populations studied (Lee et al., 2012).

According to a narrative review, the highest prevalence of betel-quid use was in Papua New Guinea, followed by Bangladesh, India, Pakistan, Myanmar, and Sri Lanka, whereas prevalence was relatively lower in Cambodia, Malaysia, Indonesia, and Taiwan, China (Gunjal et al., 2020).

[These studies mainly described betel-quid use/abuse and did not specifically mention consumption of areca nut or arecoline.]

Areca nut and smokeless tobacco are widely consumed across Myanmar (Papke et al., 2020) and by Palauans (one of the largest immigrant groups) in Hawaii, USA (Quinn Griffin et al., 2014). Yoganathan (2002) found that different types of product containing areca nut were available in New Zealand and Australia, mainly in Asian groceries. They were primarily consumed by Indian immigrants to these countries.

Areca nut is consumed in many forms by south-east Asians: raw, mixed with some additives, and as commercially available preparations. The most popular product in India is pan masala, which is a blend of areca nut powder and additives. Another commonly consumed areca-nut product is *gutkha*, which contains tobacco as a major constituent. These products are readily available with attractive packaging at affordable prices and are therefore popular among young people (Dutta et al., 2017).

Javed et al. (2008, 2010) interviewed 1000 adults (aged 45–64 years) from Karachi, Pakistan, to study the reasons why people used the areca-nut product *gutkha*. Of the study participants, 24% of those with type 2 diabetes and 8% of those without type 2 diabetes reported that they chewed *gutkha* to control hunger. A cross-sectional study conducted by Leghari et al. (2016) among schoolchildren of Karachi, Pakistan, found a high frequency of areca nut (78%) and *gutkha* chewing (60%).

A significant number of studies in humans have identified the presence of arecoline in samples of saliva, blood, urine, hair, and breast milk (Table 1.4); [these data might be useful for qualitative exposure assessments].

Salivary concentrations of arecoline in areca-nut chewers were measured during chewing and at different post-chewing intervals. Arecoline was undetectable at baseline. During chewing, mean arecoline concentrations were 77 ng/mL (regular users of areca nut) and 65 ng/mL (control group of occasional users) in areca-nut chewers and 130 ng/mL in the placebo group (regular users of areca nut who were given rubber base to chew). After chewing, mean arecoline concentrations were 196 ng/mL, 321 ng/mL, and 44 ng/mL, respectively. Arecoline concentrations were higher after chewing areca nut than during chewing. Arecoline concentrations were significantly higher in the areca-nut chewers than in the placebo group. During chewing, the highest arecoline concentrations were reached during the first minute. After chewing, arecoline concentrations were high until 10 minutes post-chewing, after which they started to decline in both groups of areca-nut chewers (Venkatesh et al., 2018). A study conducted by Franke et al. (2016) looked for the presence of arecoline in the hair, saliva, and urine of areca-nut chewers. No arecoline was detected in the hair samples tested. Arecoline was detected in the saliva and urine within 2–8 hours post-chewing; concentrations were highest up to 2 hours post-chewing, declined with time, and returned to baseline 8 hours post-chewing. Marchei et al. (2005) reported mean arecoline concentrations of 0.61–1.27 ng/mg in the hair of long-term areca-nut consumers.

High concentrations of salivary arecoline (up to 140 µg/mL) in betel-quid chewers (mean values, 52 µg/mL and 30 µg/mL with and without tobacco, respectively) were reported by Nair et al. (1985). García-Algar et al. (2005) reported the detection of arecoline in the meconium of six Asian newborn babies whose mothers consumed areca nut during pregnancy. Placental tissue from these mothers also showed the presence of arecoline. The observed ranges of arecoline concentrations measured in this study were 0.006–0.022 µg/g in meconium and not detected to 0.015 µg/g in placenta. [The Working Group noted that due to the lack of a baseline arecoline range in mothers who were not areca-nut chewers at the time, it was not possible to arrive at any conclusion.]

Wu et al. (2010) reported a statistically significant correlation between betel-quid consumption and blood arecoline concentrations (Spearman correlation coefficient, $r = 0.81$; $P < 0.01$). [Therefore, serum arecoline is a promising short-term indicator of betel-quid consumption.] Franke et al. (2020) studied the presence of arecoline in the saliva and hair of areca-nut chewers. Arecoline was only detected in hair samples from men. Conversely, sex did not influence salivary arecoline concentrations determined within 5–24 hours of chewing areca nut. These alkaloids were found to be present in hair months after the cessation of areca-nut chewing. A study conducted by Pellegrini et al. (2007) in Italy reported the presence of arecoline (together with other substances such as nicotine, caffeine, and cotinine) in human breast milk. Cox et al. (2010) estimated arecoline concentrations in the saliva of areca-nut chewers ($n = 32$) and non-chewers/controls ($n = 6$). Arecoline was detected in all areca-nut chewers. Maximum concentrations of arecoline were measured in the

Table 1.4 Concentrations of arecoline in biological samples from humans

Sample	Location	Concentration (mean, median, or range)	Method of estimation	Comments	References
Plasma	USA	Range, ND–5 ng/mL	GC-MS	Healthy volunteers, transdermal administration of arecoline 3 mg/h, measurements during 30 h after dose.	Hayes et al. (1989)
Plasma	USA	LOQ, 0.16 ng/mL C_{max} mean ± SD, 27.8 ± 20.5 ng/mL C_{max} range, 7.8–83.3 ng/mL	GC-MS	Pharmacological use of intravenous arecoline 5 mg/30 min; up to 40 mg/day for memory enhancement in 15 patients with Alzheimer disease.	Asthana et al. (1996)
Blood	Taiwan, China	Betel quid chewers: mean ± SD, 7.0 ± 10.7 ng/mL Never-chewers: mean ± SD, 0.3 ± 0.2 ng/mL; range, 0–0.63 ng/mL	LC-MS/MS	Blood from 13 betel-quid chewers and 5 never-chewers.	Wu et al. (2010)
Hair	Italy, India, Spain	Women: mean ± SD, 1.27 ± 0.20 ng/mg hair Men: mean ± SD, 0.61 ± 0.52 ng/mg hair Range reported 300 pg/mg to 1.70 ng/mg hair	HPLC-MS	First report measuring arecoline in hair samples from 11 long-term areca-nut users (2–35 years); arecoline can be used as a non-invasive biomarker for areca-nut use.	Marchei et al. (2005)
Hair	Papua New Guinea	Mean, 3.56 ng/mg hair; median, 2.24 ng/mg hair; range, 60 pg/mg to 18 ng/mg hair	UPLC-MS/MS and UPLC-Q-Tof-MS	Included 11 men and 8 women who had been abusing areca nut for more than 6 mo before hair sampling. Hair seems to be a promising marker of long-term exposure to areca nut.	Gheddar et al. (2020)
Saliva	Australia	Maximum concentration during chewing: range, 5.6–97 µg/mL	HPLC-MS	Samples collected from 32 habitual areca-nut chewers before, during and after chewing.	Cox et al. (2010)
Saliva	Taiwan, China	Range, 0–80 µg/mL C_{max} mean ± SD, 44 ± 32 µg/mL	LC-MS/MS	Saliva samples of 5 men after chewing one 5 g of areca nut. The highest concentrations were measured after 5 min of chewing.	Lee et al. (2015)
Saliva	India	Areca-nut chewers during chewing: C_{max} mean, 77 ng/mL C_{max} range, 49–280 ng/mL Areca-nut chewers after chewing: C_{max} mean, 196 ng/mL C_{max} range, 154–333 ng/mL Placebo group after chewing: C_{max} mean, 321 ng/mL	HPLC-MS	Salivary arecoline concentrations for 20 individuals before and after during chewing of 0.5 g of fresh areca nut. Baseline levels were ND. The placebo group had higher arecoline levels than the areca nut-chewing group.	Venkatesh et al. (2018)
Neonatal urine	Spain	Urine: range, ND–0.01 µg/mL		Neonatal urine samples from 2 babies born at the Hospital de Mar of Barcelona, Spain.	Pichini et al. (2003)

Table 1.4 (continued)

Sample	Location	Concentration (mean, median, or range)	Method of estimation	Comments	References
Urine	Taiwan, China	Mean ± SD, 23.9 ± 39.3 ng/mg creatinine; range, ND–142 ng/mg creatinine	Solid-phase extraction LC-TMS	Included 33 regular areca-nut chewers who were also cigarette smokers. First study to report the presence of *N*-methylnipecotic acid and arecaidine besides arecoline in urine of areca-nut chewers.	Hu et al. (2010)
Breast milk	Spain, Italy, India	Range, 18–150 µg/L	LC-MS/MS	Included 4 betel-quid-consuming breastfeeding mothers. Levels of arecoline in breast-fed infants and relationship with clinical outcomes.	Pellegrini et al. (2007)
Meconium and placenta	Spain	Meconium: range, 0.006–0.022 µg/g Placenta: range, ND–0.015 µg/g	HPLC/electrospray quadrupole-MS	First study to detect arecoline in meconium of newborns and placental tissue of 6 Asian mothers who were areca-nut chewers during pregnancy.	García-Algar et al. (2005)
Meconium	Spain	Meconium: range, 0.006–0.008 µg/g	HPLC/electrospray quadrupole-MS	Arecoline measured in biological samples from 2 newborns whose mothers consumed areca nuts; attending Hospital del Mar, Barcelona, Spain.	Pichini et al. (2003)

C_{max}, maximum concentration; GC-MS, gas chromatography-mass spectrometry; h, hour; HPLC-MS, high-performance liquid chromatography-mass spectrometry; LC-MS, liquid-chromatography-mass spectrometry; LC-MS/MS, liquid-chromatography-tandem mass spectrometry; LOQ, limit of quantification; min, minute; ND, not detected; SD, standard deviation; UPLC-MS/MS, ultra-performance liquid chromatography-tandem mass spectrometry; UPLC-Q-Tof-MS, ultraperformance liquid chromatography coupled with quadrupole time-of-flight mass spectrometry.

range of 6 to 97 µg/mL. Salivary arecoline could not be detected in controls.

[The Working Group noted that, considering the available data, arecoline levels in blood and saliva may act as short-term markers, while arecoline levels in hair may act as a long-term marker of arecoline exposure. After successful validation, these markers may have potential applications in future exposure assessments.]

1.4.3 Occupational exposure

No information was available on occupational exposure to arecoline, either directly (e.g. during the purification processes) or indirectly (due to areca-nut exposure). However, it has been suggested that people of lower socioeconomic status, specifically women in India engaged in intensive labour, use smokeless tobacco products including areca nut to suppress their hunger during working hours (Government of India, 2020a). A report from Sri Lanka showed that approximately 53% of the rural population, particularly drivers and labourers, consume areca nut as it is believed to reduce hunger and tiredness (Selvananthan et al., 2018). [The Working Group noted that the areca nut may be consumed to suppress hunger or as a stimulant during long work shifts, but the consumption of areca nut is not related to a particular occupation.]

1.5 Regulations and guidelines

1.5.1 Exposure limits and guidelines

For arecoline itself, only a few regulations are available worldwide. The European Chemicals Agency (ECHA) within its Registration, Evaluation, Authorization and Restriction of Chemicals Regulation (REACH) assessment classified arecoline and arecoline hydrochloride as "1–10 tonne registered substances" likely to meet criteria for category 1A or 1B carcinogenicity, mutagenicity, or reproductive toxicity (ECHA, 2020a, b). Since 2010, arecoline is also included in the List of Substances Prohibited in Cosmetic Products in the European Union (European Commission, 2020).

For areca nut, few regulations are in place worldwide, especially when compared with tobacco and alcohol. An urgent need for regional and global policies to mitigate the misuse of areca nut has been highlighted by various researchers (Mehrtash et al., 2017; Thakur et al., 2020). The import and inter-state transport of areca nut has been restricted by the United States Food and Drug Administration (US FDA). It is also designated as a toxic and carcinogenic substance by the California Environmental Protection Agency (CalEPA, 2006). The sale of products containing areca nut has been banned in Canada. In the European Union, areca nut is treated as an unauthorized novel food, which may not be placed on the market (European Commission, 2019).

In 2018, the Indian government made a strong move to regulate the food safety and import of areca nut through the Food Safety and Standards Authority of India (FSSAI). Previous Indian health policies were based on warnings on the packaging of areca-nut products (FSSAI, 2004), whereas the latest policies enforce the ban on the manufacturing and sale of areca-nut products containing tobacco or nicotine (FSSAI, 2011). Many Indian states have banned areca-nut products under the FSSAI (2006).

Recently, the Indian Council of Medical Research, and the Department of Health Research, Ministry of Health and Family Welfare (Government of India, 2020b) appealed to the public not to consume or spit smokeless tobacco, areca nut, or betel quid in public places in order to contain the spread of SARS-CoV-2, the cause of novel coronavirus disease (COVID-19), under the Epidemic Disease Act, 1897, the Disaster Management Act, 2005 and the Indian Penal Code 1860 and Code of Criminal Procedure. The appeal states that: "Chewing smokeless tobacco products, paan masala and areca nut (*supari*)

increases the production of saliva followed by a very strong urge to spit. Spitting in public places could enhance the spread of the COVID19 virus."

Various other Asian countries, including Bhutan, Myanmar, Taiwan, China, and Sri Lanka also have policies and regulations regarding use of betel quid and areca nut. These include bans on chewing, spitting in public places, and the sale of areca nut (Gunjal et al., 2020).

[These policies are not specific to areca nut/arecoline, but common to smokeless tobacco products and areca nut or betel quid. The Working Group expected that policies regarding areca nut/betel quid would also reduce exposure to arecoline.]

1.5.2 Reference values for biological monitoring of exposure

No validated reference values for arecoline or arecoline biomarkers to quantitatively monitor exposure were available to the Working Group.

2. Cancer in Humans

No data were available to the Working Group.

3. Cancer in Experimental Animals

In previous evaluations, the *IARC Monographs* programme concluded that there was *limited evidence* in experimental animals for the carcinogenicity of arecoline (IARC, 2004).

Studies of carcinogenicity with arecoline and its metabolite arecaidine in experimental animals are summarized in Table 3.1.

3.1 Mouse

3.1.1 Oral administration (gavage)

In the first experiment in a study by Bhide et al. (1984), two groups of male (n = 20–35) and female (n = 18–20) Swiss mice (age, 6 weeks) were treated with vehicle (control) or 1 mg of arecoline hydrochloride [purity not reported; rationale for dose not specified] dissolved in distilled water (adjusted to pH 7) per animal, by gavage, five times per week "throughout the lifespan." Additional groups of male (n = 16–19) and female (n = 8–14) mice were treated with 1 mg of arecoline hydrochloride and 1 mg of laboratory-grade potassium nitrate (KNO_3) [purity not reported; rationale for dose not reported] in distilled water per animal, with 1 mg of arecoline hydrochloride, 1 mg of KNO_3, and 1 mg of slaked lime (commercial brand used to prepare betel quid) in distilled water per animal, or with 1 mg of KNO_3 and 1 mg of slaked lime in distilled water per animal. [Data on body weight or survival were not reported.] Histopathological examination was performed on the liver, lungs, and stomach, and any other abnormal tissue of all animals.

When assessed during 25 months [it was unclear whether this was 25 months of age or 25 months of treatment], male mice treated with arecoline hydrochloride only showed a significant increase [P = 0.0023; Fisher exact test] in the incidence of total tumours compared with the vehicle control group. In the group (n = 35) treated with arecoline hydrochloride, eight mice developed liver haemangioma, four developed lung adenocarcinoma, and three developed squamous cell carcinoma of the stomach. The total tumour incidence in the other treated groups of males did not differ significantly from that in the vehicle control group. No tumours were observed in female mice (Bhide et al., 1984). [The Working Group noted the limited histopathology, the single dose used, the small

Table 3.1 Studies of carcinogenicity with arecoline or its metabolite, arecaidine, in experimental animals

Study design Species, strain (sex) Age at start Duration Reference	Route Agent tested, purity Vehicle Dose(s) No. of animals at start No. of surviving animals	Incidence (%), multiplicity, or no. of tumours	Significance	Comments
Full carcinogenicity Mouse, Swiss (M) 6 wk 25 mo Bhide et al. (1984)	Oral administration (gavage) Arecoline hydrochloride, KNO_3, and slaked lime; purity, NR Distilled water (adjusted to pH 7) Control, 1 mg arecoline hydrochloride, 1 mg arecoline hydrochloride + 1 mg KNO_3, 1 mg arecoline hydrochloride + 1 mg KNO_3 + 1 mg lime, 1 mg KNO_3 + 1 mg lime (mg/application per mouse) 5×/wk for 25 mo 20, 35, 19, 16, 17 NR	*All sites* (including liver, lung, and stomach): Tumour incidence: 1/20 (5%), 15/35 (43%)*, 3/19 (16%), 1/16 (6%), 2/17 (12%) *Liver*: haemangioma Tumour incidence: NR, 8/35 (23%), NR, NR, NR *Lung*: adenocarcinoma Tumour incidence: NR, 4/35 (11%), NR, NR, NR *Stomach*: squamous cell carcinoma Tumour incidence: NR, 3/35 (9%), NR, NR, NR	*[$P = 0.0023$, 1 mg arecoline hydrochloride vs control; one-tailed Fisher exact test; all other groups, NS vs control] NA NA NA	Principal limitations: body-weight changes, NR; lack of KNO_3-only control group; survival, NR; rationale for doses, NR; tumours observed in the vehicle control group not further specified; limited histopathology; use of a single dose; small number of mice per group; purity of test articles, NR. Other comments: it was unclear whether 25 mo (see duration and dosing regimen) was the age of the mice or the duration of treatment; KNO_3, laboratory grade; slaked lime, commercial brand used to prepare betel quid.
Full carcinogenicity Mouse, Swiss (F) 6 wk 25 mo Bhide et al. (1984)	Oral administration (gavage) Arecoline hydrochloride (+/– KNO_3 and slaked lime); purity, NR Distilled water Control, 1 mg arecoline hydrochloride, 1 mg arecoline hydrochloride + 1 mg KNO_3, 1 mg arecoline hydrochloride + 1 mg KNO_3 + 1 mg lime, 1 mg KNO_3 + 1 mg lime (mg/application/mouse) 5×/wk for 25 mo 20, 18, 14, 12, 8 NR	*All sites* (including liver, lung, and stomach): Tumour incidence: 0/20, 0/18, 0/14, 0/12, 0/8	NA	Principal limitations: body-weight changes, NR; lack of KNO_3-only control group; survival, NR; rationale for doses, NR; limited histopathology; use of a single dose; small number of mice per group; purity of test articles, NR. Other comments: it was unclear whether 25 mo (see duration and dosing regimen) was the age of the mice or the duration of treatment; KNO_3, laboratory grade; slaked lime, commercial brand used to prepare betel quid.

Table 3.1 (continued)

Study design Species, strain (sex) Age at start Duration Reference	Route Agent tested, purity Vehicle Dose(s) No. of animals at start No. of surviving animals	Incidence (%), multiplicity, or no. of tumours	Significance	Comments
Full carcinogenicity Mouse, Swiss (M) 8 wk 25 mo Bhide et al. (1984)	Oral administration (gavage) Arecoline hydrochloride (+/– KNO_3 and slaked lime), in mice fed a vitamin B complex-deficient diet; purity, NR Distilled water Control, 1 mg arecoline hydrochloride, 1 mg arecoline hydrochloride + 1 mg KNO_3, 1 mg arecoline hydrochloride + 1 mg KNO_3 + 1 mg lime (mg/application per mouse) 5×/wk for 25 mo 21, 21, 16, 18 NR	*All sites* (including liver, lung, and stomach): Tumour incidence: 2/21 (10%), 7/21 (33%), 1/16 (6%), 7/18 (39%)*	*[$P = 0.0361$, 1 mg arecoline hydrochloride + 1 mg KNO_3 + 1 mg lime vs control; one-tailed Fisher exact test; all other groups, NS vs control]	Principal limitations: body-weight changes, NR; lack of KNO_3-only control group; survival, NR; rationale for doses, NR; lack of KNO_3 + lime control group; tumours observed in the vehicle control group not further specified; limited histopathology; use of a single dose; small number of mice per group; purity of test articles, NR. Other comments: it was unclear whether 25 mo (see duration and dosing regimen) was the age of the mice or the duration of treatment; KNO_3, laboratory grade; slaked lime, commercial brand used to prepare betel quid.
		Liver: haemangioma Tumour incidence: NR, 3/21 (14%), NR, 2/18 (11%)	NA	
		Lung: adenocarcinoma Tumour incidence: NR, 3/21 (14%), NR, 3/18 (17%)	NA	
		Stomach: squamous cell carcinoma Tumour incidence: NR, 0/21, NR, 2/18 (11%)	NA	

Table 3.1 (continued)

Study design Species, strain (sex) Age at start Duration Reference	Route Agent tested, purity Vehicle Dose(s) No. of animals at start No. of surviving animals	Incidence (%), multiplicity, or no. of tumours	Significance	Comments
Full carcinogenicity Mouse, Swiss (F) 8 wk 25 mo Bhide et al. (1984)	Oral administration (gavage) Arecoline hydrochloride (+/– KNO_3 and slaked lime), in mice fed a vitamin B complex-deficient diet; purity, NR Distilled water Control, 1 mg arecoline hydrochloride, 1 mg arecoline hydrochloride + 1 mg KNO_3, 1 mg arecoline hydrochloride + 1 mg KNO_3 + 1 mg lime (mg/application/mouse) 5×/wk for 25 mo 16, 12, 16, 18 NR	*All sites* (including liver, lung, and stomach): Tumour incidence: 1/16 (6%), 6/12 (50%)*, 2/16 (13%), 8/18 (44%)**	*[P = 0.0132, 1 mg arecoline hydrochloride vs control; **P = 0.0143, 1 mg arecoline hydrochloride + 1 mg KNO_3 + 1 mg lime vs control; one-tailed Fisher exact test; other group NS vs control]	Principal limitations: body-weight changes, NR; lack of KNO_3-only control group; rationale for doses, NR; lack of KNO_3 + lime control group; tumours observed in the vehicle control group not further specified; limited histopathology; use of a single dose; small number of mice per group; purity of test articles, NR. Other comments: it was unclear whether 25 mo (see duration and dosing regimen) was the age of the mice or the duration of treatment; KNO_3, laboratory grade; slaked lime, commercial brand used to prepare betel quid.
		Liver Haemangioma Tumour incidence: NR, 2/12 (17%), NR, 2/18 (11%)	NA	
		Cholangiocarcinoma Tumour incidence: NR, 0/12, NR, 1/18 (6%)	NA	
		Lung: adenocarcinoma Tumour incidence: NR, 4/12 (33%), NR, 3/18 (17%)	NA	
		Stomach: squamous cell carcinoma Tumour incidence: NR, 0/12, NR, 2/18 (11%)	NA	
Full carcinogenicity Mouse, Swiss (M) NR Lifetime Shivapurkar et al. (1980)	Intraperitoneal injection Arecoline, analytical reagent grade; purity, NR 0 (0.1 mL distilled water), 1.5 mg 1×/wk for 13 wk 10, 10 NR	*All organs*: Tumour incidence: 0/10, 0/10	NA	Principal limitations: body-weight changes, NR; survival, NR; vehicle and volume, NR; starting age, NR; precise duration of experiment, NR; use of a single dose; small number of mice per group; short duration of treatment; use of one sex only; justification for the dose, NR.

Table 3.1 (continued)

Study design Species, strain (sex) Age at start Duration Reference	Route Agent tested, purity Vehicle Dose(s) No. of animals at start No. of surviving animals	Incidence (%), multiplicity, or no. of tumours	Significance	Comments
Full carcinogenicity Mouse, Swiss (M) NR Lifetime Shivapurkar et al. (1980)	Subcutaneous injection Arecoline, analytical reagent grade; purity, NR 0 (0.1 mL distilled water), 1.5 mg 1×/wk for 13 wk 20, 10 NR	*At injection site or of other organs*: Tumour incidence: 0/20, 0/10	NA	Principal limitations: starting age, NR; body-weight changes, NR; survival, NR; vehicle and volume, NR; precise duration of experiment, NR; use of a single dose; small number of mice per group; short duration of treatment; use of one sex only; justification of the dose, NR.
Co-carcinogenicity Mouse, C57BL/6JNarl (M) 6 wk 28 wk Chang et al. (2010)	Oral administration (drinking-water) Arecoline hydrobromide and 4-NQO; purity, NR Water 0 (control), 100 (4-NQO), 200 (4-NQO), 250 (arecoline hydrobromide), 500 (arecoline hydrobromide), 100 (4-NQO) + 250 (arecoline hydrobromide), 100 (4-NQO) + 500 (arecoline hydrobromide), 200 (4-NQO) + 250 (arecoline hydrobromide), 200 (4-NQO) + 500 (arecoline hydrobromide) µg/mL drinking-water for 8 wk, followed by drinking-water only for 20 wk 10, 10, 10, 10, 10, 11, 11, 11, 11 7, 7, 7, 7, 7, 8, 8, 8, 8	*Tongue*: hyperplasia, dysplasia, papilloma, or invasive squamous cell carcinoma (combined) Lesion incidence: 0/7, 2/7 (29%), 4/7 (57%), 0/7, 0/7, 4/8 (50%), 3/8 (38%), 4/8 (50%), 8/8 (100%) *Oesophagus*: hyperplasia, dysplasia, papilloma, or invasive squamous cell carcinoma (combined) Lesion incidence: 0/7, 1/7 (14%), 1/7 (14%), 0/7, 0/7, 4/8 (50%), 1/8 (13%), 1/8 (13%), 0/8	[No significant effect of arecoline hydrobromide] [No significant effect of arecoline hydrobromide]	Principal limitations: short duration of exposure; short duration of follow-up; rationale for doses, NR; small number of mice per group; pre-neoplastic and neoplastic lesions combined. Other comments: three mice per group were killed at 8 wk for histopathological examination, and no lesions were observed.

Table 3.1 (continued)

Study design Species, strain (sex) Age at start Duration Reference	Route Agent tested, purity Vehicle Dose(s) No. of animals at start No. of surviving animals	Incidence (%), multiplicity, or no. of tumours	Significance	Comments
Co-carcinogenicity Mouse, C57BL/6 (M) 6 wk 28 wk Chen et al. (2017)	Oral administration (drinking-water) Arecoline hydrobromide and 4-NQO; purity, NR Water 0 (control), 100 (4-NQO) for 8 wk, 100 (4-NQO) for 16 wk, 500 (arecoline hydrobromide) for 16 wk, 100 (4-NQO) + 500 (arecoline hydrobromide) for 8 wk, 100 (4-NQO) + 500 (arecoline hydrobromide) µg/mL drinking-water for 16 wk followed by drinking-water up to experimental wk 28 8, 16, 16, 8, 16, 16 8, 16, 14, 8, 15, 13	*Oesophagus* Invasive squamous cell carcinoma Tumour incidence: 0/8, 1/16 (6%), 7/14 (50%)†, 0/8, 6/15 (40%)*,††, 9/13 (69%)‡ Papilloma Tumour incidence: 0/8, 5/16 (31%), 11/14 (79%)†, 0/8, 10/15 (67%)††,‡, 12/13 (92%)‡‡	*[$P = 0.0329$, vs 100 µg/mL 4-NQO for 8 wk; †$P = 0.0201$, vs control; ††$P = 0.0496$, vs control; ‡$P = 0.0024$, vs control; one-tailed Fisher test] †[$P = 0.0005$, vs control; one-tailed Fisher exact test];††(NS; $P = 0.049$, vs 100 µg/mL 4-NQO; one-tailed χ^2 test [but $P = 0.0528$, one-tailed Fisher exact test]); ‡[$P = 0.0026$, vs control; one-tailed Fisher test]; ‡‡[$P < 0.0001$, vs control; one-tailed Fisher exact test]	Principal limitations: body-weight changes, NR; rationale for doses, NR.

Table 3.1 (continued)

Study design Species, strain (sex) Age at start Duration Reference	Route Agent tested, purity Vehicle Dose(s) No. of animals at start No. of surviving animals	Incidence (%), multiplicity, or no. of tumours	Significance	Comments
Co-carcinogenicity Rat, F344 (M) 6 wk 25 wk Wu et al. (2016)	Oral administration (drinking-water) Arecoline and NMBA; purity, NR Water 0 (control), 500 (NMBA) + 0 (arecoline), 0 (NMBA) + 500 (arecoline), 500 (NMBA) + 500 (arecoline) [μg/kg bw (for NMBA) or μg/mL (for arecoline)] Subcutaneous injection of NMBA, 3×/wk for 5 wk; arecoline in drinking-water for 25 wk 7, 7, 7, 7 7, 7, 7, 7	*Oesophagus*: papilloma Tumour incidence: 0/7, 0/7, 0/7, 7/7 (100%)* Tumour multiplicity: 0, 0, 0, 1.86 ± 0.10* Total tumours: 0, 0, 0, 13 *Tongue*: papilloma Tumour incidence: 0/7, 1/7 (14%), 0/7, 3/7 (43%) Tumour multiplicity: 0, 0.29 ± 0.17, 0, 0.43 ± 0.17 Total tumours: 0, 2, 0, 3	*[$P = 0.0003$, vs other three groups; one-tailed Fisher exact test] *$P < 0.0001$, vs other three groups; one-way ANOVA, followed by Fisher LSD test NR NA NA NR	Principal limitations: rationale for doses, NR; small number of rats per group; short duration of the carcinogenicity study. Other comments: NMBA in 0.2 mL 20% DMSO; body-weight variation was studied, but data NR.

Table 3.1 (continued)

Study design Species, strain (sex) Age at start Duration Reference	Route Agent tested, purity Vehicle Dose(s) No. of animals at start No. of surviving animals	Incidence (%), multiplicity, or no. of tumours	Significance	Comments
Co-carcinogenicity Rat, F344 (M) 6 wk 30 wk Wu et al. (2016)	Oral administration (drinking-water) Arecoline and NMBA; purity, NR Water 0 (control), 500 (NMBA) + 0 (arecoline), 0 (NMBA) + 500 (arecoline), 500 (NMBA) + 500 (arecoline) [μg/kg bw (for NMBA) or μg/mL (for arecoline)] Subcutaneous injection of NMBA, 3×/wk for 5 wk; arecoline in drinking-water for 30 wk 15, 15, 15, 15 15, 15, 15, 15	*Oesophagus*: papilloma Tumour incidence: 0/15, 8/15 (53%), 2/15 (13%), 11/15 (73%) Tumour multiplicity: 0, 1.07 ± 0.11, 0.13 ± 0.02, 2.27 ± 0.16* Total tumours: 0, 16, 2, 34 *Tongue*: papilloma Tumour incidence: 0/15, 5/15 (33%), 1/15 (7%), 10/15 (67%) Tumour multiplicity: 0, 0.53 ± 0.22, 0.07 ± 0.07, 1.07 ± 0.30* Total tumours: 0, 8, 1, 16	NA *P = 0.0260, vs NMBA group; one-way ANOVA, followed by Fisher LSD test NR NS *P = 0.0494, vs NMBA group; one-way ANOVA, followed by Fisher LSD test NR	Principal limitations: rationale for doses, NR; small number of rats per group; short duration of the carcinogenicity study. Other comments: NMBA in 0.2 mL 20% DMSO; body-weight variation was studied, but data NR.
Full carcinogenicity Hamster, Syrian golden (M+F) (combined) 6–7 wk NR (lifetime) Dunham et al. (1974)	Topical application in the cheek pouch Arecoline, NR Distilled water 0 (control), ~1 mg/day 5×/wk for life 8, 9 NR	*Oesophagus*: papilloma Tumour incidence: 0/8, 1/9 (11%) *Cheek pouch*: Tumour incidence: 0/8, 0/9	[NS] NA	Principal limitations: body-weight changes, NR; survival, NR; small number of hamsters per group; purity of test articles, NR. Other comments: 3–4 h before application of the 1.5% solution of arecoline or the vehicle, 30 mg of calcium hydroxide, as a 0.5% solution in DMSO [6 mL] was administered to the cheek pouch.

Table 3.1 (continued)

Study design Species, strain (sex) Age at start Duration Reference	Route Agent tested, purity Vehicle Dose(s) No. of animals at start No. of surviving animals	Incidence (%), multiplicity, or no. of tumours	Significance	Comments
Full carcinogenicity Hamster, Syrian golden (M+F) (combined) 6–7 wk NR [lifetime, assumed] Dunham et al. (1974)	Topical application in the cheek pouch Arecoline, NR DMSO 0 (control), ~1 mg/day 3×/wk for 5 mo 8, 8 NR	*Oesophagus or cheek pouch*: Tumour incidence: 0/8, 0/8	NA	Principal limitations: body-weight changes, NR; survival, NR; small number of hamsters per group; purity of test articles, NR. Other comments: 3–4 h before application of the 1.5% solution of arecoline or the vehicle, 30 mg of calcium hydroxide, as a 0.5% solution in DMSO [6 mL] was administered to the cheek pouch.
Full carcinogenicity Hamster, Syrian golden (M+F) (combined) 6–7 wk NR (lifetime) Dunham et al. (1974)	Oral administration (topical application to base of tongue) Arecoline, NR Distilled water 0 (control), ~2 mg/day drops, 2×/day, 5 days/wk for life 4, 8 NR	*Oesophagus or cheek pouch*: Tumour incidence: 0/4, 0/8	NA	Principal limitations: body-weight changes, NR; survival, NR; small number of hamsters per group; purity of test articles, NR. Other comments: application of 2% solution of arecoline; controls received 0.5% calcium hydroxide in distilled water to base of tongue.
Full carcinogenicity Hamster, Syrian golden (M+F) (combined) 6–7 wk NR [lifetime, assumed] Dunham et al. (1974)	Oral administration (feed) Arecoline, NR Feed 0 (control), ~6 mg/day 5 days/wk for 16 mo 4, 4 NR	*Oesophagus or cheek pouch*: Tumour incidence: 0/4, 0/4	NA	Principal limitations: body-weight changes, NR; survival, NR; very small number of hamsters per group; purity of test articles, NR. Other comments: controls and treated group were fed 2.5% calcium hydroxide in the feed (approximately 150 mg/day).

Table 3.1 (continued)

Study design Species, strain (sex) Age at start Duration Reference	Route Agent tested, purity Vehicle Dose(s) No. of animals at start No. of surviving animals	Incidence (%), multiplicity, or no. of tumours	Significance	Comments
Full carcinogenicity Hamster, Syrian golden (NR) 1.5 mo 24 mo Dunham et al. (1975)	Oral administration (feed) Arecoline, NR Feed 0 (control), ~6 mg/day 5 days/wk for 12 mo 5, 4 0, 0	*Glandular stomach*: argyrophilic carcinoid Tumour incidence: 0/5, 1/4 (25%)	[NS]	Principal limitations: body-weight changes, NR; very small number of hamsters per group; purity of test articles, NR; control group given feed containing calcium hydroxide for 16 mo (and not 12 mo). Other comments: controls and treated group were given feed containing 2.5% calcium hydroxide (approximately 150 mg/day).
Full carcinogenicity Hamster, Syrian golden (M) 8 wk 22 wk MacDonald (1987)	Topical application in the cheek pouch Arecaidine, NR Distilled water 25 µg/mL 3×/wk for 12 wk 24 13	*Cheek pouch*: Tumour incidence: 0%	NA	Principal limitations: short duration of exposure; short duration of follow-up; body-weight changes, NR; lack of control group; volume applied, NR. Other comments: applications to a 1 cm^2 area of the anterior part of the medial wall of the cheek pouch.
Initiation–promotion (tested as initiator) Hamster, Syrian golden (M) 8 wk 59 wk MacDonald (1987)	Topical application in the cheek pouch Arecaidine, NR Distilled water 25 µg/mL Arecaidine 3×/wk for 12 wk, observed for 10 wk, then croton oil (1% in acetone) 3×/wk for 3 wk 13 3	*Cheek pouch*: Tumour incidence: 0%	NA	Principal limitations: body-weight changes, NR; poor survival; lack of control group; large time gap between initiation and promotion; volume applied, NR; purity of test articles, NR. Other comments: applications to a 1 cm^2 area of the anterior part of the medial wall of the cheek pouch.

Table 3.1 (continued)

Study design Species, strain (sex) Age at start Duration Reference	Route Agent tested, purity Vehicle Dose(s) No. of animals at start No. of surviving animals	Incidence (%), multiplicity, or no. of tumours	Significance	Comments
Full carcinogenicity Hamster, Syrian golden (M) 11–13 wk 12 wk Lin et al. (1996)	Topical application in the cheek pouch Arecaidine, NR Polyethylene glycol 0 (control), 1000, 2000, 3000 µg/mL Arecaidine, 6×/wk for 12 wk 7, 7, 7, 7 NR	*Cheek pouch*: exophytic squamous cell papilloma or carcinoma (combined) Tumour incidence: 0/7, 0/7, 0/7, 0/7	NA	Principal limitations: body-weight changes, NR; survival, NR; short duration of exposure; short duration of follow-up; rationale for doses, NR; small number of hamsters per group; volumes applied, NR. Other comments: painting using a no. 4 camel-hair brush.
Initiation–promotion (tested as promoter) Hamster, Syrian golden (M) 11–13 wk 12 wk Lin et al. (1996)	Topical application on the cheek pouch Arecaidine, NR Polyethylene glycol 0 (control), 200, 300, 400, 500 µg/mL 0.5% DMBA (in mineral oil), 3×/wk for 8 wk, then arecaidine, 6×/wk for 4 wk 7, 7, 7, 7, 7 NR	*Cheek pouch*: exophytic squamous cell papilloma or carcinoma (combined) Tumour incidence: 5/7 (71%), 5/7 (71%), 5/7 (71%), 7/7 (100%), 7/7 (100%)	[NS; one-tailed Fisher exact test vs control]	Principal limitations: body-weight changes, NR; survival, NR; rationale for doses, NR; volumes applied, NR. Other comments: painting using a no. 4 camel-hair brush.
		Tumour multiplicity: 1.00 ± 0.76, 1.09 ± 1.02, 1.14 ± 0.99, 1.86 ± 0.63, 1.86 ± 0.93	[NS; one-way ANOVA test]	
		Total tumours: 7, 9, 8, 13, 13	NR	

Table 3.1 (continued)

Study design Species, strain (sex) Age at start Duration Reference	Route Agent tested, purity Vehicle Dose(s) No. of animals at start No. of surviving animals	Incidence (%), multiplicity, or no. of tumours	Significance	Comments
Initiation-promotion (tested as promoter) Hamster, Syrian golden (M) 11–13 wk 12 wk Lin et al. (1996)	Topical application in the cheek pouch Arecaidine, NR Polyethylene glycol 0 (control), 600, 700, 800, 900, 1000 µg/mL 0.5% DMBA (in mineral oil), 3×/wk for 4 wk, then arecaidine, 6×/wk for 8 wk 7, 7, 7, 7, 7, 7 NR	*Cheek pouch*: exophytic squamous cell papilloma or carcinoma (combined) Tumour incidence: 0/7, 5/7 (71%)*, 5/7 (71%)*, 4/7 (57%)*, 7/7 (100%)*, 7/7 (100%)* Tumour multiplicity: 0.00 ± 0.00, 1.00 ± 0.75, 1.14 ± 0.99*, 1.00 ± 0.65, 1.86 ± 0.82*, 2.14 ± 1.09* Total tumours: 0, 7, 8, 7, 13, 15	*[P = 0.0350–0.0003]; one-tailed Fisher exact test, vs control] *[$P < 0.05$; one-way ANOVA followed by Dunnett's test, vs control] NR	Principal limitations: body-weight changes, NR; survival, NR; rationale for doses, NR; volumes applied, NR. Other comments: painting using a no. 4 camel-hair brush.

4-NQO, 4-nitroquinoline 1-oxide; ANOVA, analysis of variance; bw, body weight; DMBA, 7,12-dimethylbenz[*a*]anthracene; DMSO, dimethyl sulfoxide; F, female; KNO_3, potassium nitrate; LSD, least significant difference; M, male; mo, month; NMBA, *N*-benzyl-*N*-methylnitrosamine; NA, not applicable; NR, not reported; NS, not significant; vs, versus; wk, week.

number of animals per group, the fact that the tumours observed in the vehicle control groups were not otherwise specified, and the lack of a KNO_3-only control group.]

In a second experiment in the same study (Bhide et al., 1984), groups of male (n = 16–21) and female (n = 12–18) Swiss mice (age, 6 weeks) were placed on a diet deficient in vitamin B complex. After 2 weeks, they were treated by gavage five times per week "throughout the life-span" with vehicle (control) or 1 mg of arecoline hydrochloride dissolved in distilled water per animal, with 1 mg arecoline hydrochloride and 1 mg KNO_3 in distilled water per animal, or with 1 mg arecoline hydrochloride, 1 mg KNO_3, and 1 mg slaked lime in distilled water per animal.

When assessed over 25 months [it was unclear whether this was 25 months of age or 25 months of treatment], male mice treated with arecoline hydrochloride, KNO_3, and slaked lime showed a significant increase [P = 0.0361] in the incidence of total tumours of the liver, stomach, and lung (two mice developed liver haemangioma, three developed lung adenocarcinoma, and two developed stomach squamous cell carcinoma) compared with the vehicle control group. The total tumour incidence in the other treated groups of males did not differ significantly from that in the vehicle control group. Female mice treated with arecoline hydrochloride showed a significant increase [P = 0.0132] in the incidence of total tumours (two mice developed liver haemangioma and four developed lung adenocarcinoma) compared with the vehicle control group. Likewise, female mice treated with arecoline hydrochloride, KNO_3, and slaked lime showed a significant increase [P = 0.0143] in the incidence of total tumours of the liver, stomach, and lung (two mice developed liver haemangioma, three developed lung adenocarcinoma, two developed stomach squamous cell carcinoma, and one developed a cholangiocarcinoma) compared with the vehicle control group. The total tumour incidence in the other treated group of females (arecoline hydrochloride plus KNO_3) did not differ significantly from that in the vehicle control group (Bhide et al., 1984). [The Working Group noted the limited histopathology, the single dose used, the small number of animals per group, the fact that tumours observed in the vehicle control groups were not further specified, and the lack of a KNO_3-only control group and a KNO_3 plus lime control group.]

3.1.2 Intraperitoneal injection

A group of 10 male Swiss mice [age not reported] was treated with 1.5 mg of arecoline (analytical reagent grade) per animal [rationale for dose not reported; vehicle and volume not reported] by intraperitoneal injection once per week for 13 weeks. A control group of 10 mice was treated with 0.1 mL of distilled water by intraperitoneal injection once per week for 13 weeks. Both groups were monitored for their "lifetime" [the precise duration was not reported]. Neither the control group nor the group treated with arecoline developed any tumours (Shivapurkar et al., 1980). [The Working Group noted the small number of animals per group, the use of male animals only, the single dose used, the lack of survival and body-weight data, and the short duration of treatment. The study was considered inadequate for the evaluation of the carcinogenicity of arecoline in experimental animals.]

3.1.3 Subcutaneous injection

A group of 10 male Swiss mice [age not reported] was treated with 1.5 mg of arecoline (analytical reagent grade) per animal [rationale for dose not reported; vehicle and volume not reported] dorsally by subcutaneous injection once per week for 13 weeks. A control group of 20 mice was treated with 0.1 mL of distilled water by subcutaneous injection once per week for 13 weeks. Both groups were monitored for their "lifetime" [the precise duration was not

reported]. Neither the control group nor the group treated with arecoline developed tumours at the site of injection or in any other organs (Shivapurkar et al., 1980). [The Working Group noted the small number of animals per group, the use of male animals only, the single dose used, the lack of survival and body-weight data, and the short duration of treatment. The study was considered inadequate for the evaluation of the carcinogenicity of arecoline in experimental animals.]

3.1.4 Co-administration with known carcinogens

In a study by Chang et al. (2010), two groups of 10 male C57BL/6JNarl mice (age, 6 weeks) were given drinking-water containing 4-nitroquinoline 1-oxide (4-NQO) [purity not reported] at a concentration of 100 or 200 µg/mL [rationale for the doses not reported] for 8 weeks. Two additional groups of 10 mice received drinking-water containing arecoline hydrobromide [purity not reported] at a concentration of 250 or 500 µg/mL [rationale for the doses not reported] for 8 weeks. Four additional groups of 11 mice received drinking-water containing 4-NQO at 100 µg/mL and arecoline hydrobromide at 250 or 500 µg/mL; or 4-NQO at 200 µg/mL and arecoline hydrobromide at 250 or 500 µg/mL. A control drinking-water group of 10 mice was included. After 8 weeks of treatment, no histopathological evidence of disease was reported in mice (three per group) that were killed to assess lesions of the tongue, oesophagus, liver, colon, kidney, spleen, or stomach. The remaining mice continued to receive control drinking-water for an additional 20 weeks. Mice receiving 4-NQO showed a dose-related decrease in water consumption. Drinking-water consumption was not affected by treatment with arecoline hydrobromide. When assessed at 28 weeks, mice receiving drinking-water containing 4-NQO at 200 µg/mL, or 4-NQO at 100 µg/mL plus arecoline hydrobromide at 500 µg/mL, or 4-NQO at 200 µg/mL plus arecoline hydrobromide at 250 µg/mL, showed significant decreases in body weight [body-weight values being 75–85% of those of the control group]. Histopathological examination was performed and reported for the tongue and the oesophagus.

There were no lesions of the tongue or oesophagus (hyperplasia, dysplasia, papilloma, or invasive squamous cell carcinoma, combined) in mice treated only with drinking-water containing arecoline hydrobromide at 250 or 500 µg/mL for 28 weeks. Arecoline hydrobromide had no significant effect on the incidence of lesions of the tongue or oesophagus (hyperplasia, dysplasia, papilloma, or invasive squamous cell carcinoma, combined) (Chang et al., 2010). [The Working Group noted the small number of animals per group, the short durations of exposure and follow-up, and the combination of pre-neoplastic and neoplastic lesions. The study was considered inadequate for the evaluation of the carcinogenicity of arecoline in experimental animals.]

In a study by Chen et al. (2017), two groups of 16 male C57BL/6 mice (age, 6 weeks) received drinking-water containing 4-NQO at a concentration of 100 µg/mL [purity not reported; rationale for dose not reported] for 8 or 16 weeks. A group of 8 mice received drinking-water containing arecoline hydrobromide at 500 µg/mL [purity not reported; rationale for dose not reported] for 16 weeks. Two groups of 16 mice received drinking-water containing 4-NQO at 100 µg/mL plus arecoline hydrobromide at 500 µg/mL for 8 or 16 weeks. A control drinking-water group of 8 mice was included. After 8 or 16 weeks of treatment, mice were placed on control drinking-water. All surviving mice were killed 28 weeks after the initiation of treatment. Up to three mice per group exposed to 4-NQO died before the end of the experiment. [Treatment-related changes in body weight, and data on drinking-water consumption were not

reported.] Histopathological examination was performed and reported for the oesophagus. The incidence of papilloma and invasive squamous cell carcinoma of the oesophagus was only reported for mice alive at 28 weeks.

In mice given drinking-water containing 4-NQO at 100 µg/mL plus arecoline hydrobromide at 500 µg/mL for 8 weeks, there was a significant increase [$P = 0.0329$] in the incidence of invasive squamous cell carcinoma of the oesophagus compared with mice given only 4-NQO for 8 weeks. In mice given drinking-water containing 4-NQO at 100 µg/mL for 16 weeks or 4-NQO at 100 µg/mL plus arecoline hydrobromide at 500 µg/mL for 8 or 16 weeks, there was a significant increase [$P < 0.05$] in the incidence of invasive squamous cell carcinoma of the oesophagus compared with the control group.

In mice given drinking-water containing 4-NQO at 100 µg/mL for 16 weeks or 4-NQO at 100 µg/mL plus arecoline hydrobromide at 500 µg/mL for 8 or 16 weeks, there was a significant increase [$P < 0.003$] in the incidence of oesophageal papilloma compared with the control group. In mice given drinking-water containing 4-NQO at 100 µg/mL plus arecoline hydrobromide at 500 µg/mL for 8 weeks, there was a non-significant increase [$P = 0.0528$, one-tailed Fisher exact test] in the incidence of oesophageal papilloma compared with mice receiving 4-NQO at 100 µg/mL for 8 weeks. No tumours were observed in mice given only arecoline hydrobromide (Chen et al., 2017).

3.2 Rat

Oral administration (drinking-water)

In a carcinogenicity and co-carcinogenicity study by Wu et al. (2016), male F344 rats (age, 6 weeks) were allocated to one of four groups of 22 rats. A first group received a subcutaneous injection of 0.2 mL of 20% dimethyl sulfoxide (DMSO) three times per week for 5 weeks. This group received control drinking-water for up to 30 weeks. A second group received a subcutaneous injection of *N*-benzyl-*N*-methylnitrosamine (NMBA) [purity not reported] at 500 µg/kg body weight (bw) in 0.2 mL of 20% DMSO three times per week for 5 weeks. This dose was selected based on data from the literature. This group also received control drinking-water for up to 30 weeks. A third group received a subcutaneous injection of 0.2 mL of 20% DMSO three times per week for 5 weeks. This group received drinking-water containing arecoline [purity not reported; rationale for dose not reported] at 500 µg/mL for up to 30 weeks. A fourth group received a subcutaneous injection of NMBA at 500 µg/kg bw in 0.2 mL of 20% DMSO three times per week for 5 weeks. This group received drinking-water containing arecoline at 500 µg/mL for up to 30 weeks. Papillomas of the oesophagus and of the tongue were assessed by macroscopic examination, and confirmed by microscopic examination for all tongue lesions, and only half of the oesophageal lesions.

Twenty-five weeks after the initiation of the study, the incidence of papilloma of the oesophagus and of the tongue was assessed in seven rats from each group. Oesophageal papilloma only occurred in the group treated with NMBA and arecoline, with the incidence (7/7) [$P = 0.0003$] and multiplicity (1.86 ± 0.10) ($P < 0.0001$) significantly increased compared with that in the group treated only with NMBA (incidence, 0/7). Tongue papilloma occurred in rats treated with NMBA and with NMBA plus arecoline; but neither incidence nor multiplicity differed significantly between the two groups.

Thirty weeks after the initiation of the study, the incidence of papilloma of the oesophagus and of the tongue was assessed in the remaining 15 rats from each group. Oesophageal papilloma occurred in groups treated with NMBA, arecoline, and NMBA plus

arecoline; multiplicity was significantly increased (P = 0.0260) in rats treated with NMBA plus arecoline compared with those treated with only NMBA. Tongue papilloma occurred in groups treated with NMBA, arecoline, and NMBA plus arecoline; multiplicity was significantly increased (P = 0.0494) in rats treated with NMBA plus arecoline compared with those treated with only NMBA (Wu et al., 2016). [The Working Group noted the small number of animals per group, and the short duration of the carcinogenicity study.]

3.3 Hamster

Topical application in the cheek pouch or to the base of the tongue

In a study by Dunham et al. (1974), an approximately equal number of male and female Syrian hamsters (age, 6–7 weeks; 9 hamsters total) were treated topically in the right cheek pouch with a 1.5% solution of arecoline [purity not reported] in distilled water five times per week for their lifespan [the duration was not specified]. This dose (approximately 1 mg/day) was anticipated to produce some physiological effects but not kill the animals. Three to four hours before application of the arecoline, 30 mg of calcium hydroxide [purity not reported], as a 0.5% solution in DMSO [6 mL] was applied to the cheek pouch. A second group of eight hamsters (with an approximately equal number of males and females) was treated similarly with 1.5% arecoline (approximately 1 mg/day) in DMSO three times per week for 5 months. This group was also pre-treated with calcium hydroxide. A control group for these two treated groups consisted of eight hamsters given 0.5% calcium hydroxide applied in DMSO.

A third group of eight hamsters (with an approximately equal number of males and females) was treated by oral administration with drops of 2% arecoline (approximately 2 mg/day) applied in distilled water to the base of the tongue twice daily, 5 days per week, for the lifespan. A fourth group of eight hamsters (with an approximately equal number of males and females) was treated with 2% arecoline and 0.5% calcium hydroxide in distilled water in a similar manner. A control group for these two groups consisted of four hamsters and was treated with only 0.5% calcium hydroxide applied in distilled water.

A fifth group of four hamsters (with an approximately equal number of males and females) was given feed containing arecoline at approximately 6 mg/day (prepared by adding a 0.1% aqueous solution to the feed) and 2.5% calcium hydroxide [approximately 150 mg/day] 5 days per week for 16 months. A control group for this treatment group consisted of four hamsters fed only 2.5% calcium hydroxide.

Microscopic examination was performed, and neoplastic lesions were only reported for the cheek pouch or the oesophagus. One female hamster treated with arecoline (in distilled water) in the cheek pouch for life developed an oesophageal papilloma. No hamsters in the control groups or the other arecoline-treated groups developed neoplasms in the cheek pouch or oesophagus (Dunham et al., 1974). [The Working Group noted the small number of hamsters per group and lack of survival and body-weight data. The study was considered inadequate for the evaluation of the carcinogenicity of arecoline in experimental animals.]

Four Syrian golden hamsters (age, 1.5 months) [sex not reported] were given feed containing arecoline [purity not reported] at approximately 6 mg/day (prepared by adding a 0.1% aqueous solution to the feed) and 2.5% calcium hydroxide [purity not reported; approximately 150 mg calcium hydroxide/day], 5 days per week for 12 months. This dose was not anticipated to affect the hamsters' health or survival. A control group consisted of five hamsters given only feed containing 2.5% calcium hydroxide for 16 months. The experiment ended when the last hamster died at age 25.5 months, and

microscopic examination was performed. The only tumour reported was an argyrophilic carcinoid of the glandular stomach, which occurred in one hamster fed arecoline. This is a very rare tumour in Syrian golden hamsters (Dunham et al., 1975). [The Working Group noted the very small number of hamsters per group and lack of body-weight data. The study was considered inadequate for the evaluation of the carcinogenicity of arecoline in experimental animals.]

3.4 Carcinogenicity of metabolites

The available studies on the carcinogenicity of arecoline metabolites concerned topical appplication in the cheek pouch of arecaidine to Syrian golden hamsters.

Twenty-four male Syrian golden hamsters (age, 8 weeks) were treated with arecaidine at 25 µg/mL in distilled water (the pH of the solution was approximately 3) [purity not reported; volume administered not reported] applied topically in one cheek pouch, three times per week for 12 weeks. This dose was based upon the results obtained from an "in vitro" carcinogenicity study with arecaidine. At the end of 12 weeks, 13 hamsters were still alive. These animals were observed for 10 additional weeks. After the 10-week observation period, the 13 hamsters were treated topically in the same cheek pouch with a 1% solution of croton oil [purity, characterization, and stability, not reported; volume administered, not reported; rationale for dose, not specified] three times per week for 3 weeks. The hamsters were monitored for 34 weeks after completion of the croton-oil treatment, at which time three hamsters were still alive. There were no tumours evident upon macroscopic and microscopic examination of the cheek pouch of hamsters treated with only arecaidine or with arecaidine followed by croton oil (MacDonald, 1987). [The Working Group noted the lack of control groups and body-weight data, and the short durations of exposure and follow-up in the carcinogenicity experiment; and the large time gap between initiation and promotion, lack of body-weight data, and poor survival in the initiation–promotion experiment. The Working Group considered the study inadequate for the evaluation of the carcinogenicity of arecaidine.]

In a first initiation–promotion experiment in the study by Lin et al. (1996), groups of seven male Syrian golden hamsters (age, 11–13-weeks) were treated topically in the right cheek pouch with 0.5% 7,12-dimethylbenz[*a*]anthracene (DMBA) [purity not reported; rationale for doses not specified] in heavy mineral oil [volume administered not reported], three times per week for 8 weeks. At the end of the 8-week period, one group was left untreated (control) while the remaining groups were treated topically in the right cheek pouch with arecaidine at 200, 300, 400, or 500 µg/mL [purity not reported; rationale for doses not specified] in polyethylene glycol [volume administered not reported], six times per week for 4 weeks. In another initiation–promotion experiment in the same study, additional groups of seven male Syrian golden hamsters were treated topically in the right cheek pouch with 0.5% DMBA in heavy mineral oil, three times per week for 4 weeks. At the end of the 4-week period, one group was left untreated (control) while the remaining groups were treated topically in their right cheek pouch with arecaidine at 600, 700, 800, 900, or 1000 µg/mL, six times per week for 8 weeks. In a full carcinogenicity experiment in the same study, further groups were treated in the right cheek pouch with arecaidine at 0 (control), 1000, 2000, or 3000 µg/mL, six times per week for 12 weeks. Twelve weeks after the initiation of dosing, the extent of tumorigenesis was assessed in the buccal pouches.

In the first initiation–promotion experiment, the incidence of tumours (exophytic squamous cell papilloma or carcinoma, combined) in the cheek pouch in the group of hamsters treated with DMBA for 8 weeks and then held for an additional 4 weeks was 71%. Neither the tumour

incidence nor the average number of tumours was significantly increased by the subsequent administration of arecaidine for 4 weeks. In the other initiation–promotion experiment, control hamsters treated with DMBA for 4 weeks did not develop cheek pouch tumours when assessed at 12 weeks. The subsequent administration of arecaidine for 8 weeks resulted in a significant increase in the incidence of tumours [range, 57–100%; P = 0.0350–0.0003, versus control] in all treatment groups, coupled with a significant increase in the average number of tumours [$P < 0.05$, versus control] in the groups at 700, 900, and 1000 µg/mL. In the full carcinogenicity experiment, there were no cheek pouch tumours in hamsters treated only with arecaidine (Lin et al., 1996). [The Working Group noted the short durations of exposure and follow-up for the full carcinogenicity experiment, the lack of survival and body-weight data, and the small number of hamsters per group for the full carcinogenicity experiment. The full carcinogenicity experiment was considered inadequate for the evaluation of the carcinogenicity of arecaidine in experimental animals.]

3.5 Evidence synthesis for cancer in experimental animals

The carcinogenicity of arecoline has been assessed in two studies in male and female mice treated by oral administration (gavage). One study in male mice treated by intraperitoneal injection and one study in male mice treated by subcutaneous injection were considered inadequate for assessing the carcinogenicity of arecoline in experimental animals. The carcinogenicity of arecoline has also been assessed in male mice upon co-administration with known carcinogens in two studies, one of which was considered inadequate for the evaluation of the carcinogenicity of arecoline in experimental animals. Additionally, arecoline was assessed in one study in male rats upon administration with known carcinogens. The carcinogenicity of arecoline has been investigated in hamsters, in two studies of oral administration and in studies of topical application to the cheek pouch that were considered inadequate for the evaluation of the carcinogenicity of arecoline in experimental animals. Other studies in hamsters evaluated the carcinogenicity of the arecoline metabolite arecaidine.

In one study, male Swiss mice treated with arecoline hydrochloride by oral administration (gavage) showed a significant increase in the incidence of total tumours. In another study, female Swiss mice fed a vitamin B complex-deficient diet and treated with arecoline hydrochloride by gavage showed a significant increase in the incidence of total tumours (Bhide et al., 1984). No tumours were observed in male Swiss mice treated with arecoline by intraperitoneal or subcutaneous injection in studies considered inadequate for assessing the carcinogenicity of arecoline in experimental animals (Shivapurkar et al., 1980).

In a co-carcinogenicity study in which arecoline hydrobromide was administered by oral administration (in the drinking-water) in combination with the carcinogen 4-NQO to male C57BL/6 mice, arecoline hydrobromide plus 4-NQO increased the incidence of invasive squamous cell carcinoma of the oesophagus compared with mice receiving only 4-NQO (Chen et al., 2017). In a second co-carcinogenicity study that was considered inadequate for the evaluation of the carcinogenicity of arecoline in experimental animals, arecoline hydrobromide was administered in combination with the carcinogen 4-NQO by oral administration (in the drinking-water) to male C57BL/6JNarl mice and had no significant effect upon the incidence of tongue or oesophageal lesions (hyperplasia, dysplasia, papilloma, or invasive squamous cell carcinoma, combined) compared with mice receiving only 4-NQO (Chang et al., 2010).

In one co-carcinogenicity study, male F344 rats given NMBA by subcutaneous injection and drinking-water containing arecoline showed a significant increase in the incidence and multiplicity of oesophageal papilloma and a significant increase in multiplicity of tongue papilloma, compared with rats receiving only NMBA (Wu et al., 2016).

One study was conducted in which male and female Syrian golden hamsters were treated with arecoline applied to the cheek pouch or to the base of the tongue (Dunham et al., 1974). In this study and another, male and female Syrian golden hamsters were also fed a diet containing arecoline (Dunham et al., 1974, 1975). Both studies were judged to be inadequate for assessing the carcinogenicity of arecoline in experimental animals.

Other studies in male Syrian golden hamsters evaluated the carcinogenicity of the arecoline metabolite arecaidine as a complete carcinogen, as a tumour initiator in one experiment, and as a tumour promoter in two experiments. Male Syrian golden hamsters were used in a study to assess the ability of arecaidine to act as a complete carcinogen in one experiment or as a tumour promoter in two experiments. In one experiment, treating hamsters in the cheek pouch with the carcinogen DMBA in the initiation phase followed by arecaidine in the promotion phase resulted in a significant increase in the incidence and multiplicity of exophytic squamous cell papilloma or carcinoma (combined) compared with hamsters treated with DMBA only; hamsters administered only arecaidine did not develop tumours (Lin et al., 1996). In a separate study judged to be inadequate for assessing the carcinogenicity of arecaidine in experimental animals, the ability of arecaidine to act as a complete carcinogen or as a tumour initiator was assessed by applying arecaidine by itself or with croton oil (in the promotion phase) to the cheek pouch of male Syrian golden hamsters (MacDonald, 1987).

4. Mechanistic Evidence

4.1 Absorption, distribution, metabolism, and excretion

4.1.1 Humans

(a) Exposed humans

Arecoline is readily absorbed in the oral cavity. It has been found in saliva (Nair et al., 1985; Cox et al., 2010; Lee et al., 2015; Franke et al., 2016; Venkatesh et al., 2018) as well as in urine (Franke et al., 2016) from areca-nut and betel-quid chewers. In habitual chewers, arecoline was detected in saliva not only during 25 minutes of chewing areca nut but also before and 15 minutes after removing nut particles from the mouth. In 20 out of 22 chewers, arecoline concentrations in saliva were above 0.1 µg/mL, and in 11 chewers (50%) a salivary concentration of 10 µg/mL was exceeded during chewing and after spitting out the areca nut (Cox et al., 2010). Somewhat lower salivary arecoline concentrations (mean during chewing, 77 ng/mL) were detected under similar conditions by Venkatesh et al. (2018).

Pharmacokinetic parameters of arecoline were measured in a study of 15 Alzheimer patients (9 women and 6 men) who received 5 mg of arecoline each by intravenous infusion during 30 minutes. Plasma concentrations quickly decreased following two-phase (biexponential) kinetics. In the plasma, the half-lives of arecoline were 0.95 ± 0.54 minutes and 9.33 ± 4.5 minutes for the first and second phase, respectively. Other kinetic parameters were as follows: maximum plasma concentration, C_{max}, 27.8 ± 20.5 ng/mL; clearance, 13.6 ± 5.8 L/kg; and steady-state apparent volume of distribution, V_d, 2.55 ± 2.05 L/kg (Asthana et al., 1996).

Lee et al. (2015) identified *N*-methylnipecotic acid (MNPA) (see Fig. 4.1) in saliva during and shortly after chewing areca nut. Its concentration and ratio to both arecoline and arecaidine (which

were also detected in saliva) rose in the course of and after chewing, indicating that MNPA is a metabolite of arecoline. MNPA, together with arecaidine and arecoline, was also found in the urine after ingestion of an aqueous extract of areca nut as well as after areca-nut chewing. The major urinary metabolite was arecaidine, with an elimination half-life of 4.3 hours, followed by MNPA, with an elimination half-life of 7.9 hours, and very low levels of arecoline, with an elimination half-life of 0.97 hours. Mean urinary concentrations of arecoline, arecaidine, and MNPA in regular areca-nut chewers (who were also smokers) were 23.9, 5816, and 1298 ng/mg creatinine, respectively (Hu et al., 2010).

Arecoline and its metabolite arecaidine were also found in the plasma of betel-quid chewers. Amounts correlated significantly with self-reported amounts of betel quid chewed the day before blood sampling (Wu et al., 2010).

In a pilot pharmacokinetic study, arecoline, together with three other areca alkaloids, was detected in the saliva of four occasional betel-nut chewers. Arecoline concentrations in saliva peaked within the first 2 hours post-chewing before returning to baseline levels after 8 hours and paralleled urinary excretion in one volunteer (Franke et al., 2016).

Arecoline was detected at concentrations ranging from 18 to 159.9 µg/L in the breast milk of four betel-quid chewers (Pellegrini et al., 2007), as well as in meconium and urine of infants of mothers who used betel nut during pregnancy (Pichini et al., 2003).

Two nitrosamines, namely *N*-nitrosoguvacoline and *N*-nitrosoguvacine, were found in the saliva but not in the urine of betel-quid chewers (Fig. 4.2; Nair et al., 1985). [The Working Group noted that these nitrosamines may have been formed from arecoline as well as from other alkaloids of betel quid, namely, guvacoline and guvacine.]

(b) Human enzymes in acellular systems in vitro

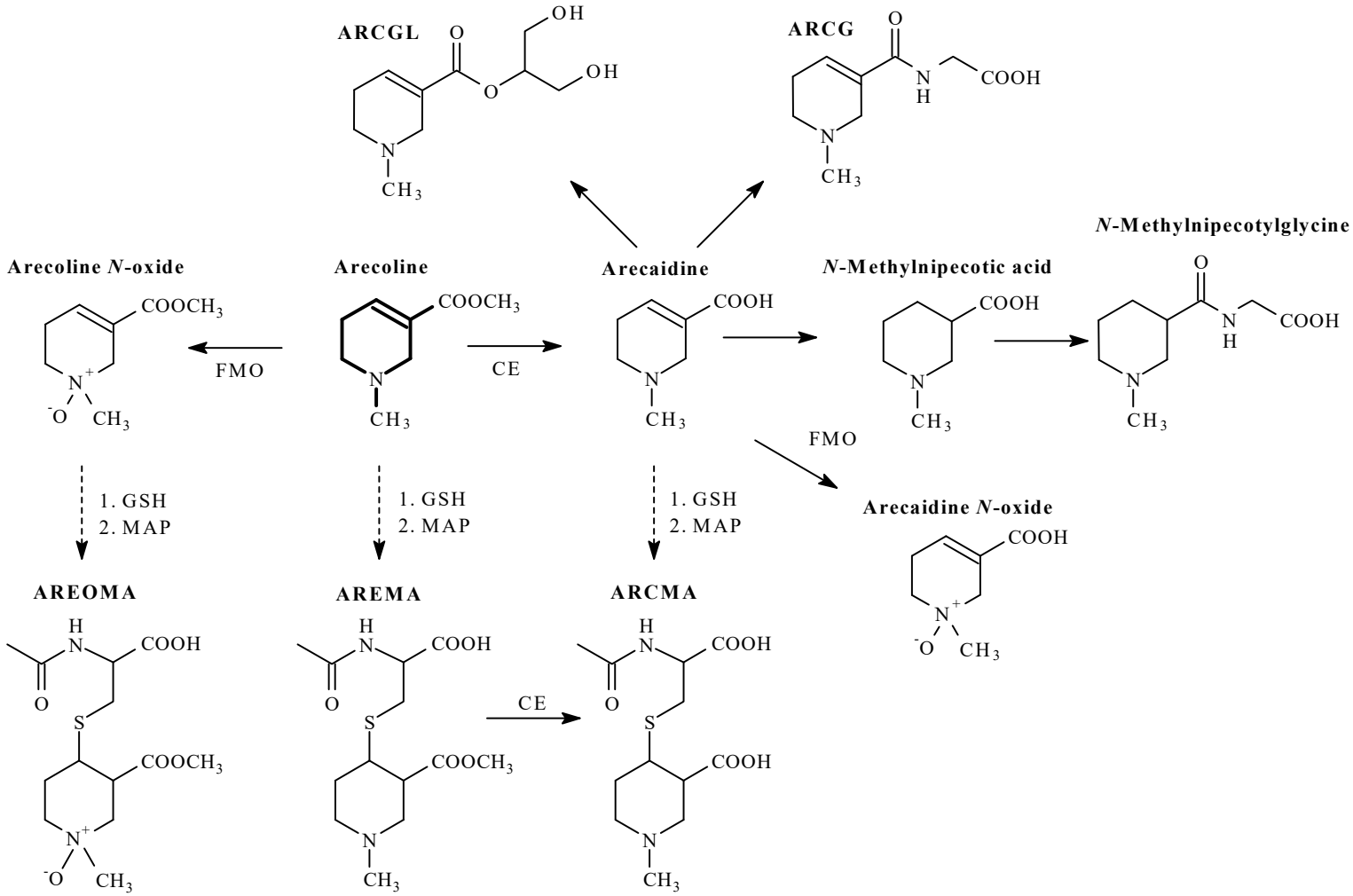
Arecoline was converted to arecoline *N*-oxide in vitro by recombinant human flavin-containing monooxygenases FMO1 (K_m, 13.6 ± 4.9 µM; V_{max}, 0.114 ± 0.01 nmol/min per µg protein) and FMO3 (K_m, 44.5 ± 8.0 µM; V_{max}, 0.014 ± 0.001 nmol/min per µg protein) but not by FMO5 or any of 11 recombinant human cytochrome P450s (CYPs) (Giri et al., 2007).

4.1.2 Experimental systems

(a) Absorption and distribution

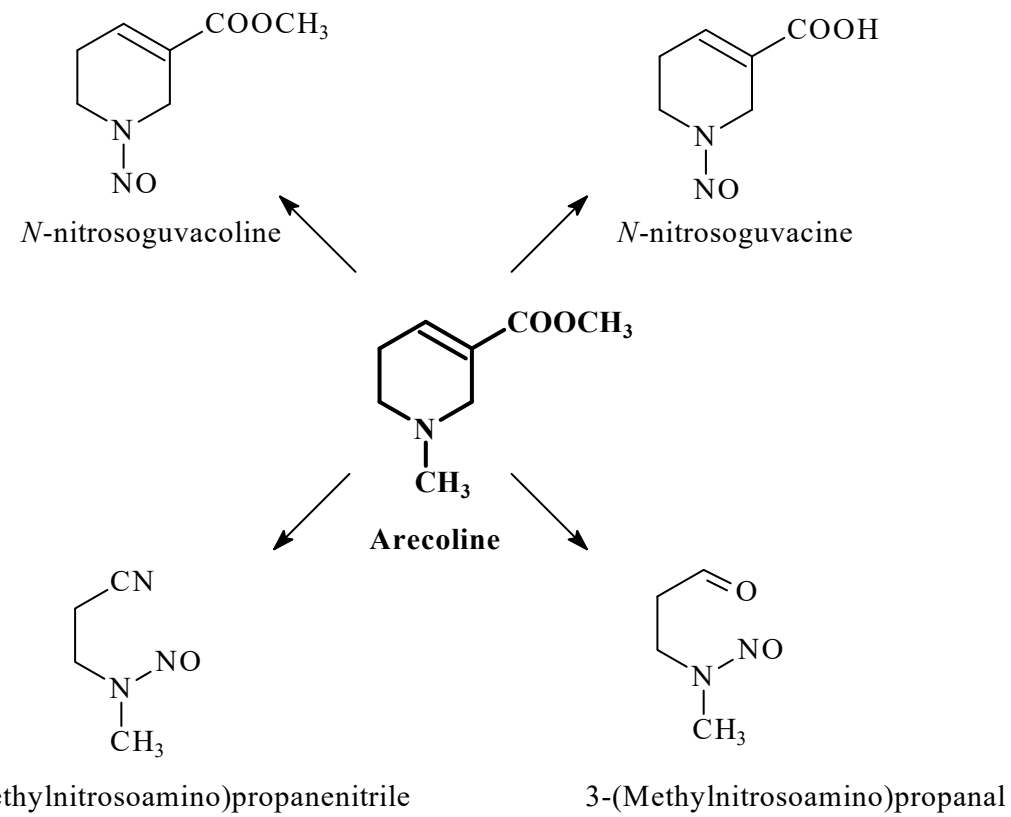
Arecoline was rapidly absorbed in dogs after oral administration (Li et al., 2014), in rats after oral (Pan et al., 2017, 2018), intraperitoneal (Soncrant et al., 1989), intranasal, or intramuscular application (Hussain & Mollica, 1991), and in mice after intraperitoneal (Patterson & Kosh, 1994), intravenous, or oral administration (Sethy & Francis, 1990). When dogs were dosed orally with arecoline hydrobromide at 3 mg/kg bw, the concentration of arecoline in the plasma peaked after 120 minutes, reaching 60.6 ng/mL. Absorption and elimination half-lives were 50 and 69 minutes, respectively, and plasma clearance was 0.19 L/minute per kg (Li et al., 2014). A much shorter elimination time of arecoline ($t_{1/2}$, 6.5 ± 0.4 minutes; n = 5) was found in rats dosed orally with arecoline hydrobromide at 150 mg/kg bw. In this case, plasma concentration peaked 5 minutes after dosing and reached approximately 175 ng/mL (Pan et al., 2017). At a lower oral dose (20 mg/kg bw) the maximum plasma concentration was 15 ng/mL at 0.25 hour. The sensitivity of the analytical method used was not sufficient to determine pharmacokinetic parameters for arecoline, therefore the pharmacokinetics of its abundant metabolites, arecaidine and arecoline *N*-oxide, were followed. For arecaidine and arecoline *N*-oxide, respectively, maximum plasma concentrations

Fig. 4.1 Metabolism of arecoline



ARCG, *O*-arecaidinylglycine; ARCGL, *O*-arecaidinylglycerol; AREMA, *N*-acetyl-*S*-(3-methoxycarbonyl-1-methylpiperid-4-yl)-L-cysteine (arecoline mercapturic acid); ARCMA, *N*-acetyl-*S*-(3-carboxy-1-methylpiperid-4-yl)-L-cysteine (arecaidine mercapturic acid); AREOMA, arecoline *N*-oxide mercapturic acid; CE, carboxylesterases, FMO, flavine-monooxygenases; GSH, glutathione; MAP, mercapturic acid pathway.
Compiled by the Working Group with data from Giri et al. (2006), Boyland & Nery (1969) and Patterson & Kosh (1993).

Fig. 4.2 Nitrosation and cleavage of arecoline



Created by the Working Group.

were 2130 ± 611 and 2761 ± 138 ng/mL at 1.83 ± 0.29 and 1.33 ± 0.58 hours, plasma clearance was 2.02 ± 0.21 and 1.6 ± 0.25 L/hour per kg and apparent volume of distribution was 6.75 ± 2.0 and 6.01 ± 1.85 L/kg, respectively (Pan et al., 2018). Because of its cholinergic activity via binding to muscarine receptors, distribution of arecoline into the brain is of special interest. In a study in Fischer 344 rats (aged 3 and 24 months) given a single intraperitoneal injection of arecoline hydrobromide at 5 mg/kg bw, peak plasma concentrations of 1142 ± 554 ng/mL and 923 ± 368 ng/mL (mean ± standard error; $n \geq 3$), respectively, were measured after 1 minute. Thereafter, arecoline was eliminated with $t_{1/2}$ values of 5.8 and 3.5 minutes, respectively. Arecoline rapidly entered the brain where it reached peak concentrations of 1558 ± 588 ng/g and 1830 ± 317 ng/g (mean ± standard error; $n \geq 3$) in 3 minutes in the cerebral cortex of rats aged 3 and 24 months, respectively. Thereafter, the brain concentrations in all rats declined rapidly, with $t_{1/2}$ values of 3.6 and 2.9 minutes, respectively. Hence, small but statistically significant differences in pharmacokinetic parameters were found between young and old rats (Soncrant et al., 1989). When mice were given a single intraperitoneal dose of arecoline at 15 mg/kg bw, the concentration of arecoline in the brain reached a maximum of 7.9 nmol/g after 3 minutes and decreased to 1 nmol/g after 30 minutes. The highest arecoline concentration was found in

the cortex followed by the subcortex and cerebellum (Patterson & Kosh, 1994). Somewhat slower distribution into and elimination from the brain was reported in mice after an intravenous injection of 10 μmol/kg as indicated indirectly by an ex vivo [^{3}H]-oxotremorine binding assay. The inhibition of oxotremorine binding reached a maximum 10 minutes after injection (Sethy & Francis, 1990). [The Working Group noted that the slower time course observed after intravenous injection might be explained by the much lower dose and/or methodological issues.]

(b) Metabolism and excretion

Primary metabolic pathways of arecoline are ester hydrolysis to arecadine and oxidation to arecoline *N*-oxide, glutathione conjugation to yield *N*-acetyl-*S*-(3-methoxycarbonyl-1-methylpiperid-4-yl)-L-cysteine (arecoline mercapturic acid; AREMA) (Boyland & Nery, 1969; Nery, 1971; Patterson & Kosh, 1993; Giri et al., 2006). Double-bond reduction eventually yielding MNPA, a major arecoline metabolite in mice, was found to be another important metabolic pathway (Giri et al., 2006).

In an early study in female rats dosed with arecoline hydrochloride at 20 mg/kg bw per day by intraperitoneal injection for 3 weeks, three urinary metabolites were identified as arecaidine, AREMA, and *N*-acetyl-*S*-(3-carboxy-1-methylpiperid-4-yl)-L-cysteine (arecaidine mercapturic acid; ARCMA) (Boyland & Nery, 1969). The same metabolites in different relative amounts were also obtained when arecoline hydrochloride was administered orally (0.2% arecoline hydrochloride w/v in water ad libitum for 10 days) (Boyland & Nery, 1969).

In a study in mice dosed with either arecoline hydrobromide or arecaidine (both at 20 mg/kg bw orally and by intraperitoneal injection), 10 arecoline metabolites were identified in the urine, namely: arecaidine, arecoline *N*-oxide, arecaidine *N*-oxide, MNPA, *N*-methylnipecotylglycine, *O*-arecaidinylglycine, *O*-arecaidinylglycerol, ARCMA, AREMA, and arecoline *N*-oxide mercapturic acid (AREOMA) (Giri et al., 2006). Six of these metabolites were formed from arecaidine, namely, arecaidine *N*-oxide, MNPA, *N*-methylnipecotylglycine, ARCMA, *O*-arecaidinylglycine, and *O*-arecaidinylglycerol. Unchanged arecoline excreted in the urine within 12 hours after dosing comprised 0.3–0.4% of the administered dose, major metabolites being arecaidine (7.1–13.1%), arecoline *N*-oxide (7.4–19.0%), and MNPA (13.5–30. 3% of the administered dose) (Giri et al., 2006).

(c) Metabolism in vitro

Arecoline was hydrolysed with mouse liver and kidney homogenates, and a supernatant obtained by centrifugation of the liver homogenate at 10 000 g. In the supernatant, a V_{max} of 4.7 nmol/min per mg protein and K_m of 9.6 mM were obtained. Experiments with several enzyme inhibitors indicated that carboxylesterase EC 3.1.1.1 was primarily responsible for the rapid metabolism of arecoline in the mouse (Patterson & Kosh, 1993).

Arecoline reacted readily with glutathione in a pH-dependent manner (Boyland & Nery, 1969). At pH 7.4 and 37 °C, the reaction of arecoline with glutathione and L-cysteine proceeded following second-order kinetics with the apparent rates of glutathione depletion of 0.0619 ± 0.009 μM^{-1} min^{-1} and 0.2834 ± 0.0637 μM^{-1} min^{-1} for glutathione and L-cysteine, respectively (Hoang et al., 2020). An addition of rat liver homogenate to the incubation mixture of glutathione and arecoline significantly decreased the yield (Boyland & Nery, 1969). [The Working Group noted that this effect can be explained by enzymatic hydrolysis of the methyl ester moiety.]

Induction of CYP2E1 was observed when rats were dosed orally with arecoline at 20 and 100 mg/kg bw per day for 7 days. The induction was attenuated with increasing dose (Huang et al., 2016a). [The Working Group noted that this

suggests possible metabolic interactions between numerous CYP2E1 substrates and arecoline in betel-quid chewers.]

In model experiments of nitrosation under conditions similar to those prevailing in the oral cavity or stomach of betel chewers (37 °C, pH ranging from 2 to 7), three nitrosamines were identified: *N*-nitrosoguvacoline, 3-(methylnitrosoamino)propanenitrile, and 3-(methylnitrosoamino)propanal (Fig. 4.2; Wenke & Hoffmann, 1983).

4.2 Evidence relevant to key characteristics of carcinogens

This section summarizes the evidence for the key characteristics of carcinogens (Smith et al., 2016), including whether arecoline is electrophilic or can be metabolically activated to an electrophile; is genotoxic; alters DNA repair or causes genomic instability; induces epigenetic alterations; induces oxidative stress; is immunosuppressive; modulates receptor-mediated effects; and alters cell proliferation, cell death, or nutrient supply.

4.2.1 Is electrophilic or can be metabolically activated to an electrophile

Arecoline contains an α,β-unsaturated carbonyl moiety in its molecule. This moiety is known to undergo Michael addition reactions by nucleophilic attack at the electrophilic β-carbon. Arecoline electrophilicity was manifested by reaction with glutathione in vitro with and without rat liver homogenate (Boyland & Nery, 1969; Hoang et al., 2020) and in vivo by formation of urinary AREMA (Boyland & Nery, 1969; Giri et al., 2006). Carboxylesterase-catalysed hydrolysis of arecoline to arecaidine and subsequent ionization of the latter under physiological pH diminishes the electrophilic reactivity due to the electron-donating carboxylate ion; however, arecaidine is itself electrophilic and reacts with glutathione. This reaction results in the formation of arecaidine mercapturic acid (Giri et al., 2006). Metabolic activation of arecoline to the corresponding epoxide has not been reported but N-oxidation also produces an electrophilic metabolite capable of reaction with glutathione (Giri et al., 2007) and, possibly, with other nucleophilic sites in biomolecules.

No data on DNA adducts were available to the Working Group. Protein adducts of arecoline and arecaidine were detected by LC-MS at cysteine, lysine, histidine, and the N-terminal amino acid of bovine serum albumin in vitro. Analogous adducts were also found in multiple proteins when rat renal proximal tubular cells (NRK-52E) were incubated with arecoline, and when human plasma was incubated with excess arecoline or arecaidine to mimic high exposure (Chou et al., 2012). Protein adducts at cysteine, lysine, and N-terminal amino acids with arecoline *N*-oxide and arecaidine *N*-oxide were found in human oral keratinocytes treated with areca-nut extract (Kuo et al., 2015). [The Working Group noted that, under physiological conditions, arecoline may form *N*-nitrosamines, which are known to yield alkylating agents upon activation.]

4.2.2 Is genotoxic

(a) Humans

(i) Exposed humans

No data were available to the Working Group.

(ii) Human cells in vitro

Arecoline

See Table 4.1.

Arecoline induced a minor increase in the frequency of DNA strand breaks in buccal epithelial cells in the DNA alkaline elution assay (Sundqvist et al., 1989, 1991), but did not induce DNA strand breaks in oral mucosal fibroblasts (Jeng et al., 1994) or buccal fibroblasts (Chang et al., 1998) in the DNA precipitation assay.

It induced phosphorylation of histone H2AX (γH2AX), a biomarker of DNA double-strand breaks, in a human oral cancer cell line (KB), human embryonic kidney cells (HEK293) (Tsai et al., 2008), an oral squamous cell carcinoma cell line (OC2) (Ji et al., 2012), and human gingival epithelial Smulow–Glickman cells and human oral squamous cell carcinoma cell lines (OEC-M1 and SAS) (Tu et al., 2019). Treatment of normal human gingival fibroblast cells (HGF-1) with arecoline hydrobromide resulted in a slight increase in γH2AX (Kuo et al., 2015). Arecoline induced DNA damage in human epithelial squamous carcinoma cells (HEp-2) analysed by the alkaline comet assay (Huang et al., 2016b).

Arecoline induced unscheduled DNA synthesis in HEp-2 cells (Sharan & Wary, 1992) and chromosomal aberration and sister-chromatid exchange in human lymphocytes (Kumpawat et al., 2003). Arecoline hydrobromide induced micronucleus formation in HEp-2 cells and in human lymphocytes (Kevekordes et al., 2001).

Arecoline N-*oxide*

See Table 4.1.

Treatment of normal human gingival fibroblast cells (HGF-1) with arecoline *N*-oxide resulted in an increase in γH2AX (Kuo et al., 2015, 2019).

(b) Experimental systems

(i) Arecoline

Non-human mammals in vivo

See Table 4.2.

Arecoline induced unscheduled DNA synthesis in Swiss albino mouse spermatids after a single intraperitoneal dose (Sinha & Rao, 1985a).

In C57BL/6J mice given drinking-water containing arecoline hydrobromide for 6 weeks, the transgenic rodent gene mutation assay did not show significant positive results. However, in the oral tissues from several individual animals, mutation frequencies were much higher than in untreated mice and mutation spectra exhibited (G:C→T:A transversion mutations) were unique compared with the untreated controls (Wu et al., 2012).

Several cytogenetic studies have been conducted with arecoline, arecoline hydrobromide, or arecoline hydrochloride administered by intraperitoneal or oral routes. The studies gave consistent positive results for chromosomal aberration, micronucleus formation, and sister-chromatid exchange in bone marrow (Panigrahi & Rao, 1982, 1983; Shirname et al., 1984; Deb & Chatterjee, 1998; Chatterjee & Deb, 1999). Arecoline hydrobromide administered as a single intraperitoneal dose to pregnant Swiss albino mice on day 17 of gestation gave positive results for micronucleus formation as measured in fetal blood (Sinha & Rao, 1985b).

Non-human mammalian cells in vitro

See Table 4.3.

Arecoline induced DNA strand breaks in mouse kidney cells as assessed by the alkaline DNA-unwinding assay (Wary & Sharan, 1988). Arecoline in the hydrobromide form induced DNA damage in rat liver clone-9 cells as shown by the alkaline comet assay (Chou et al., 2008), whereas arecoline did not induce DNA damage in rat liver clone-9 cells as shown by the alkaline comet assay (Wang et al., 2018).

Arecoline hydrochloride induced a significant increase in the frequency of hypoxanthine-guanine phosphoribosyltransferase (*Hprt*) gene mutations with and without metabolic activation in Chinese hamster V79 cells (Shirname et al., 1984).

Several in vitro cytogenetics studies on arecoline or arecoline hydrobromide have been conducted without metabolic activation in Chinese hamster ovary (CHO) cells. Most studies gave positive results for chromosomal aberrations, micronucleus formation, and

Table 4.1 Genetic and related effects of arecoline and arecoline *N*-oxide in human cells in vitro

End-point	Tissue, cell line (sex)	Results[a]		Agent, concentration (LEC or HIC)	Comments	Reference
		Without metabolic activation	With metabolic activation			
Arecoline						
DNA strand breaks (SSBs, DNA alkaline elution assay)	Primary buccal epithelial cells	(+)	NT	Arecoline, 5 mM [776 µg/mL]	Limited reporting of material and methods. Minor increase.	Sundqvist et al. (1989, 1991)
DNA strand breaks (SSBs, DNA precipitation assay)	Primary oral mucosal fibroblasts	(–)	NT	Arecoline, 400 µg/mL, [2.57 mM]	Data not shown for SSBs.	Jeng et al. (1994)
DNA strand breaks (SSBs, DNA precipitation assay)	Primary oral mucosal fibroblasts	(–)	NT	Arecoline, 400 µg/mL [2.57 mM]	Data not shown for SSBs. No positive control.	Chang et al. (1998)
DNA strand breaks (DSBs, γH2AX)	Human oral cancer cell line, KB, and human embryonic kidney cell line, 293	+	NT	Arecoline, 0.3 mM [46.5 µg/mL]		Tsai et al. (2008)
DNA strand breaks (DSBs, γH2AX)	Normal human gingival fibroblast cell line, HGF-1	+	NT	Arecoline hydrobromide, 200 µM [47.2 µg/mL]		Kuo et al. (2015)
DNA strand breaks (DSBs, γH2AX)	Human oral SCC cell line, OC2	+	NT	Arecoline, 0.5 mM [77.6 µg/mL]		Ji et al. (2012)
DNA strand breaks (DSBs, γH2AX)	Human gingival epithelial Smulow–Glickman cells and human oral SCC cell lines OEC-M1 and SAS	+	NT	Arecoline, 200 µg/mL [1.29 mM]		Tu et al. (2019)
DNA damage (alkaline comet assay)	Human laryngeal SCC cell line, HEp-2	+	NT	Arecoline, 0.1 mM [15.5 µg/mL]		Huang et al. (2016b)
Unscheduled DNA synthesis	Human laryngeal SCC cell line, HEp-2	(+)	NT	Arecoline, 0.04 mM [6.2 µg/mL]	Dose-dependent increase; however, not data shown for vehicle control.	Sharan & Wary (1992)
Chromosomal aberration	Primary human lymphocytes (M)	+	NT	Arecoline, 0.43 mM [67 µg/mL]		Kumpawat et al. (2003)
Micronucleus formation	Primary human lymphocytes	+	+	Arecoline hydrobromide, 12.5 µM [3 µg/mL]		Kevekordes et al. (2001)
Micronucleus formation	Human hepatoma cell line, HEp-G2	+	NT	Arecoline hydrobromide, 25 µM [6 µg/mL]		Kevekordes et al. (2001)

Table 4.1 (continued)

End-point	Tissue, cell line (sex)	Results[a]		Agent, concentration (LEC or HIC)	Comments	Reference
		Without metabolic activation	With metabolic activation			
Sister-chromatid exchange	Primary human lymphocytes (M)	+	NT	Arecoline, 0.43 mM [67 µg/mL]		Kumpawat et al. (2003)
Arecoline N-*oxide*						
DNA strand breaks (DSBs, γH2AX)	Normal human gingival fibroblasts, HGF-1	+	NT	200 µM [34.5 µg/mL]	Greater increase than with arecoline hydrobromide.	Kuo et al. (2015, 2019)

DSB, double-strand breaks; γH2AX, phosphorylated histone H2AX; HIC, highest ineffective concentration; LEC, lowest effective concentration: M, male; NT, not tested; SSBs, single strand breaks; SCC, squamous cell carcinoma.

[a] +, positive; –, negative; (+) or (–), positive/negative in a study of limited quality.

Table 4.2 Genetic and related effects of arecoline and its metabolites in non-human mammals in vivo

End-point	Species, strain (sex)	Tissue	Results[a]	Agent, dose (LED or HID)	Route, duration, dosing regimen	Comments	Reference
Arecoline							
Unscheduled DNA synthesis	Mice, Swiss albino (M)	Spermatid (early stage)	+	Arecoline, 20 mg/kg bw	Intraperitoneal; 1×; sampled after 16 h		Sinha & Rao (1985a)
Gene mutation	Mice, *Gpt* delta transgenic mice (C57BL/6J) (M)	Oral (gingival, buccal, pharyngeal, and sublingual)/ liver	–	Arecoline hydrobromide 700 µg/mL, 101 mg/kg bw per day	Oral (drinking-water), 6 wk, sampled after 2 wk	Unique mutation spectra (G:C→T:A transversion mutations) compared to spontaneous mutations.	Wu et al. (2012)
Chromosomal aberration	Mice, Swiss albino (M, F)	Bone marrow	+	Arecoline, 0.25 mg/animal	Intraperitoneal; daily for 10, 20 or 30 days; sampled after treatment		Panigrahi & Rao (1982)
Chromosomal aberration	Mice, Swiss albino (M)	Bone marrow	+	Arecoline, 40 mg/kg bw	Intraperitoneal; single administration; sampled after 20 h		Deb & Chatterjee (1998)
Chromosomal aberration	Mice, Swiss albino (M)	Bone marrow	(+)	Arecoline, 40 mg/kg bw (intraperitoneal), 170 µg/mL (oral)	Intraperitoneal and oral (ad libitum drinking-water); Intraperitoneal daily for 1, 5 or 15 days; sampled after 20 h	Only one concentration. No positive control.	Chatterjee & Deb (1999)
Micronucleus formation	Mice, Swiss albino (M)	Bone marrow	(+)	Arecoline hydrochloride, 2 mg/animal	Intraperitoneal; daily for 2 days, sampled after 6 h	Dose levels appear very high (LD_{50} reported, 2 mg/kg). No body weight reported.	Shirname et al. (1984)

Table 4.2 (continued)

End-point	Species, strain (sex)	Tissue	Results[a]	Agent, dose (LED or HID)	Route, duration, dosing regimen	Comments	Reference
Micronucleus formation	Mice, Swiss albino (F)	Fetal blood	(+)	Arecoline hydrobromide, 20 mg/kg bw	Intraperitoneal; 1× (day 17 of gestation); sampled after 45 h	Limited details reported in materials and methods.	Sinha & Rao (1985b)
Sister-chromatid exchange	Mice, Swiss albino (M, F)	Bone marrow	+	Arecoline, 0.5 mg/animal	Intraperitoneal, daily for 5, 10, or 15 days; sampled after treatment		Panigrahi & Rao (1983)
Sister-chromatid exchange	Mice, Swiss albino, (M, F)	Bone marrow	(+)	2.5 mg/animal	Intraperitoneal; daily for 5, 15, or 20 days; sample after treatment	No positive control. Dose levels not justified. Toxicity and cytotoxicity not reported.	Panigrahi & Rao. (1984)
Sister-chromatid exchange	Mice, Swiss albino (M)	Bone marrow	+	Arecoline, 20 mg/kg bw	Intraperitoneal; 1×; sampled after 20 h		Deb & Chatterjee (1998)
Sister-chromatid exchange	Mice, Swiss albino (M)	Bone marrow	(+)	Arecoline, 40 mg/kg (intraperitoneal), 170 µg/mL (oral)	Intraperitoneal and oral (drinking-water); daily for 1, 5, or 15 days; sampled after 20 h	Only one concentration. No positive control.	Chatterjee & Deb (1999)
Arecaidine							
Micronucleus formation	Mice, Swiss (M)	Bone marrow	(–)	14 mg/animal	Intraperitoneal; daily for 2 days, sampled after 6 h	Dose levels appear very high (LD_{50} reported 14 mg/kg). No body weight reported.	Shirname et al. (1984)
Sister-chromatid exchange	Mice, Swiss albino, (M)	Bone marrow	+	2.5 mg/animal	Intraperitoneal; daily for 1, 5, or 15 days; sampled after 19 h		Panigrahi & Rao (1984)

Table 4.2 (continued)

End-point	Species, strain (sex)	Tissue	Results[a]	Agent, dose (LED or HID)	Route, duration, dosing regimen	Comments	Reference
Arecoline N-*oxide*							
DNA strand breaks (DSBs, γH2AX)	Mice, NOD. CB17-*Prkdc*^{scid}/NcrCrl (NOD SCID)	Tongue	(+)	500 µg/mL (cotton swab smearing)	Topical; daily for 5 days, sampled after last treatment	One dose level only. Dose level not justified. No positive control. No clinical signs reported.	Kuo et al. (2015)

bw, body weight; DSB, DNA-strand breaks; F, female; Gpt, glutamic pyruvic transaminase; h, hour; γH2AX, phosphorylated histone H2AX; HID, highest ineffective dose; LD_{50}, median lethal dose; LED, lowest effective dose; M, male; wk, week.

[a] +, positive; –, negative; (+), positive in a study of limited quality.

Table 4.3 Genetic and related effects of arecoline and its metabolites in non-human mammals in vitro

End-point	Species, tissue, cell line	Results		Agent, concentration (LEC or HIC)	Comments	Reference
		Without metabolic activation	With metabolic activation			
Arecoline, arecoline hydrobromide, arecoline hydrochloride						
DNA strand breaks/ alkaline DNA-unwinding assay	Mice kidney primary cell	(+)	NT	Arecoline, [0.065 mM], 10 µg/mL	Source not reported. Only one concentration, no positive control.	Wary & Sharan (1988)
DNA damage/alkaline comet assay	Rat liver epithelial cells, clone-9 (cell line) (CRL-1439)	+	NT	Arecoline hydrobromide, 0.5 mM [118 µg/mL]		Chou et al. (2008)
DNA damage/alkaline comet assay	Rat liver clone-9 cell line	–	NT	Arecoline, 0.5 mM [77. 5 µg/mL]		Wang et al. (2018)
Gene mutation, *Hprt*	Chinese hamster lung V79 cells	+	+	Arecoline hydrochloride, [0.03 mM], 5 µg/mL		Shirname et al. (1984)
Chromosomal aberration	Chinese hamster ovary cells	+	NT	Arecoline hydrobromide, [0.32 mM], 75 µg/mL		Dave et al. (1992)
Chromosomal aberration	Chinese hamster ovary cells	+	NT	Arecoline hydrobromide, 0.85 mM [200 µg/mL]		Stich et al. (1981)
Chromosomal aberration	Chinese hamster ovary cells	(–)	NT	Arecoline hydrobromide, 0.05 mM [12.5 µg/mL]	Number of replicates not reported. Only one concentration. No cytotoxicity measured.	Trivedi et al. (1993)
Micronucleus formation	Chinese hamster ovary cells	+	NT	Arecoline, 0.2 µM [0.031 µg/mL]		Lee et al. (1996)
Sister-chromatid exchange	Chinese hamster ovary cells	+	NT	Arecoline hydrobromide, 0.05 mM, 12.5 µg/mL		Dave et al. (1992)
Sister-chromatid exchange	Chinese hamster ovary cells	(+)	NT	Arecoline hydrobromide, 0.05 mM, 12.5 µg/mL	Number of replicates not reported. Only one concentration. No cytotoxicity measured.	Trivedi et al. (1993)
Arecaidine						
Gene mutation, *Hprt*	Chinese hamster lung V79 cells	–	+	[0.071 mM], 10 µg/mL	Only one dose tested; No positive control.	Shirname et al. (1984)
Arecoline N-*oxide*						
DNA damage/comet assay	Rat liver clone-9 cells	+	NT	Arecoline, 125 µM [19.5 µg/mL]		Wang et al. (2018)

HIC, highest ineffective concentration; Hprt, hypoxanthine-guanine phosphoribosyltransferase; LEC, lowest effective concentration, NT, not tested.

[a] +, positive; –, negative; (+) or (–), positive in a study of limited quality.

sister-chromatid exchange (Stich et al., 1981; Dave et al., 1992; Lee et al., 1996). The study by Trivedi et al. (1993) gave positive results for sister-chromatid exchange and negative results for chromosomal aberration.

Non-mammalian experimental systems

See Table 4.4.

Arecoline hydrobromide induced DNA damage in *Drosophila melanogaster (hsp70-lacZ) Bg*^{9} as assessed by the comet assay (Shakya & Siddique, 2018; Shakya et al., 2019).

Arecoline hydrobromide did not induce DNA damage in the SOS chromotest in *Escherichia coli* PQ37 (Kevekordes et al., 1999).

Several assays for bacterial gene mutation, mainly in *Salmonella typhimurium* TA98 and TA100, have been conducted with arecoline, arecoline hydrobromide, or arecoline hydrochloride. Most studies gave positive results with and without metabolic activation (Shirname et al., 1983; Wang & Peng, 1996; Lin et al., 2011a). The study by Wang et al. (2018) performed only without metabolic activation gave negative results.

(ii) Arecaidine

See Table 4.2; Table 4.3 and Table 4.4.

Arecaidine did not induce micronucleus formation (Shirname et al., 1984) but induced sister-chromatid exchange in bone marrow after repeated intraperitoneal administration in Swiss albino mice (Panigrahi & Rao, 1984).

An in vitro assay for *Hprt* gene mutation with arecaidine gave positive results in the presence, but not the absence, of metabolic activation (Shirname et al., 1984).

In assays for bacterial gene mutation in *Salmonella typhimurium* TA100, TA1535, TA98, and TA1538, positive results were reported with and without metabolic activation (Shirname et al., 1983).

(iii) Arecoline N*-oxide*

See Table 4.2, Table 4.3 and Table 4.4.

Arecoline *N*-oxide induced γH2AX in mouse (NOD SCID) tongue tissues after 5-day topical application (once daily) (Kuo et al., 2015).

In rat liver clone-9 cells, arecoline *N*-oxide induced DNA damage as assessed by the comet assay (Wang et al., 2018).

Assays for bacterial gene mutation in *Salmonella typhimurium* TA98 and TA100 performed only without metabolic activation gave positive results (Lin et al., 2011a; Wang et al., 2018).

(iv) N*-nitrosoguvacoline*

See Table 4.4.

Assays for bacterial gene mutation in *Salmonella typhimurium* TA98 and TA100 with and without metabolic activation gave positive results (Wang & Peng, 1996).

4.2.3 Alters DNA repair or causes genomic instability

(a) Exposed humans

No data were available to the Working Group.

(b) Human cells in vitro

See Table 4.5.

Arecoline increased O^{6}-methylguanine-DNA methyltransferase activity in primary human oral keratinocytes (Lee et al., 2013a).

Arecoline increased DNA repair at 12.5 μg/mL [0.08 mM] but decreased repair at a higher concentration (i.e. 50 μg/mL [0.32 mM]) in two human oral squamous carcinoma cell lines (i.e. OEC-M1 and SAS) according to the host cell reactivation assay (Tu et al., 2019). Arecoline decreased DNA repair in a human oral cancer cell line (KB), a laryngeal squamous carcinoma cell line that may be a HeLa cervical cancer cell-line derivative (HEp-2), and human embryonic kidney cells (HEK293) assessed by means of

Table 4.4 Genetic and related effects of arecoline, arecaidine, arecoline *N*-oxide and *N*-nitrosoguvacoline in non-mammalian experimental systems

Test system (species, strain)	End-point	Results[a]		Agent, concentration (LEC or HIC)	Comments on study quality	Reference
		Without metabolic activation	With metabolic activation			
Arecoline						
Drosophila melanogaster (*hsp70-lacZ*) *Bg*^{9}	DNA damage (comet assay) on midgut cells	+	NA	Arecoline hydrobromide, 20 µM [5 µg/mL] (in feed)		Shakya & Siddique (2018)
Drosophila melanogaster (*hsp70-lacZ*) *Bg*^{9}	DNA damage (comet assay) on midgut cells	(+)	NA	Arecoline hydrobromide, 80 µM [19 µg/mL] (in feed)	Only one concentration, combined effects with geraniol investigated. No positive control.	Shakya et al. (2019)
SOS chromotest on *Escherichia. coli* PQ37	DNA damage (β-galactosidase activity)	–	–	Arecoline hydrobromide, 156.8 µg/assay		Kevekordes et al. (1999)
Salmonella typhimurium TA100, preincubation method	Reverse mutation	–	+	Arecoline hydrobromide, 1 µg/plate		Lin et al. (2011a)
Salmonella typhimurium TA98, preincubation method	Reverse mutation	–	–	Arecoline hydrobromide, 200 µg/plate		Lin et al. (2011a)
Salmonella typhimurium TA98, plate-incorporation method	Reverse mutation	–	–	Arecoline, 39.0 µmol/plate [6046 µg/plate]		Wang & Peng (1996)
Salmonella typhimurium TA98, plate-incorporation method	Reverse mutation	–	NT	Arecoline, 1 mM [155 µg/mL]		Wang et al. (2018)
Salmonella typhimurium TA1535, TA98, and TA1538, plate-incorporation method	Reverse mutation	(+)	(+)	Arecoline hydrochloride (concentration not reported)	Data not shown for TA1535, TA98, and TA1538 (reported to be positive).	Shirname et al. (1983)

Table 4.4 (continued)

Test system (species, strain)	End-point	Results[a]		Agent, concentration (LEC or HIC)	Comments on study quality	Reference
		Without metabolic activation	With metabolic activation			
Salmonella typhimurium TA100, plate-incorporation method	Reverse mutation	+	+	Arecoline hydrochloride, 10 µg/plate (+S9)		Shirname et al. (1983)
Salmonella typhimurium TA100, plate-incorporation method	Reverse mutation	+	+	Arecoline, 6.5 µmol/plate (–S9) [1009 µg/plate]		Wang & Peng (1996)
Salmonella typhimurium TA100, plate-incorporation method	Reverse mutation	–	NT	Arecoline, 1000 µM [155 µg/mL]		Wang et al. (2018)
Arecaidine						
Salmonella typhimurium TA1535, TA98, and TA1538, plate-incorporation method	Reverse mutation	(+)	(+)	NR	Data not shown for TA1535, TA98, and TA1538 (reported to be positive).	Shirname et al. (1983)
Salmonella typhimurium TA100, plate-incorporation method	Reverse mutation	+	+	100 µg/plate (–S9); 10 µg/plate (+S9)		Shirname et al. (1983)
Arecoline N-*oxide*						
Salmonella typhimurium TA98, plate-incorporation method	Reverse mutation	+	NT	125 µM [21 µg/mL]		Wang et al. (2018)
Salmonella typhimurium TA98, preincubation method	Reverse mutation	+	NT	1 µg/plate		Lin et al. (2011a)

Table 4.4 (continued)

Test system (species, strain)	End-point	Results[a]		Agent, concentration (LEC or HIC)	Comments on study quality	Reference
		Without metabolic activation	With metabolic activation			
Salmonella typhimurium TA100, plate-incorporation method	Reverse mutation	+	NT	500 µM [85 µg/mL]		Wang et al. (2018)
Salmonella typhimurium TA100, preincubation method	Reverse mutation	+	NT	50 µg/plate		Lin et al. (2011a)
N-*Nitrosoguvacoline*						
Salmonella typhimurium TA100, plate-incorporation method	Reverse mutation	+	+	24 µmol/plate (± S9) [4084 µg/plate]		Wang & Peng (1996)
Salmonella typhimurium TA98, plate-incorporation method	Reverse mutation	+	+	15 µmol/plate (–S9) [2552 µg/plate]		Wang & Peng (1996)

HIC, highest ineffective concentration; LEC, lowest effective concentration; NA, not applicable; NR, not reported; NT, not tested; S9, 9000 × *g* supernatant.

[a] +, positive; –, negative; (+), positive in a study of limited quality.

Table 4.5 Altered DNA repair and genomic instability in human cells exposed to arecoline in vitro

End-point	Tissue, cell line	Results[a]		Concentration (LEC or HIC)	Comments	Reference
		Without metabolic activation	With metabolic activation			
DNA repair/O^6-methylguanine-DNA methyltransferase (protein activity)	Primary human oral keratinocytes	↑	NT	[0.13 mM], 20 μg/mL		Lee et al. (2013a)
DNA repair/host cell reactivation assay	Human oral squamous SCC, OEC-M1 and SAS (cell lines)	↑/↓	NT	[0.08 mM] 12.5 μg/mL (increase); [0.32 mM] 50 μg/mL (decrease)		Tu et al. (2019)
DNA repair/host cell reactivation assay	Human oral cancer, KB (cell line); Human laryngeal SCC, HEp-2 (cell line) and human embryonic kidney, HEK 293 (cell line)	↓	NT	0.3 mM [46 μg/mL]		Tsai et al. (2008)
DNA repair/host cell reactivation assay	Human laryngeal SCC, HEp-2 (cell line)	↓	NT	0.1 mM [15.5 μg/mL]		Huang et al. (2016b)
Genomic (chromosomal instability)/biomarker (Bub1, Mad2, Mps1, cyclin B1, Aurora A, BubR1, α-tubulin, histone H3)	Human oral cancer, KB (cell line) and human SCC, HEp-2 (cell line)	(+)	NT	0.3 mM [46 μg/mL]	Only one concentration. No positive control. No cytotoxicity measured.	Wang et al. (2010)

HIC, highest ineffective concentration; LEC, lowest effective concentration: NT, not tested; SCC, squamous cell carcinoma.

[a] ↑, increase; ↓, decrease; ↑/↓, no dose–response with two concentrations; (+), positive in a study of limited quality.

the host cell reactivation assay (Tsai et al., 2008; Huang et al., 2016b).

Arecoline treatment of human oral cancer cells (KB) and laryngeal carcinoma cells (HEp-2) stabilized mitotic spindle assembly, which led to distorted organization of mitotic spindles, misalignment of chromosomes, and upregulation of spindle-assembly checkpoint genes (Wang et al., 2010).

4.2.4 Induces epigenetic alterations

(a) Humans

(i) Exposed humans

No data were available to the Working Group.

(ii) Human cells in vitro

The expression of multiple genes catalysing histone methylation (*MLL*, *SETDB1*, and *SUV39H2*), acetylation (*ATF2*), or demethylation (*JMJD6*) were altered in human leukaemia cells (K-562) treated with arecoline (Lin et al., 2011b).

TP53 promoter methylation and expression reduction were associated with arecoline treatment in primary cultures of oral mucosal fibroblasts. Arecoline treatment was also associated with proliferation and epithelial–mesenchymal transition (EMT) of these cells (Zheng et al., 2018).

Several studies investigated the effect of arecoline on microRNAs (miRs) and EMT-related genes in human cells in vitro (see Table 4.6). In two buccal mucosal fibroblast cell lines (BMF-1 and BMF-2, between passages 3 and 8), arecoline was associated with a dose-responsive decrease in *hsa-mir-200c* expression. Overexpression of miR-200c in these cells reduced arecoline-induced collagen gel contraction, migration, invasion, and wound-healing capacities (Lu et al., 2018). [The Working Group noted that the cells were derived from areca-nut chewing subjects with oral submucous fibrosis.]

Arecoline reduced *hsa-mir-203* expression in immortalized oral keratinocyte (HaCaT) cells, and hsa-mir-203 downregulated secreted frizzled-related protein 4 (SFRP4) mRNA and protein expression, and upregulated transmembrane-4 L six family member 1 (TM4SF1) mRNA and protein expression. SFRP4 is involved in EMT and TM4SF1 is involved in motility and proliferation. Increased expression of hsa-mir-203 was associated with reduced cell proliferation and upregulated CK19 and E-cadherin (CDH1) protein expression, and downregulated N-cadherin (CDH2) and vimentin (VIM) protein expression in control untreated cells (Zheng et al., 2015).

Arecoline treatment of two oral epithelial cell lines (Smulow–Glickman normal gingival cells and FaDu oral squamous cell carcinoma cells) was associated with the acquisition of stemness, EMT, migratory and invasive properties, tumour formation potential in vivo, and chemoresistance, and hsa-mir-145 was found to play an arecoline-dependent albeit partial role (Wang et al., 2016). In particular, hsa-mir-145 overexpression inhibited cancer stemness: hsa-mir-145 was found to directly target stemness-related transcription factors OCT4 and SOX2, whereas SOX2 and OCT4 overexpression was associated with the reversal of hsa-mir-145 stemness features.

Four miRs associated with cancer (hsa-mir-26a, hsa-mir-23a, hsa-mir-30a-5p, and hsa-mir-143) were overexpressed in arecoline-treated gingival fibroblast CRL-2014 cells and gingival carcinoma Ca9-22 cells (Tsai et al., 2011).

After treatment with arecoline, hsa-mir-211 was upregulated in oral squamous cell carcinoma cells (SAS) (Chen et al., 2016).

In ORL-48(T) and ORL-136(T) oral squamous cell carcinoma cells treated with arecoline, hsa-mir-22 was reduced and inversely correlated with *IL6* and *STAT3* gene expression and *MYC* gene and protein expression. Arecoline also increased cell viability, proliferation, and G2/M proportion in ORL-48(T) and increased *MYC* promoter activity. In these same cells, oncostatin

Table 4.6 Effect of arecoline on microRNAs and genes related to the epithelial–mesenchymal transition in human cells in vitro

End-point	Tissue, cell line	Result[a]	Concentration (LEC or HIC), duration	Comments	Reference
hsa-miR-200c	Buccal mucosal fibroblasts BMF-1 and BMF-2	↓	5 µg/mL for 48 h	Cells derived from areca-nut chewing OSF subjects.	Lu et al. (2018)
hsa-miR-203	Oral keratinocyte cell line, HaCaT	↓	0.08 mM for 72 h		Zheng et al. (2015)
CK19		↑	0.04 mM for 72 h		
CDH2		↓	0.04 mM for 72 h		
CDH1		↑	0.04 mM for 72 h		
VIM		↓	0.02 mM for 72 h		
SFRP4		↓	0.08 mM for 72 h		
TM4SF1		↑	0.08 mM for 72 h		
hsa-miR-145	Oral epithelial cell lines: SG (Smulow-Glickman normal gingival) and FaDu (oral SCC) cells	↑	10 µg/mL for 90 days		Wang et al. (2016)
Oct4		↑			
Nanog		↑			
Sox2		↑			
Snail		↑			
Twist		↑			
Slug		↑			
VIM		↑			
E-Cadherin		↓			
hsa-miR-26a	Normal gingival fibroblasts (CRL-2014); gingival carcinoma Ca9-22 cells	↑	100 µg/mL for 24 h		Tsai et al. (2011)
hsa-miR-23a		↑			
hsa-miR-30a-5p		↑			
hsa-miR-143		↑			
hsa-miR-211	Oral SCC cell line (SAS)	↑	2.5 µg/mL for 24 h		Chen et al. (2016)
hsa-miR-22	ORL-48(T) and ORL-136(T) oral SCC cells	↓	0.025 µg/mL for 24 h		Chuerduangphui et al. (2018)
hsa-miR-486-3p	Primary oral SCC cells	↓	100 µM for 5 days		Chou et al. (2012)
MEG3 (hsa-miR-329 and hsa-miR-R410)	Human oral keratinocytes (HOK)	↓	100 µmol/L, up to 9 days	Chemical form of arecoline not specified.	Shiah et al. (2014)

CDH1, E-cadherin; CDH2, N-cadherin; CK19, cytokeratin 19; EMT, epithelial mesenchymal transition; h, hour; HIC, higher ineffective concentration; LEC, lowest effective concentration; miR, microRNA; OSF, oral submucous fibrosis; SCC, squamous cell carcinoma; TM4SF1, transmembrane-4 L six family member 1; VIM, vimentin.

[a] ↑, increase; ↓, decrease.

M (*OSM*) was upregulated and inversely correlated with hsa-mir-22, which was found to be directly targeted and suppressed in hsa-mir-22-transfected oral squamous cell carcinoma and 293FT cells, via a luciferase reporter assay (Chuerduangphui et al., 2018).

Arecoline treatment was associated with recruitment of DNA methyltransferase 3B (DNMT3B) to the ANK1 promoter, and reduction in the expression of hsa-mir-486-3p in oral squamous cell carcinoma cells. miR-486-3p was shown to decrease discoidin domain receptor-1 (DDR1) expression in vitro by directly targeting its 3′-UTR. Overexpression of hsa-mir-486-3p resulted in growth inhibition and apoptosis by knockdown of DDR1 via MTT and annexin V assays (Chou et al., 2019).

Arecoline reduced the expression of MEG3, and of the 14q32.2 miRs hsa-mir-329 and hsa-mir-R410 (silenced by arecoline-induced DNA methylation of the MEG3 DMR) in a time-dependent manner in human oral keratinocyte (HOK) cells. Arecoline upregulated *WNT7B* and increased the phosphorylation of GSK3β and active-β-catenin in oral squamous cell carcinoma (HOK) cells, with a concomitant upregulation of cyclin D and Myc proteins (Shiah et al., 2014). [The Working Group noted that the chemical form of arecoline was not specified.]

(b) Experimental systems

Histone proteins H1 in spleen cells and H2B in bone-marrow cells of male Swiss albino mice exhibited an increase in poly-ADP-ribosylation after treatment with arecoline hydrobromide (10 μg/mL in drinking-water for up to 5 weeks), while all other tested proteins exhibited a reduction. The chromatin of spleen and bone-marrow cells was also increasingly open with increasing arecoline doses (Saikia et al., 1999). [The Working Group noted that the data were sparse and showed no clear overall pattern.]

4.2.5 Induces oxidative stress

(a) Humans

See Table 4.7.

(i) Exposed humans

No data were available to the Working Group.

(ii) Human cells in vitro

Nine reports have provided direct evidence for production of reactive oxygen species (ROS) after treatment with arecoline (see Table 4.7) in normal cells, such as BMF cell cultures (Lee et al., 2016; Hsieh et al., 2018), primary oral keratinocytes and the oral epithelial cell line OECM-1 (Lee et al., 2013b), the spontaneously immortalized keratinocyte cell line HaCaT (Thangjam & Kondaiah, 2009), and human umbilical vein endothelial cells (HUVEC, passages 3 to 5) (Hung et al., 2011) [the Working Group noted that the chemical form of arecoline was not specified]; and from studies in cancer cells, such as two oral squamous cell carcinoma cell lines (Ji et al., 2012; Shih et al., 2020) and two oesophageal squamous cell carcinoma cell lines (Wang et al., 2019). In the study by Shih et al. (2020), the antioxidant-responsive element (ARE)-containing genes *NRF2*, *HO1*, *SOD1*, and *NQO1* were upregulated.

N-acetyl-L-cysteine (NAC) or epigallocatechin-3-gallate was used to support findings in six of these studies. (Hung et al., 2011; Ji et al., 2012; Lee et al., 2013b, 2016; Hsieh et al., 2018; Wang et al., 2019). [The Working Group noted that the chemical form of arecoline was not specified by Hung et al. (2011).] One study found that arecoline exposure and ROS production are inversely correlated in human polymorphonuclear lymphocytes (Lai et al., 2007).

In addition, one study reported that the arecoline metabolite arecoline *N*-oxide induced ROS production in the liver WRL 68 cell line (Wang et al., 2018). [The Working Group noted that WRL 68 may be a HeLa cervical cancer cell line derivative with hepatocyte morphology

Table 4.7 Effect of arecoline and arecoline *N*-oxide on production of reactive oxygen species in human cells in vitro

Cell line	Result[a]	Concentration (LEC or HIC), duration	Comments	Reference
Arecoline				
Normal buccal mucosal fibroblasts	↑	20 µg/mL for 8 h		Lee et al. (2016)
Normal buccal mucosal fibroblasts	↑	0.2 mM for 1 h	Single concentration.	Hsieh et al. (2018)
Oral keratinocytes (HOK)	↑	20 µg/mL for 24 h		Lee et al. (2013b)
Oral SCC cell line (OECM-1)	↑	40 µg/mL for 24 h		
Keratinocyte cell line (HaCaT)	↑	50 µg/mL for 48 h		Thangjam & Kondaiah (2009)
HUVECs (passages 3 to 5)	↑	10 µg/mL for 2 h	Chemical form of arecoline not stated.	Hung et al. (2011)
Oral SCC cell line (OC2)	↑	0.3 mM [46.8 µg/mL] for 2 h		Ji et al. (2012)
Oral SCC cell line (OEC-M1)	↑	100 µg/mL for 24 h		Shih et al. (2020)
Oesophageal cell lines (OE21 and CE81T)	↑	15.6 µM for 24 h	Single concentration.	Wang et al. (2019)
Arecoline N-*oxide*				
Liver cell line (WRL 68)	↑	31.25 µM for 24 h	WRL 68 may be a HeLa cervical cancer cell line derivative.	Wang et al. (2018)

h, hour; HIC, highest ineffective concentration; HUVECs, human umbilical vein endothelial cells; LEC, lowest effective concentration; SCC, squamous cell carcinoma.

[a] ↑, increase.

and liver-enzyme expression profile, as noted by the European Collection of Authenticated Cell Cultures.]

Regarding indirect evidence, based on ROS-induced changes in gene expression, the available data concerned heat shock protein 70 (HSP70) and haem oxygenase-1 (HO1). Regarding HSP70, it was found to be expressed via Western-blot analysis in a time- and dose-dependent manner in response to arecoline treatment in an oral epithelial cell line of gingival carcinoma provenance (GNM). NAC protected the cells from this effect, as did curcumin, PD98059, and staurosporine (Lee et al., 2008). Regarding HO1, mRNA and protein levels were found to be correlated with arecoline concentration in human umbilical vein endothelial cells (HUVECs) and only partly due to increased ROS levels. Arecoline treatment also increased intercellular adhesion molecule-1 (ICAM1) and vascular cell adhesion molecule-1 (VCAM1) protein expression. Pre-treatment with glutathione prevented ROS production and VCAM1 expression, but not ICAM1 expression (Hung et al., 2011) [The Working Group noted that the chemical form of arecoline was not specified.] HO1, as well as other genes including glucose-6-phosphate dehydrogenase (*G6PDH*), and glutathione reductase (*GRD*), were found to be upregulated at the mRNA level in HaCaT cells after arecoline treatment, and co-treatment with NAC prevented this upregulation. ROS production and cell-cycle arrest at the G1/G0 phase, and reduced catalase activity, were found to be associated with arecoline treatment (Thangjam & Kondaiah, 2009).

(b) Experimental systems

See Table 4.8.

Experiments in at least three species of experimental animal support a link between arecoline

treatment and the induction of ROS: the rat (Run-mei et al., 2014; You et al., 2019); the mouse (Dasgupta et al., 2006; Laskar et al., 2019) [the Working Group noted that the chemical form of arecoline was not specified. The Working Group also noted that only female mice were tested]; and the fruit fly (Shakya & Siddique, 2018).

Male Sprague-Dawley rats exposed daily to drinking-water containing arecoline hydrobromide at 10 mg/mL n for 49 days displayed increased protein levels of α-collagen-1 (COL1A), α-smooth muscle actin (ASMA), NADPH oxidase 4 (NOX4), nucleotide-binding domain leucine-rich repeat containing pathway 3 (NLRP3), and interleukin-1β (IL1B) in oral submucosa tissues (You et al., 2019). [The Working Group noted that only one dose level of arecoline was tested.] The levels of superoxide dismutase (SOD), CAT, GSH-Px, and glutathione were decreased in male Wistar rat liver after oral administration of arecoline hydrobromide at 100 mg/kg bw per day for 7 days (Run-mei et al., 2014).

One report of a study in Swiss albino mice [sex not reported] showed that reductions in liver enzyme levels of SOD, glutathione, and glutathione *S*-transferase were all dose-responsive after treatment with arecoline doses inferior or equal to 20 mg/kg bw per day for 14 days (Dasgupta et al., 2006). Increased levels of glutathione *S*-transferase, CYPB5, CYP450, and malondialdehyde were seen in Swiss albino mice given arecoline intraperitoneally (10, 20, or 40 mg/kg per day) for 10 or 30 days. Reduced sulfhydryl content was seen after administration of arecoline at 40 mg/kg per day for 10 or 30 days (Singh & Rao, 1993).

A study in Swiss albino mice exposed daily to drinking-water containing arecoline hydrobromide at 10 μg/mL for 24 weeks showed that arecoline treatment was associated with pre-neoplastic nodulation in the liver and white patches in the lungs of the mice, along with increased lipid peroxidation in the liver, lungs, and intestines, increased thiobarbituric acid-reactive substances in the liver, lungs, and intestines, and reduced protein carbonylation (Laskar et al., 2019). [The Working Group noted that the chemical form of arecoline was not specified, and that only one dose level of arecoline was tested.]

The metabolite arecoline *N*-oxide (31.25 μM, 2 hours) induced ROS production in rat liver clone-9 cells and this increase was relieved by NAC (Wang et al., 2018)

4.2.6 Modulates receptor-mediated effects

(a) Humans

No data were available to the Working Group.

(b) Experimental systems

Reduced T3 and T4 thyroid hormones and increased thyroid-stimulating hormone, increased pineal and seral serotonin, reduced pineal and seral *N*-acetyl serotonin, reduced pineal and seral melatonin and increased testosterone, fructose, and sialic acid, were observed in male Wistar rats injected with arecoline intraperitoneally twice per day for 10 days (10 mg/kg bw per day) (Saha et al., 2018, 2020). [The Working Group noted that only one dose was tested.]

4.2.7 Alters cell proliferation, cell death, or nutrient supply

(a) Humans

No data in exposed humans were available to the Working Group.

A significant decrease in TP53 and the downstream p21 (CDKN1) protein levels was observed in primary cultured oral mucosal fibroblasts treated with arecoline, indicating a loss of tumour-suppression activity (Zheng et al., 2018). Arecoline treatment also decreased the expression of E-cadherin protein but increased the expression of *N*-cadherin and vimentin, overall suggesting an arecoline-mediated cellular proliferative and EMT response in oral mucosal fibroblasts (Zheng et al., 2018). Arecoline treatment

Table 4.8 Effect of arecoline and arecoline *N*-oxide on production of reactive oxygen species in experimental systems

End-point	Species, strain (sex)	Tissue	Results[a]	Dose (LED, HID)	Route, duration, dosing regimen	Comments	Reference
Arecoline							
COL1A, αSMA, CTGF, NOX4, NLRP3, and IL1β protein levels	Rats, Sprague-Dawley (M)	Oral submucous tissues	↑	10 mg/mL	Injection into oral mucosa, 1×	Single exposure; Males only.	You et al. (2019)
SOD, CAT, GSH-Px, and GSH	Rats, Wistar (M)	Liver	↓	100 mg/kg per day for 7 days	Oral		Run-mei et al. (2014)
SOD GSH GST	Mice, Swiss albino (NR)	Liver	↓ ↓ ↓	10 mg/kg bw per day for SOD 5 mg/kg bw per day for GSH 10 mg/kg bw per day for GST	Intraperitoneal, 1×/day for 14 days	Sex of mice not stated.	Dasgupta et al. (2006)
GST, CYPB5, CYP450, and MDA SH content	Mice, Swiss albino (M, F)	Liver	↑ ↓	10 mg/kg per day for 10 or 30 days 40 mg/kg per day for 10 or 30 days	Intraperitoneal, 1×/day for 10 or 30 days	The chemical form of arecoline was not stated.	Singh & Rao (1993)
TBARS Protein carbonylation	Mice, LACA Swiss albino (F)	Liver, lung, and intestine	↑ ↑	10 µg/mL	Oral (drinking-water), 24 weeks	The amount of drinking-water ingested was not reported.	Laskar et al. (2019)
GSH content GST activity, lipid peroxidation, protein carbonyl content	Fruit fly *Drosophila melanogaster* (hsp70-lacZ) Bg third instar larvae)	Fruit fly homogenate	↓ ↑	20 µM	Oral (feed), 24 h		Shakya & Siddique (2018)
Arecoline N-*oxide*							
ROS	Rat liver clone-9 cells			31.25 µM	2 h		Wang et al. (2018)

bw, body weight; COL1A, α-collagen 1A; CTGF, connective tissue growth factor; CYP450, cytochrome P450; F, female; GSH, glutathione; GST, glutathione *S*-transferase; h, hour; HID, highest ineffective dose; IL, interleukin; LED, lowest effective dose; M, male; MDA, malondialdehyde; NOX4, nicotinamide adenine dinucleotide phosphate oxidase 4; NLRP3, NOD (nucleotide oligomerization domain)-like receptor family pyrin domain containing 3 inflammasome complex; NR, not reported; ROS, reactive oxygen species; SH, sulfhydryl; α-SMA, α-smooth muscle actin; SOD, superoxide dismutase; TBARS, thiobarbituric acid-reactive substances.

[a] ↑, increase; ↓, decrease.

in Smulow–Glickman human gingival epithelial cells and FaDu pharynx epithelial squamous cell carcinoma cells led to a dose-dependent elevation in expression of the zinc-finger transcription factor ZEB1, which was associated with migration ability, cell invasiveness, and anchorage-dependent growth (Ho et al., 2015). Lin et al. (2015) also demonstrated arecoline-induced proliferation, migration, invasiveness, and anchorage-independent growth in Smulow–Glickman and FaDu cells, citing Lin28B, an RNA-binding protein that was dose-dependently expressed, as a key player.

Arecoline alone, and in combination with nicotine, activated epidermal growth factor receptor/protein kinase B (EGFR/AKT) signalling in head and neck squamous cell carcinoma cells, which resulted in enhanced anti-apoptotic and pro-EMT signalling (Yang et al., 2018). Furthermore, reverse transcription-polymerase chain reaction analysis confirmed by Western blotting showed increased expression of transcripts of mesenchymal markers, including vimentin and E-cadherin (Wang et al., 2016). Bronchial smooth-muscle cells were shown to proliferate and migrate after exposure to cytokines in cultured media taken from human bronchial epithelial cells treated with arecoline (Kuo et al., 2011).

Oral fibroblasts treated with arecoline and its metabolite, arecoline *N*-oxide, showed an increase in the expression of the fibrotic-related genes transforming growth factor β 1 (*TGFB1*), S100 calcium-binding protein A4 (*S100A4*), matrix metallopeptidase-9 (*MMP-9*), interleukin 6 (*IL6*) and fibronectin, and a decrease in the expression of E-cadherin (Kuo et al., 2015). Arecoline *N*-oxide elicited a significantly stronger response than did the parent compound (Kuo et al., 2015).

Fibroblasts cultured from human gingival, normal buccal mucosa, and oral submucous fibrosis showed dose-dependent increases in cell proliferation with arecoline treatment (≤ 10 μg/mL) as measured by 5-bromo-2′-deoxyuridine labelling (Chen et al., 1995). [The Working Group noted the inhibition of cell proliferation with arecoline at 100 μg/mL.] These findings are consistent with arecoline-induced cell proliferation in oesophageal squamous cell carcinoma cells (arecoline concentration, ≤ 31.2 μM) (Wang et al., 2019) and oral squamous cell carcinoma cells (arecoline concentration, ≤ 0.25 μg/mL) (Chuerduangphui et al., 2018), shown to be activated through AKT and extracellular signal-regulated kinase 1/2 (ERK1/2) phosphorylation or induction of c-MYC and reduction of miR-22, respectively.

(b) Experimental systems

Early work showed that treatment of mouse kidney cells with arecoline at 10 μg/mL increased cell proliferation (Wary & Sharan, 1988). In a nude mouse assay, SCC-9 xenografts showed increased Ki-67 staining after arecoline treatment (Zheng et al., 2018).

4.3 Data relevant to comparisons across agents and end-points

Arecoline is one of the approximately 1000 chemicals tested across the full assay battery of the Toxicity Testing in the 21st Century (Tox21) and Toxicity Forecaster (ToxCast) programmes supported by the United States Environmental Protection Agency (US EPA, 2020). In vitro assay descriptions to map Tox21 and ToxCast screening data in the context of the 10 key characteristics were previously summarized by Chiu et al. (2018). Results in this data set only show 7 active hits out of 235 assays. Six out of the 7 assays with active hits tested for cell viability after 0, 8, 16, 24, 32, and 40 hours of exposure in human liver cells using a dead-cell DNA stain. Results indicated that treatment with arecoline led to a loss of cell viability. [The Working Group noted the flag for less than 50% efficiency with this assay

at 0, 24, 32, and 40 hours; only one concentration above baseline was active in the assay tested after 8 hours of exposure.] Arecoline was active in the thyrotropin-releasing hormone receptor (TRHR)-Hek293 cell-based assay. Intracellular calcium is measured using fluorescence detection as an indicator of Gq-protein-coupled TRHR activation. These results suggested arecoline involvement in modulating receptor-mediated effects through potential agonism of the TRHR. [The Working Group noted one flag for < 50% efficiency with this assay.]

5. Summary of Data Reported

5.1 Exposure characterization

Arecoline is the primary active ingredient of the areca nut, which has been previously classified by the *IARC Monographs* programme as *carcinogenic to humans (Group 1)*. Areca nut is widely cultivated in Asia. It has been estimated that more than 10% of the world's population, primarily in south-eastern Asia, chew areca nut for its mild psychoactive effects. Arecoline is commonly traded and used in the hydrobromide form, but the hydrochloride form is also commercially available. Arecoline is now rarely used medicinally as an antiparasitic drug, but it is still applied indirectly in the form of areca nut as an ingredient in traditional Chinese and Ayurveda medicines. Exposure to arecoline in occupational settings is expected from the use of areca nut to suppress hunger during intensive labour and as a stimulant, but no exposure data were found for workers directly handling arecoline or areca nut. After exposure, arecoline can be detected in saliva, blood, urine, hair, and breast milk from humans. However, short-term markers in blood and saliva provide only qualitative information.

5.2 Cancer in humans

No data were available to the Working Group.

5.3 Cancer in experimental animals

5.3.1 Arecoline

Arecoline increased the incidence of tumours in one co-carcinogenicity study, increased the incidence and multiplicity of tumours in another co-carcinogenicity study, and caused an increase in the incidence of total tumours in two studies.

In one co-carcinogenicity study in which arecoline hydrobromide was administered orally (in the drinking-water) to male C57BL/6 mice in combination with the carcinogen 4-nitroquinoline 1-oxide (4-NQO), arecoline in combination with 4-NQO increased the incidence of invasive squamous cell carcinoma of the oesophagus compared with mice receiving only 4-NQO.

In another co-carcinogenicity study, male F344 rats given *N*-benzyl-*N*-methylnitrosamine (NMBA) by subcutaneous injection and arecoline by oral administration (in the drinking-water) showed a significant increase in the incidence and multiplicity of papilloma of the oesophagus and a significant increase in multiplicity of papilloma of the tongue, compared with rats receiving only NMBA.

Male Swiss mice treated with arecoline hydrochloride by oral administration (gavage) had a significant increase in the incidence of total tumours in one study. In another study, female Swiss mice fed a vitamin B complex-deficient diet and treated with arecoline hydrochloride by oral administration (gavage) showed a significant increase in the incidence of total tumours.

5.3.2 Arecaidine

Arecaidine caused an increase in the incidence of tumours in one initiation–promotion study.

Male Syrian golden hamsters treated in the cheek pouch with the carcinogen 7,12-dimethylbenz[*a*]anthracene (DMBA) in the initiation phase followed by arecaidine in the promotion phase showed a significant increase in the incidence and multiplicity of exophytic squamous cell papilloma or carcinoma (combined) compared with hamsters treated with DMBA only.

5.4 Mechanistic evidence

Arecoline is readily absorbed in the oral cavity. It has been found in the saliva, blood, and urine of areca-nut chewers and can readily enter the brain of rats. It is rapidly metabolized. The main metabolic pathways are hydrolysis to arecaidine, oxidation to arecoline *N*-oxide catalysed by human flavin-containing monooxygenases, and conjugation with glutathione leading eventually to arecoline and arecaidine mercapturic acids. In mice, the metabolic pathways are similar and altogether 10 metabolites have been identified. Under physiological conditions, arecoline can react with sodium nitrite to form *N*-nitrosamines, a class of carcinogenic agents that are known to be metabolically activated to alkylating agents. Two nitrosamines, namely *N*-nitrosoguvacoline and *N*-nitrosoguvacine, were found in the saliva of betel-quid chewers.

There is consistent and coherent evidence that arecoline exhibits multiple key characteristics of carcinogens. Arecoline is an electrophilic α,β-unsaturated ester that can undergo Michael addition with cellular nucleophiles. No data were available regarding DNA-adduct formation by arecoline. Arecoline reacts with glutathione, ultimately yielding mercapturic acids, and forms adducts at multiple sites within proteins in human plasma and in human primary cells in vitro.

Arecoline is genotoxic. Data in humans were not available. Arecoline showed mixed results for DNA strand breaks in human primary cells and in several human cell lines in vitro using different assays, but there were consistently positive results for unscheduled DNA synthesis, micronucleus formation, chromosomal aberration, and sister-chromatid exchange. Similarly, arecoline showed consistent positive results for chromosomal aberration, micronucleus formation, and sister-chromatid exchange in experimental systems both in vitro and in vivo. Arecoline induced gene mutations in most assays for bacterial gene mutation and in mammalian cells in vitro. Arecoline altered the mutation spectrum in a transgenic mouse mutation assay. Results from the few available genotoxicity tests on *N*-nitrosoguvacoline, and the arecoline metabolites arecaidine and arecoline *N*-oxide, were positive.

Arecoline alters DNA repair and causes genomic instability. No data were available in exposed humans or in non-human experimental systems, and data in human primary cells were scarce. In human cell lines in vitro, arecoline altered DNA-repair enzyme activity. Arecoline increased O^6-methylguanine-DNA methyltransferase activity in primary human oral keratinocytes. In human cell lines, arecoline altered DNA repair and stabilized mitotic spindle assembly, which led to distorted organization of mitotic spindles, misalignment of chromosomes, and upregulation of spindle assembly checkpoint genes.

Arecoline induces oxidative stress. No data in humans were available. In several studies in various human primary cells, arecoline increased production of reactive oxygen species. Various reports of production of reactive oxygen species in vivo were also available in different experimental species (mouse, rat, and fruit fly) and in rat liver clone-9 cells.

There is suggestive evidence that arecoline alters cell proliferation in experimental systems. In vitro studies using human-derived cells have shown that arecoline is capable of inducing proliferation, migration, invasiveness, and anchorage-independent growth. In one study

in primary cultured oral mucosal fibroblasts, arecoline decreased TP53 and the downstream CDKN1A protein levels, indicating a loss of tumour-suppression activity.

There is sparse evidence regarding other key characteristics of carcinogens. The available data that arecoline induces epigenetic alterations mainly concerned microRNAs (miRs). Arecoline treatment reduced the expression of four miRs (hsa-mir-200c, hsa-mir-203, hsa-mir-329, and hsa-mir-410), and increased the expression of six miRs (hsa-mir-145, hsa-mir-26a, hsa-mir-23a, hsa-mir-30a-5p, hsa-mir-143, and hsa-mir-211) in human cells.

Arecoline was essentially without effects in the assay battery of the Toxicity Testing in the 21st Century and Toxicity Forecaster research programmes.

6. Evaluation and Rationale

6.1 Cancer in humans

There is *inadequate evidence* in humans regarding the carcinogenicity of arecoline.

6.2 Cancer in experimental animals

There is *limited evidence* in experimental animals for the carcinogenicity of arecoline.

6.3 Mechanistic evidence

There is *strong* evidence in human primary cells and in various experimental systems that arecoline exhibits multiple key characteristics of carcinogens.

6.4 Overall evaluation

Arecoline is *possibly carcinogenic to humans (Group 2B).*

6.5 Rationale

The *Group 2B* evaluation for arecoline is based on *strong* mechanistic evidence. There is *strong* evidence in human primary cells that arecoline exhibits multiple key characteristics of carcinogens; arecoline is electrophilic; it is genotoxic; and it induces oxidative stress. It also alters DNA repair or causes genomic instability in experimental systems.

There is also *limited evidence* for cancer in experimental animals, on the basis of increased incidence of tumours in one co-carcinogenicity study, increased incidence and multiplicity of tumours in another co-carcinogenicity study, and increased incidence of total tumours in two studies. The evidence regarding cancer in humans is *inadequate*, as no studies were available.

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