2017

Carcinogenic potential of sanguinarine, a phytochemical used in 'therapeutic' black salve and mouthwash

Andrew Croaker
Southern Cross University

Graham J. King
Southern Cross University

John H. Pyne
University of Queensland

Shailendra Anoopkumar-Dukie
Griffith University

Vilim Simanek
Palacký University

See next page for additional authors

Publication details

Postprint of: Croaker, A, King, GJ, Pyne, JH, Anoopkumar-Dukie, S, Simanek, V & Liu, L 2017, 'Carcinogenic potential of sanguinarine, a phytochemical used in 'therapeutic' black salve and mouthwash', *Mutation Research - Reviews*, vol. 774, pp. 46-56.
Published version available from:
https://doi.org/10.1016/j.mrrev.2017.09.001

Carcinogenic Potential of Sanguinarine, a Phytochemical used in 'Therapeutic' Black Salve and Mouthwash.

Andrew Croaker[a,b,c], Graham J. King[a], John H. Pyne [d], Shailendra Anoopkumar-Dukie[c,e], Vilim Simanek[f] and Lei Liu[a]*.

[a]*Southern Cross Plant Science, Southern Cross University, Lismore, NSW, Australia;* [b]*Wesley Medical Research Institute, Wesley Hospital, Auchenflower, QLD, Australia;* [c]*Quality Use of Medicines Network, Queensland, Australia;* [d]*School of Medicine, University of Queensland, St Lucia, QLD, Australia;* [e]*School of Pharmacy and Pharmacology, Griffith University, Gold Coast Campus, Gold Coast, QLD, Australia;* [f]*Department of Medical Chemistry and Biochemistry, Faculty of Medicine and Dentistry, Palacký University, Olomouc, Czech Republic.*

*corresponding author:

Dr. Lei Liu Southern Cross Plant Science, Southern Cross University.

Military Rd East Lismore NSW 2480 Australia ben.liu@scu.edu.au T: +61 2 6620 3293

Key Words

Sanguinarine; black salve; skin cancer; genotoxin; carcinogenesis; topical; bloodroot; *Sanguinaria canadensis*; escharotic.

Abstract

Black salves are escharotic skin cancer therapies in clinical use since the mid 19th century. *Sanguinaria canadensis*, a major ingredient of black salve formulations, contains a number of bioactive phytochemicals including the alkaloid sanguinarine. Despite its prolonged history of clinical use, conflicting experimental results have prevented the carcinogenic potential of sanguinarine from being definitively determined.

Sanguinarine has a molecular structure similar to known polyaromatic hydrocarbon carcinogens and is a DNA intercalator. Sanguinarine also generates oxidative and endoplasmic reticulum stress resulting in the unfolded protein response and the formation of 8-hydroxyguanine genetic lesions. Sanguinarine has been the subject of contradictory *in vitro* and *in vivo* genotoxicity and murine carcinogenesis test results that have delayed its carcinogenic classification. Despite this, epidemiological studies have linked mouthwash that contains sanguinarine with the development of oral leukoplakia, sanguinarine also proposed as an aetiological agent in gallbladder carcinoma.

This literature review investigates the carcinogenic potential of sanguinarine. Reasons for contradictory genotoxicity and carcinogenesis results are explored, knowledge gaps identified and a strategy for determining the carcinogenic potential of sanguinarine especialy relating to black salve are discussed. As patients continue to apply black salve, especially to skin regions suffering from field cancerization and skin malignancies, an understanding of sanguinarines genotoxic and carcinogenic potential is of urgent clinical relevance.

1. Introduction

Patients often associate natural therapies as being safe with a reduced risk of toxicity [1]. This can be a dangerous misconception with natural product exposures responsible for a range of toxicities and fatalities [2]. While natural products may possess anti-mutagenic properties, they also include some of the most potent carcinogens known to humankind [3]. Unlike pharmaceuticals, rigorous pre-clinical testing of natural therapies is not a regulatory requirement for their sale to consumers placing patients at risk of adverse outcomes [4].

Topical skin cancer therapies are often applied to tissue that has been chronically exposed to carcinogenic ultraviolet radiation (UVR) with both UVA and UVB inducing genetic damage to skin cells [5]. Over time UVR results in the formation of abnormal clonal cellular areas, patches of damaged skin with altered p53 tumor gene expression levels, in a process referred to as field cancerization [6]. It is therefore especially important that topical therapeutics, which may be applied to UV-induced precancerous skin regions and skin malignancies, are assessed for their mutagenic and carcinogenic potential.

Black salve is an alternative skin cancer therapy available for purchase online. It contains a number of constituents that vary in composition and concentration between vendors including bloodroot (*S. canadensis*), chaparral (*Larrea tridentata*), graviola (*Annona muricata*), oleander (*Nerium oleander*) and zinc chloride among others. A number of these botanical extracts and their constituent phytochemicals have not been assessed for mutagenic or carcinogenic potential.

Black salve has never been studied in a systematic clinical trial, with only a limited number of patient outcomes being reported in case studies [7]. Patients with melanoma [8], squamous cell carcinoma [9] and basal cell carcinoma [10] have experienced black salve treatment failures. Whether black salve altered the natural history or metastatic potential of these malignancies is currently unknown, as is the rate of new skin cancer formation in black salve treated areas.

S. canadensis is a key ingredient of black salve. It contains quaternary benzophenanthridine alkaloids, chelerythine, chelilutine, chelirubine, sanguilutine with sanguinarine being the main alkaloid in bloodroot and black salve formulations [11]. There are concerns about the carcinogenic potential of sanguinarine arising from reports of an association with mouthwash-induced leukoplakia and gallbladder carcinoma [12], [13]. The usual battery of *in-vitro* genotoxicity tests and murine studies on sanguinarine have given mixed and conflicting results [14], [15], [16], [17]. This literature review explores the evidence relating to the carcinogenic potential of sanguinarine, the main alkaloid present in black salve.

2. Mechanisms of Sanguinarine Carcinogenesis

2.1 DNA Intercalation

Sanguinarine can exist as a charged iminium (pH 2-6) or uncharged alkanolamine (pH 6.5-9.0) form, at physiological pH (7.4) both forms are present [18, 19] (Figure 1). Spectroscopic studies involving calf thymus DNA suggests the iminium form of sanguinarine binds to DNA, exhibiting GC base pair specificity [19]. Thermodynamic analysis has revealed DNA binding

is exothermic and enthalpy driven, which is indicative of intercalative binding [20].

Figure 1: Attached Separately

DNA intercalators are able to interfere with the action of DNA polymerase thus impairing DNA replication, especially in rapidly dividing cancer cells. As a result, the mechanism of DNA intercalation has been harnessed by the anthracycline chemotherapy agents doxorubicin and the topoisomerase II inhibitor mitoxantrone [21]. Sanguinarine cytotoxicity correlates with its DNA intercalating ability, with DNA single-strand breaks occurring before the initiation of apoptosis induced double strand breaks, suggesting genotoxic activity [22], [23].

DNA intercalators are usually associated with frameshift mutations as they increase the distance between adjacent DNA base pairs [24]. Paradoxically some agents used to treat cancer may result in the development of secondary malignancies. For example, anthracycline intercalating agents can cause leukemia's and myelodysplasia [25] by inducing chromosomal disruption with the subsequent development of tumor forming translocations [26]. Sanguinarine is a strong DNA intercalator [27], with a binding constant to calf thymus DNA of $1.00 \times 10^6\,M^{-1}$ [28] being similar to the binding constants of anthracycline chemotherapy agents daunorubicin ($1.27 \times 10^6\ M^{-1}$) and doxorubicin ($2.04\text{-}3.3 \times 10^6\ M^{-1}$) [29, 30]. The strong intercalative binding action of sanguinarine, as with anthracycline agents, may damage DNA with carcinogenic consequences.

2.2 Reactive Species Generation

Free radicals are chemical moieties that contain orbiting unpaired electrons. They are unstable, reactive and able to interact with and damage cellular proteins, lipids and nucleic acids [31]. Reactive Oxygen Species (ROS) contain biologically active reactive oxygen functional groups such as hydrogen peroxide (H_2O_2) and singlet oxygen [32]. Oxidative stress being an indirect mechanism through which carcinogens can exert genotoxic effects. ROS have been shown to act in cancer initiation, promotion and progression [33], [34], [35], [36].

Sanguinarine induces ROS in a range of cell lines [37], [38] and also in a murine *in vivo* model [39], by mechanisms that are not as well established as for UVA radiation. While UVA activates nicotinamide adenine dinucleotide phosphate-oxidase (NADPH) oxidase, facilitating electron transfer to molecular oxygen creating superoxide anions, sanguinarine appears to be reduced spontaneously by NADPH-producing superoxide anions without requiring a catalytic enzyme [40]. Subsequent intracellular sanguinarine re-oxidation can occur, suggesting redox cycling as a mechanism by which sanguinarine rapidly generates large amounts of ROS, in particular H_2O_2 [41]. Redox cycling is more likely to occur in proliferating cells with high NADPH concentrations that then reduce nucleotides for DNA synthesis, and less likely to occur in cells, such as the MCF breast cancer cell line, that contain higher levels of catalase [42].

When assessing the ROS effects of sanguinarine in SPCA1 human lung adenocarcinoma cells, endoplasmic reticulum (ER) stress was found to be induced [43]. It is well established that

ROS can cause the misfolding or unfolding of proteins which accumulate in the ER lumen [44], resulting in the unfolded protein response (UPR) molecular cascade [45]. While the UPR is an anti-cancer target [46], continuous ER stress creates a positive ROS feedback loop [47]. From the current evidence, sanguinarine therefore appears to activate the ROS-ER stress cycle, and amplifies the oxidative damage sustained by cells [43].

Sanguinarine has also been shown to generate reactive nitrogen species (RNS), in LNCaP prostate cancer epithelial cells exposed to 3μg/ml sanguinarine experience having increased nitric oxide (NO) production [48]. The mechanism for sanguinarine induced NO generation is currently unknown, although neoplastic prostate cancer cell lines express higher levels of inducible Nitric Oxide Synthase (iNOS) than their non-neoplastic counterparts [49]. In the epidermis, human keratinocytes express all three isoforms of NOS, including iNOS [50] [51]. Human skin fibroblasts stimulated with cytokines and lipopolysaccharides have been shown to express both constitutive and inducible NOS [52]. Although not yet studied in human keratinocytes, if sanguinarine increases RNS production then the generation of highly reactive peroxinitrite ($ONOO^-$) is likely to increase its genotoxic potential in skin.

A number of factors affect antioxidant levels in human skin. The epidermis, being the body's main barrier to environmental exposures has significantly higher levels of enzymatic (superoxide dismutase (SOD), catalase) and non-enzymatic (Vitamin C, Vitamin E, uric acid and glutathione) antioxidants than the dermis [53]. Aged skin has normal levels of enzymatic anti-oxidants but non-enzymatic antioxidant levels are 60-70% of those found in younger skin [54]. Sanguinarine exposure can result in a rapid and severe depletion of cellular

glutathione (GSH) in PC3 and L-929 cell lines. Similarly, in human hepatocytes sanguinarine has been shown to result in reduced GSH levels and cell death without an increase in malondialdehyde (MDA) production, a marker of lipid peroxidation [55]. Sanguinarine GSH depletion appears to occur via addition of GSH to iminium bond of sanguinarine in a 1:1 ratio and not due to the generation of ROS [56]. An analogous interaction is suggested with SH-enzymes [57]. Whether sanguinarine exerts a similar antioxidant depleting effect in human skin is currently not known.

UVA is a known carcinogen that exerts a significant proportion of its carcinogenic effect through the generation of reactive species [35], [34]. Sanguinarine has been found in a number of normal and malignant cell lines to generate a reactive species profile similar to that of UVA [58]. Additionally, the process of redox cycling, ROS-ER stress cycle activation and antioxidant depletion may amplify the cellular oxidative stress and carcinogenic potential that may occur from sanguinarine exposure. Cells exposed to high levels of ROS undergo apoptosis or necrosis [59], although sublethal ROS doses can result in genotoxicity that a cell is unable to repair completely [60], [61], increasing the likelihood of gene mutations that ultimately may lead to cancer [62], [63].

Despite a number of studies investigating the action of sanguinarine as an oxidant and its ability to cause genetic damage, surprisingly only one study has searched for the presence of the ROS induced 8-hydroxyguanine. This effect in sanguinarine exposed HCT116 human colon cancer cells indicates that sanguinarine can induce guanine oxidative genetic damage [58].

2.3 DNA Adduct Formation and Sanguinarine Metabolism

In a ^{32}P-postlabelling assay, sanguinarine when incubated with β-naphthoflavone (β-NF) activated rat hepatic microsomes, resulted in DNA adduct formation [64]. This was concentration dependent, a sanguinarine concentration of 100μM causing 36.3 DNA adducts per 10^8 nucleotides, 10μM causing 3.2 DNA adducts per 10^8 nucleotides and 1μM not resulting in the formation of detectable DNA adducts by using a nuclease P1 enriched ^{32}P-postlabeling assay [64]. Whether the DNA adducts detected in this study were genuine adducts or artefacts generated during oxidative DNA damage [65] has been questioned [42]. While DNA adducts may be formed by reactive oxygen species and lipid peroxidation [66], malondialdehyde generation was not detected, suggesting these processes were not responsible for the observed DNA adduct formation [55]. While adducts may have formed in a rat liver microsome system, they either do not form after an equivalent sanguinarine exposure in human hepatocytes, or any adducts that form are repaired and removed [67]. In a 90 day feeding trial of pigs, toxicity studies with 64ppm sanguinarine ingestion failed to show liver DNA adduct formation utilizing ^{32}P-postlabeling [68]. Plasma sanguinarine levels of 0.11μg/ml and liver levels of 0.13μg/g in the highest exposure group were reported, although these are below the concentration at which DNA adducts were induced in the positive rat liver microsome study [64].

Once formed, DNA adduct stability and the risk of its ability to induce a persistent somatic mutation is determined by the cells replication rate and DNA repair capacity [69], [70]. When DNA adducts are used in quantitative risk assessment, induced DNA adduct levels should be

compared to the tissues background adduct formation level in each tissue [71]. Care should be exercised when extrapolating animal adduct results for assessing human risk, as humans have greater sensitivity to tumor induction than mice when exposed to some carcinogens [72].

With sanguinarine not resulting in definite adduct formation, research has subsequently focused on whether the metabolism of sanguinarine generates a DNA adduct forming metabolite. Observations that sanguinarine toxicity in mice is reduced by 3-methylcholanthrene, a CYP450 inducer, suggests sanguinarine is metabolized by CYP450 [73]. CYP1A, a member of the CYP450 family, has been found to metabolize polycyclic aromatic hydrocarbons (PAH) to generate reactive epoxides that have carcinogenic potential [74]. Sanguinarine shares a similar structure to PAHs, raising the possibility of sanguinarine also being metabolized by CYP450 to an epoxide metabolite [75].

Sanguinarine has been shown to undergo dihydro derivative formation by *in vitro* UV-Vis spectrometry and fluorimetry [76], this being confirmed by an *in vivo* rodent study [77]. Kosina et al in 2011 [78], further elucidated the multi-step metabolism of sanguinarine in human hepatocytes using electrospray quadrupole ion-trap mass spectrometry with reversed phase chromatographic analysis. They found that dihydrosanguinarine (DHSG) is oxidized by cytochrome P450 enzymes resulting in O-demethyl and hydroxyl metabolite formation. These metabolites subsequently appeared to undergo Phase II biotransformation, being conjugated by glucuronidases and sulfotransferases, as determined by the respective MS mass measurements and fragmentation patterns of the resultant compounds. Sanguinarine epoxide

metabolites suspected of having carcinogenic potential were not detected in this analysis [78].

Dihydrosanguinarine is the main phase I metabolite of sanguinarine [78]. In a 90 day rat feeding study [79] DHSG reached a maximal plasma concentration of 28ng/ml with a maximal liver concentration of 130ng/ml. No liver adduct formation was detected following exposure to these low DHSG concentrations.

2.4 DNA Repair Mechanisms

Alkaloids may potentially disrupt DNA repair mechanisms. The catalytic subunit of human telomerase (hTERT) upregulates DNA repair genes and increases the nucleoside triphosphate (NTP) pool available for correcting DNA lesions [80]. A number of alkaloids inhibit telomerase activity by suppressing hTERT mRNA expression, including the isoquinoline alkaloids, chelidonine [81] and papaverine [82]. Sanguinarine has been shown to disrupt telomerase activity through G-quadruplex binding with an 8μM concentration resulting in a 76% reduction in telomerase activity [83] but its effect on hTERT is not currently known. While telomerase is a valid anticancer target being overexpressed in 85% of human malignancies [84], the impact of telomerase targeting compounds on DNA repair processes is unclear. By impairing DNA repair, such agents may pose a mutagenic risk for normal and malignant cells.

Inflammation has also been shown to inhibit DNA repair by up to 70% in cholangiocarcinoma cells via NO mediated DNA repair enzyme nitrosylation [85]. While sanguinarine has been shown to induce NO production in prostate carcinoma cells [48], a number of studies have found sanguinarine to have an anti-inflammatory effect [86], [87],

[88]. Black salve however has been associated with histological evidence of significant inflammation in humans [89], either due to the effect of sanguinarine or other salve constituents. This inflammation may impair DNA repair, aggravating any genotoxic effects.

Current genotoxicity testing protocols do not directly assess the impact of a compound on DNA repair processes. Due to human inter-individual variation in DNA repair capabilities, the developing field of personalized medicine may in the future incorporate an assessment of an individual's genetic repair capacity when determining toxicity risk. The effect of sanguinarine on DNA repair mechanisms is currently unknown.

2.5 Tumor Immune Surveillance

Organ transplant patients can have a 100-fold increased risk of non-melanoma skin cancer compared to the general population [90] arising from the use of anti-rejection medications [91]. Nearly 100% of patients diagnosed with non-melanoma skin cancers develop immune suppressive effects from UV exposure compared to 40% in the general population [92], [93]. These findings highlight the importance that immune surveillance plays in limiting skin carcinogenesis.

UV-induced immunosuppressant cellular and cytokine effects are triggered either by DNA damage to skin immune cells or by oxidative stress [94], [95]. Both mechanisms may result from sanguinarine exposure. Currently the effect of sanguinarine on skin immune cell numbers and function is unknown, as is its potential impact on skin immune surveillance and carcinogenesis.

3. Assessment of Sanguinarine Genotoxicity

In order to register a pharmaceutical compound there is a regulatory requirement to carry out and report genotoxicity testing to assess carcinogenicity, and in some instances to assess the risk of heritable mutation [96], [97]. A battery of genotoxicity tests based on micro-organism and mammalian systems are routinely employed to detect compound-induced DNA damage (Table 1).

3.1 Sanguinarine In Vitro Genotoxicity Results

Following S9 activation sanguinarine and sanguinaria extract yield positive Ames test results [98], [99]. Sanguinarine has been shown to elicit a positive mutagenic response [98] in TA98, TA100, TA1537 and TA1538 strains, suggesting it may induce frame-shift mutations [99]. Interestingly in one study where sanguinaria extract, derived from *S. canadensis* rhizomes, contained the full complement of bloodroot phytochemicals, a positive Ames test result was observed with metabolic activation only in the one TA1538 tester strain [99]. Despite these positive Ames test results, sanguinarine has returned negative genotoxicity test results in the SOS chromotest [100] and *Saccharomyces cerevisiae* mutation test [98]. While the Ames test has a greater capacity for identifying carcinogens, the SOS chromotest has a lower false positive rate [101]. Unfortunately, the majority of these were industry studies where experimental methodology was not available for peer-reviewed scrutiny [99].

Sanguinarine has also produced conflicting *in vitro* mammalian results with Comet assays being positive while Micronucleus assays have yielded negative genotoxicity results. Positive Comet assays following sanguinarine exposure were reported from several studies in murine

and human cell lines [102], [40], [103], [104] at low concentrations from 1μg/ml [102]. Human gingival fibroblasts develop 2 to 3-fold higher single DNA strand break levels in the Comet assay in response to sanguinarine compared to prostate cancer cell lines LNCaP and DU-145 [105]. This was observed in the absence of a significant increase in gingival fibroblast apoptosis, suggesting gingival fibroblasts may be more susceptible to sanguinarine genotoxic injury than prostate cancer cells [105].

There is evidence that sanguinarine can induce rapid *in vitro* genetic damage. In one study, after a one hour exposure a concentration of 4μg/ml caused 10% of human CEM leukemia T cells to have comet assay detectable DNA damage, and this increased to 60% of cells by 3 h. These cells showed a bimodal mechanism of cytotoxicity with some displaying poly (ADP-ribose) polymerase-1 (PARP-1) fragmentation indicative of apoptosis and others being propidium iodide positive indicative of necrosis [14]. Another study investigated the timing of comet assay DNA single and double strand break formation in HCT116 colon cancer cells treated with 3μM sanguinarine. Single strand breaks developed after 45 min, while double strand breaks developed after 90 min, around the time apoptosis markers such as PARP cleavage appeared, suggesting sanguinarine possesses reactive species genotoxicity apart from its cytotoxic apoptotic effects. The single strand breaks showed guanine oxidation to 8-Oxo-2'-deoxyguanosine characteristic of oxidative stress with antioxidant treatment preventing DNA single strand breaks and reducing DNA double strand breaks to control levels [58].

Due to the significant cytotoxicity displayed by sanguarine against a number of normal and

295 malignant cell types [7], the comet assay may be prone to potentially false positive
296 genotoxicity test results. [106]. Human keratinocytes and hepatocytes have shown greater
297 resistance to sanguinarine cytotoxicity than other cell types [107], [108] and may represent
298 better models for the *in vitro* Comet assay testing of sanguinarine.

299 Contradictory negative *in vitro* genotoxicity results for sanguinarine have however been
300 reported using the micronucleus assay. In the presence of rat liver S9, human lymphocytes
301 and the metabolically competent Hep-G2 human hepatoma cell line showed no micronucleus
302 formation when exposed to sanguinarine concentrations up to 5μM [15]. This study tested a
303 total of fifteen natural products including five established rodent carcinogens arecoline, plant
304 extract aristolochic acid, B-asarone, psoralen and safrole, promoters of carcinogenesis
305 coumarin, isatidine dehydrate, monocrotaline, retrorsine, tannin and thiourea, in addition to
306 compounds suspected of carcinogenic activity benzyl acetate, emodine, reserpine and
307 sanguinarine chloride. All of the established carcinogens in this study with the exception of
308 safrole showed genotoxicity, the suspected carcinogens monocrotaline and retrorsine also had
309 negative genotoxicity results. While other groups also reported negative safrole results with
310 rat hepatocytes [109], they did show safrole as clearly positive in the Hep-G2 system [110].
311 This suggested that the Hep-G2 subclone used by the authors may differ in its metabolic
312 activation capacity and raises questions regarding the accuracy of sanguinarines negative
313 micronucleus assay result.

314 The lack of clarity surrounding inferences from *in vitro* testing of sanguinarine include the
315 equivocal results obtained when tested in the Chinese hamster ovary (CHO-HPRT)

316 mutagenesis assay [99]. In one industry study relayed by Munro et al [99] a significant
317 increase in mutant CHO cell numbers occurred in the presence of S9 at a sanguinarine
318 concentration of 10μg/ml. This was in the absence of mutant frequency increases at lower
319 dose levels. An industry study also reported by Munro et al [99] produced similar results with
320 a concentration of 10μg/ml increasing mutant frequency with no mutants forming below this
321 concentration. The CHO-HPRT mutagenesis assay results were considered inconclusive. The
322 selective use of such data by Munro et al [99] to refute concerns about the safety of
323 sanguinarine, without the company that conducted the studies making the research available
324 for public scrutiny, is a disappointing display where commercial interests seem to have taken
325 precedence over patient safety.

326 The inconclusive assessment from sanguinarines *in vitro* genotoxicity testing has led to a
327 need for clarification with *in vivo* toxicity studies. *In vitro* metabolism systems contain
328 restricted Phase I and II enzyme subsets that preferentially enable oxidative cytochrome
329 P450-mediated reactions [111]. Alternate metabolic processes are utilized by a number of
330 mutagens to form genotoxic metabolites that are often not assessed by current *in vitro* assays
331 [112]. *In vivo* toxicity studies however operate in a physiological system that utilizes
332 reduction, hydrolysis and conjugation in addition to oxidation with a full complement of
333 Phase I and II enzymes [113]. Current *in vitro* genotoxicity assays have high false positive
334 rates, this being another reason for use of *in vivo* assessment of compounds. One analysis
335 found that of 700 compounds that were known rodent non-carcinogens, 75-95% generated a
336 positive result in at least one *in vitro* genotoxicity test [114]. This outcome is supported by a

study assessing over 1,000 pharmaceutical compounds [115], [116]. Chemicals that are genotoxic *in vitro* therefore require further evaluation *in vivo* in order to establish their potential risk to humans.

Table 1: See End of Document

3.2 Sanguinarine In Vivo Genotoxicity Assessment

Several *in vivo* assays have been used in an attempt to determine the genotoxicity and carcinogenic potential of sanguinarine (Table 2). These have unfortunately not clarified the compound's toxicity status, with methodology problems, unpublished non-peer reviewed industry studies and contradictory results all contributing to uncertainty and a lack of consensus.

Regulatory agencies have increasingly accepted the *in vivo* Comet assay as a reliable genotoxicity test [117], [118] following the development of a standardized protocol [119]. It has been validated showing 73.7% to 78.9% sensitivity with 19 known genotoxins in liver and stomach tissues respectively [120]. As with the *in vitro* comet assay, the *in vivo* assay may detect cytotoxic rather than genotoxic compounds. To reduce false positive results, histopathological correlation and cell analysis for cytotoxicity are often performed.

Male Swiss albino mice given a single intra-peritoneal injection of sanguinarine starting at a dose of 2.7mg alkaloid/ kg body weight, developed bone marrow and blood cell signs of

genotoxicity starting with an increase in comet tail length. At 5.4mg/kg Olive Tail Moment (OTM) and tail length increased by 14-32% while 10.8mg/kg caused a significant increase of 33-51% OTM, tail length and tail DNA [121]. This suggests that a single sanguinarine exposure can result in cellular genetic damage in a dose dependent manner. The sanguinarine in this study however was of low purity (88%), being a precipitation product obtained from argemone oil. Argemone oil is an alternative botanical source of sanguinarine, that comes from the seeds of *Argemone mexicana* Linn, and has independently been shown to possess genotoxic potential [122, 123]. In a murine model the intraperitoneal injection of argemone oil resulted in positive micronucleus and chromosomal aberration tests at a 1ml/kg body weight dose and positive comet assays at a 0.25ml/kg body weight dose in lymphocytes/ hepatocytes and bone marrow cells [122]. Whether this is due to the sanguinarine contained within the argemone oil or other factors is yet to be determined. Therefore, any conclusions drawn from positive sanguinarine genotoxicity test results using low purity sanguinarine, with possible argemone oil contaminants, should be considered with caution. *Macleaya cordata* extract is primarily composed of quaternary benzophenanthridine alkaloids including sanguinarine 528.95 g/kg and chelerythrine 82.05 g/kg, with a minor quantity of protopine and allocryptopine [124]. A subsequent study using such an extract, orally administered to rats showed no hepatocyte adduct formation and no evidence of lymphocyte or hepatocyte genotoxicity in Comet assay [125]. Since less than 5% of ingested sanguinarine is absorbed [77], oral sanguinarine administration without the determination of plasma sanguinarine levels may translate to blood concentrations too low for comet assay positivity yielding false

negative results.

In vivo micronucleus (MN) assays have also been developed as a genotoxicity screening tool [126]. *Sanguinaria* extract at doses up to 14.2mg/kg bw administered intraperitoneally on two occasions 24 h apart were investigated by *in vivo* MN assay in CD-1 mice. While bone marrow polychromatic erythrocytes showed no increases in MN formation [99], this information derives from an industry study conducted by Vipont Pharmaceuticals Inc (manufacturer of Viadent) that is unpublished and not available for public scrutiny. This prevents an assessment of sample size and research methodology, undermining its value. Compared to the normal practice of sampling bone marrow 24 h after the second compound exposure [127], bone marrow was collected 6 h after the second dosing. This may not have allowed sufficient time for MN formation and detection. There have been no peer-reviewed reports to date of *in vivo* MN assessment of sanguinarine.

Sanguinarine has been assessed for its effect on *in vivo* chromosome aberration and sister chromatid exchange. Mice administered sanguinarine chloride intraperitoneally at 5, 10 and 15mg/kg body weight concentrations had bone marrow cell changes suggestive of genotoxicity, with a minimum effective concentration of 10mg/kg [128]. The sanguinarine in this experiment was a defined product from Sigma, which reduces concerns of purity and contamination. The chromosomal aberrations observed were largely chromatid breaks with occasional chromosome breaks. Positive control mitomycin C gave a 28-fold higher chromosomal aberration rate compared to the negative control, with a five fold higher rate than sanguinarines minimum effective concentration. The SCE induction and predominance

of chromatid-type breaks were consistent with S phase-dependent clastogen induced DNA damage [129], [130]. The 10mg/kg dose induced 5.32 sister chromatid exchanges (SCE) per cell, while the 15mg/kg dose induced 6.02 SCEs/cell.

When investigating the DNA damage caused by genotoxic carcinogens, different agents cause unique changes in the genomic sequence of cancer related genes [131]. These characteristic mutations act as carcinogen specific 'signatures', where their assessment is known as DNA-lesion footprinting or DNA-damage mapping [132]. For example ultraviolet radiation (UVR) induces dipyrimidine site C to T or CC to TT transitions within the RAS oncogene and TP53 tumor suppressor gene while polycyclic aromatic hydrocarbons from tobacco smoke tend to induce G to T transversions [133]. To date, DNA damage mapping has not been performed to determine whether sanguinarine or black salve result in characteristic genomic sequence mutations.

Table 2: See End of Document

3.3 Contradictory Murine Sanguinarine Carcinogenesis Results

Two experiments have assessed sanguinarines murine carcinogenic potential. One study in female Swiss albino mice, sought to determine whether sanguinarine could initiate or promote cancer. Mice exposed to 1,3-dimethylbutylamine (DMBA) and sanguinarine (4.5μM concentration) as a combination initiator did not develop increased tumor rates compared to DMBA initiation alone. Mice however, initiated with DMBA and subsequently exposed to topical twice weekly 1.5μM sanguinarine for 25 weeks did develop earlier tumor onset with

mean tumor numbers increasing from 5 to 7.07 [16], suggesting sanguinarine may act as a tumor promoter.

Another study sought to determine whether sanguinarine could protect mice from the effects of UVB. Female SKH-1 hairless mice had 5μM topical sanguinarine applied as a pretreatment 30 min pre-UVB exposure or 5 min post-UVB exposure, both groups showed significantly reduced skin edema, leukocyte infiltration and hyperplasia [17]. There was also a significant reduction in H_2O_2 and ornithine decarboxylase (ODC) levels, suggesting sanguinarine's anti-inflammatory action may reduce oxidative stress with low concentration topical application. Sanguinarine's mixed cancer promotion and potentially protective effects in murine models requires clarification by futher research, especially as sanguinarine containing topical therapies are in current use by patients.

In addition to these studies that have assessed isolated sanguinarine, two dental industry-funded studies have explored the carcinogenic potential of *Sanguinaria* extract in rats [99]. The first study in 50 female and 50 male CD rats at doses up to 60mg/kg body weight/ day administered by gavage was terminated before the scheduled 104-week study period was reached due to early high dose female and male control group mortality. An explanation for rat mortality was not provided, no increased incidence of pre-neoplastic or neoplastic lesions was reported [99].

A follow-up 2-year dietary feeding study administered *Sanguinaria* extract up to 200mg/kg body weight/ day to 75 male and female Charles River CD rats. The incidence of fibrosarcomas and subcutaneous fibromas was increased in high dose males (6/75 animals)

with high dose females developing an increase in uterine polyps (5/75 animals). These lesions did not occur in control animals. Despite the difference between the treatment and control groups, the authors cited the 5% historical incidence of such tumors in control animals [134] and concluded the fibrosarcomas/ fibromas and uterine polyps seen in the high dose group were not a consequence of *Sanguinaria* exposure [99]. These studies highlight the lack of transparency and independent peer-review that may occur when commercially used botanical products undergo industry funded toxicity testing [135].

4. Human Epidemiological Evidence suggesting Sanguinarine Carcinogenic Potential

4.1 Maxillary Vestibule Leukoplakia

There is epidemiological evidence linking sanguinarine exposure to the development of leukoplakia. Viadent, an antibacterial mouth rinse and toothpaste containing 0.3μM sanguinarine chloride and 0.7μM sanguinarine chloride respectively [136], [137], [138], has been associated with increased maxillary vestibule leukoplakia [12]. This area normally is rarely effected by leukoplakia [139]. The anterior portion of the maxillary vestibule has the highest concentration of intra-oral rinse residue due to a low salivary turnover this mucosal area has prolonged mucosal product contact [140]. 84% of patients that developed maxillary vestibule leucoplakia had used Viadent with the average period of use being 4.4 years. The epidemiological correlation between sanguinarine dental product use and the development of leukoplakia suggests sanguinarine may induce pre-malignant change in humans.

Histologically, Eversole et al [141] showed sanguinaria-associated leucoplakias had borderline dysplasia in 55% of cases, mild dysplasia in 42.5% of cases and moderate dysplasia in 2.5% of cases. No lesions showed severe dysplasia with no instances of carcinoma arising within a sanguinaria-associated leukoplakia. A single case of oral squamous cell carcinoma that was confluent with a sanguinaria-associated leucoplakia has been reported. Using sanguinarine-containing products increases the risk for leukoplakia development 10 fold, which is significantly higher than the 2.5 times risk of leukoplakia reported from tobacco smoking [142], [143]. The majority of sanguinaria related leukoplakias appear not to resolve even after Viadent is discontinued suggesting the possibility of a permanent alteration in the genome of epithelial cell lineages.

Based on evidence from molecular profiling, sanguinaria-related leukoplakias appear to lie between benign and dysplastic keratoses. With p53 expression, p16, proliferating cell nuclear antigen (PCNA) and cyclin D1 levels being intermediate between the two [144]. While dysplastic biopsy samples analyzed in this study were over 20 years old and this has been associated with reduced immunohistochemistry reactivity [145], another study by Eversole et al. utilizing archived pathology specimens showed similar results. In a further study PCNA levels were elevated in sanguinaria-associated leukoplakia compared to benign keratoses, while lower than those in dysplastic lesions [141]. In this analysis, whilst sanguinaria-associated leukoplakias did not display an elevation of total DNA content, 1.5% of their cell population were aneuploid, compared to 3.5% for dysplasias, and zero for benign keratoses. The authors recommended that sanguinaria-associated leukoplakia be classified as

mildly dysplastic lesions of uncertain malignant potential.

Several studies have sought to determine the mechanism by which sanguinarine may have a carcinogenic effect on oral mucosa. Sanguinarine's chemical structure shows considerable homology to polycyclic aromatic hydrocarbons (PAHs) [144]. Many PAHs undergo metabolic activation by cytochrome P450 to form mutagenic and carcinogenic compounds [146], or activate the aryl hydrocarbon receptor (AhR) causing an upregulation of AhR responsive genes that include the protocarcinogen activating enzymes CYP1A1 and CYP1B1 [147]. There has therefore been interest in determining the interactions of sanguinarine, cytochrome P450 enzymes and the AhR.

A study published in 2005 by Karp et al. suggested that sanguinarine may exert a carcinogenic effect by activating the aryl hydrocarbon receptor (AhR) in oral human keratinocytes [148]. These results were however challenged due to the inconsistency of findings and experimental control failures [149]. A subsequent study found that sanguinarine at 1μM did not alter CYP1A1 mRNA or protein expression (key features of AhR activation) in human hepatoma cells, contrary to the proposed sanguinarine AhR carcinogenesis mechanism [67]. Sanguinarine also failed to induce AhR in H4IIE.luc rat hepatoma cells at a concentration of 1μM when incubated for 48 h [150].

While sanguinarine does not appear to activate AhR, it may be metabolized to a carcinogenic compound by cytochrome P450 enzymes. CYP1A induction reduces sanguinarine *in vitro* rat hepatocyte and human hepG2 cytotoxicity, suggesting CYP1A converts sanguinarine into a less cytotoxic metabolite [151]. This reduced cytotoxic metabolite, may however have greater

genotoxicity as evidenced by rat DNA adduct levels increasing with higher microsomal cytochrome P450 sanguinarine activation [64]. Sanguinarine incubated with human liver microsomes and NADPH for 150 min failed to generate HPLC detectable metabolites [151]. Serum free human hepatocyte cell cultures exposed to sanguinarine and analyzed with electrospray ionization quadrupole time-of-flight mass spectrometry, did detect and unambiguously identify new metabolites. However, their carcinogenic potential is unknown [78]. Currently the molecular mechanism by which *Sanguinaria* containing dental products cause oral leukoplakia has not been determined.

4.2 Sanguinarine Associated Gallbladder Carcinoma

The northern Indian provinces of Uttar Pradesh and Bihar have a high incidence of gallbladder carcinoma, the disease being the most common biliary tract malignancy in these regions [152]. Indeed the highest gallbladder cancer incidence rates in the world of 21.5 per 100,000 are to be found in Indian women [153].

A number of etiological factors have been investigated in an effort to determine the cause for these increased rates. Up to 95% of gallbladder cancers have been associated with gallstones [154], with patients having gallstones >3cm carrying a 10-fold higher risk of gallbladder cancer [155], [156]. However, gallbladder carcinoma prevalence does not always correlate with cholelithiasis. In the developed world, 10% of the population has gallstones yet gallbladder carcinoma only accounts for 0.5% of all malignancies [157]. Chronic inflammation is another possibility with chronic carriers of *Salmonella typhi* infection having an 8-fold increased risk of gallbladder cancer [158].

Environmental carcinogen exposure has also been explored. Northern Indian regions are transected by the river Ganges, which provides the main source of irrigation and drinking water. Carcinogen exposure from the water supply, as Gangetic waters receive industrial effluents and untreated domestic sewage has been suggested as a possible cause for elevated gallbladder carcinoma rates [159]. Dietary carcinogen exposure has also been investigated [159]. Repeatedly boiled sunflower oil, compared to single boiled and fresh sunflower oil has a significantly increased polycyclic aromatic hydrocarbon (PAH) content [160]. PAHs are known mutagens and can be carcinogenic. Heating vegetable oil at high temperatures also results in the formation of toxic compounds [161], [162], [163].

Argemone mexicana Linn, a herb originally of West Indies origin, grows extensively in sub-tropical and tropical countries being abundant on roadsides and in wastelands, with its seeds and oil closely resembling that of mustard seeds allowing their substitution [164]. Argemone oil contains sanguinarine, with a content varying between 0.044 to 0.5% [165]. Sanguinarine accounts for 5% of argemone seed oil alkaloids, dihydrosanguinarine 87%, the remainder composed of chelerythrine, protopine and berberine [166]. Mustard oil (obtained from *Brassica* species including *B. rapa* (syn. *campestris*) and *B. nigra*) is used as a frying and cooking medium in North India [167], [168]. Unscrupulous traders, for economic gain, often adulterate mustard oil with cheap argemone oil obtained from *Argemone mexicana* [169], [170].

In those with gallbladder carcinoma the gallbladder sanguinarine concentration was 195.18 ng/mg while in those who had cholelithiasis it was 24.05 ng/mg. Blood sanguinarine levels

were also reported as elevated in those with gallbladder carcinoma compared to cholelithiasis being 230.96 ng/ml and 14.01ng/ml respectively [13]. While suggesting a causative link between sanguinarine tissue exposure and gallbladder carcinoma, the capillary gas chromatography method used by the authors is unlikely to assess sanguinarine concentration accurately. Unfortunately, no analytical data were supplied to assist with the scrutiny of this result. However, with sanguinarines ability to induce DNA damage and reactive oxygen species it may potentially be a contributing factor in gallbladder carcinogenesis [171].

5. Current Sanguinarine Carcinogen Status and Future Directions

The carcinogenic risk of sanguinarine has not been definitively determined due to its conflicting test results as represented in Figure 2. Sanguinarine has not been included in carcinogen databases, such as the IARC monographs (http://monographs.iarc.fr/ENG/Classification/index.php), the International Workshop on Genotoxicity Testing UDS List [172], the 1,547 chemicals of the Carcinogenic Potency Project Database (CPPD) (http://toxnet.nlm.nih.gov/cpdb/), and the 2,300 chemicals of the US National Toxicology Program (http://www.predictive-toxicology.org/data/ntp/original_ntp_data.txt). Further toxicology work is required to clarify its status.

Figure 2: Summary of Current Sanguinarine Carcinogenic Risk Assessment

(see end of document)

A mode of action approach is used to determine a chemical's intrinsic genotoxic properties [173] by taking all of the available genotoxicity information into account, combining it with pharmacokinetic, structure activity, ADME (absorption, distribution, metabolism and excretion) data and other biological responses to determine the risk a chemical may pose to humans. The mode of action approach assesses whether positive genotoxicity results pose a risk for human health. Currently this has not been performed for sanguinarine [174].

While the majority of genotoxicity testing has assessed compound-induced genetic damage in blood and liver cells, there is growing interest in using skin for genotoxicity testing [175], [176], [177]. This alternative is especially relevant for sanguinarine, since skin is the main target organ for black salve. The tissues used for risk assessment should have the highest toxicant exposure levels or be a site of biological action. Several skin genotoxicity assessment methods have been developed including the rodent skin *in vivo* MN assay [175], ex vivo human skin [178] and human reconstructed epidermal models [179].

Skin is the largest body organ and acts as a toxin barrier. Epidermal cells from discarded neonatal foreskin have been found to express 13 of 15 CYP1-4 gene mRNA at detectable levels [180]. CYP skin metabolism can inactivate reactive compounds having a protective role [181], [182] but can also activate procarcinogens potentially leading to toxicity and skin cancer [183], [146]. It is currently unknown whether sanguinarine interacts with cytochrome p450 metabolism in human skin.

The PAH benzo[a]pyrene (BP), which has a chemical structure similar to sanguinarine, is not toxic itself but is metabolized into mutagenic and carcinogenic metabolites. BP provides a

586 useful test compound to assess the metabolic capability of various skin genotoxicity models.
587 *Ex-vivo* human skin, cultured normal human keratinocytes and 3D human skin constructs
588 generate all the major BP derived metabolites in sufficient quantity to induce significant DNA
589 damage present in the alkaline comet assay [184]. Constructs are however not metabolically
590 equivalent to normal skin, as they are insufficient for cyclophosphamide metabolic activation
591 [185] with some constructs found to have lower basal CYP expression levels compared to
592 human skin biopsies [186].

593 In March 2009 the European Union imposed a ban on the *in vivo* genotoxicity testing of
594 cosmetics ingredients [187] which stimulated the development of animal free methods for
595 assessing toxicity. A multilayered human skin construct (EpiDerm) has been developed based
596 on differentiated foreskin-derived epidermal keratinocytes, that allows both basal and apical
597 compound exposure to mimic topical therapy application [188]. This construct has metabolic
598 gene expression similar to that in human skin, with Phase II enzymes more pronounced than
599 Phase I enzymes [186]. Micronucleus [188] and comet assay [189] protocols have been
600 developed and are undergoing validity testing. The construct has a low background frequency
601 of MN and detects lower genotoxin induced MN levels than the Japanese rodent skin MN
602 assay suggesting it may be the preferred method for testing sanguinarine [190]. False positive
603 results often generated by significantly cytotoxic compounds and those that induce oxidative
604 stress in other assays are less likely to occur [191].

605 Fresh *ex-vivo* human skin obtained as excess tissue from cosmetic surgery has provided
606 another model for genotoxicity testing. Against a range of true negative, misleading positive

607 and true positive genotoxins it was found to have a sensitivity, specificity and accuracy of
608 89%, 90% and 89% respectively [192]. Human skin has some advantage over reconstructed
609 epidermal models as all skin cell types are present and the stratum corneum has a normal
610 function [193] with comparable physiological permeability [194], [195]. To date sanguinarine
611 and black salve have not been assessed using skin genotoxicity models.

612 At present, ADME factors cannot be determined for black salve, largely due to a lack of
613 compositional data. Despite toxicity concerns, there has only been a single report in the
614 literature that analyzed black salve constituents without determining compound
615 concentrations [196]. According to ICH guidelines exposure to genotoxins or their
616 degradation products must be limited to 1.5μg/day in order to minimize the risk of
617 carcinogenicity.

618 Current genotoxicity testing strategies were developed primarily for assessing single
619 chemicals, and so applying these strategies to herbal therapies that contain multiple bioactive
620 compounds has been challenging. According to current EU herbal product guidelines (EMEA
621 2007), the Ames test is the primary endpoint for genotoxicity testing. Compounds that test
622 negative are accepted as probably non-genotoxic [197]. As discussed, the Ames test as
623 discussed fails to detect a number of genotoxins with a sensitivity of 60%, while its
624 specificity of 77% indicates a number of compounds will yield false positive results [198].
625 The lack of regulatory rigor in natural product genotoxicity testing is of ongoing concern, as
626 some of the most potent carcinogens known are natural products [199]. Since sanguinarine
627 containing black salve is currently in clinical use, accurately determining its carcinogenic

potential should be a matter of some urgency.

6. Conclusion

Currently we do not know whether sanguinarine or products that contain it are carcinogenic. This is surprising, as sanguinarine has been the subject of a significant body of scientific investigation. Sanguinarine shares molecular mechanisms of action with known carcinogens such as UVA and intercalating DNA agents, structurally resembles PAHs - a chemical class containing carcinogens, has positive *in vitro* and *in vivo* genotoxicity results, has cancer promoter action in a murine model, has a causative role in the development of mouthwash induced human leukoplakia and a suspected role in gallbladder carcinogenesis. Despite these findings other contradictory *in vitro* and *in vivo* results have prevented the carcinogenic classification of sanguinarine.

As patients are presently using *S. canadensis* containing topical therapies in areas of UV induced field cancerization, urgent research is needed to determine the carcinogen status of sanguinarine and assess the level of risk. If carcinogenic, patients using black salve may develop subsequent malignancies. If mutagenic, black salve may increase the malignancy of existing skin cancers, making them more invasive and treatment resistant. In this situation, as skin is the target organ of interest, assessing sanguinarine genotoxicity in a bio-equivalent human skin *ex vivo* model may provide the most relevant and accurate assessment of its carcinogenic risk.

Declaration of Interest: The authors report no conflicts of interest.

References

1. Efferth, T. and B. Kaina, *Toxicities by herbal medicines with emphasis to traditional Chinese medicine.* Curr Drug Metab, 2011. **12**(10): p. 989-96.

2. Byard, R.W., *A review of the potential forensic significance of traditional herbal medicines.* Journal of forensic sciences, 2010. **55**(1): p. 89-92.

3. Singer, B. and D. Grunberger, *Molecular biology of mutagens and carcinogens*. 2012: Springer Science & Business Media.

4. Bent, S. and R. Ko, *Commonly used herbal medicines in the United States: a review.* The American journal of medicine, 2004. **116**(7): p. 478-485.

5. Mancebo, S.E. and S.Q. Wang, *Skin cancer: role of ultraviolet radiation in carcinogenesis.* Reviews on environmental health, 2014. **29**(3): p. 265-273.

6. Braakhuis, B.J., et al., *A genetic explanation of Slaughter's concept of field cancerization evidence and clinical implications.* Cancer research, 2003. **63**(8): p. 1727-1730.

7. Croaker, A., et al., *A Review of Black Salve: Cancer Specificity, Cure, and Cosmesis.* Evidence-Based Complementary and Alternative Medicine, 2017. **2017**: p. 11.

8. Sivyer, G.W. and C. Rosendahl, *Application of black salve to a thin melanoma that subsequently progressed to metastatic melanoma: a case study.* Dermatol Pract Concept, 2014. **4**(3): p. 77-80.

9. Ma, L.Q., J.W. Dharamsi, and T. Vandergriff, *Black Salve as Self-Treatment for Cutaneous Squamous Cell Carcinoma.* Dermatitis, 2012. **23**(5): p. 239-240.

10. Laub, D.R., Jr., *Death from metastatic basal cell carcinoma: herbal remedy or just unlucky?* J Plast Reconstr Aesthet Surg, 2008. **61**(7): p. 846-8.

11. Croaker, A., et al., *Sanguinaria canadensis: Traditional Medicine, Phytochemical Composition, Biological Activities and Current Uses.* International Journal of Molecular Sciences, 2016. **17**(9): p. 1414.

12. Damm, D.D., et al., *Leukoplakia of the maxillary vestibule - an association with Viadent?* Oral Surgery Oral Medicine Oral Pathology Oral Radiology and Endodontics, 1999. **87**(1): p. 61-66.

13. Dixit, R., et al., *Association of mustard oil as cooking media with carcinoma of the gallbladder.* J Gastrointest Cancer, 2013. **44**(2): p. 177-81.

14. Kaminskyy, V., O. Kulachkovskyy, and R. Stoika, *A decisive role of mitochondria in*

defining rate and intensity of apoptosis induction by different alkaloids. Toxicology Letters, 2008. **177**(3): p. 168-181.

15. Kevekordes, S., et al., *Micronucleus formation in human lymphocytes and in the metabolically competent human hepatoma cell line hep-G2: Results with 15 naturally occurring substances.* Anticancer Research, 2001. **21**(1A): p. 461-469.

16. Ansari, K.M. and M. Das, *Potentiation of tumour promotion by topical application of argemone oil/isolated sanguinarine alkaloid in a model of mouse skin carcinogenesis.* Chemico-Biological Interactions, 2010. **188**(3): p. 591-597.

17. Ahsan, H., et al., *Protective effect of sanguinarine on ultraviolet B-mediated damages in SKH-1 hairless mouse skin: Implications for prevention of skin cancer.* Photochemistry and Photobiology, 2007. **83**(4): p. 986-993.

18. Maiti, M., et al., *Influence of DNA structures on the conversion of sanguinarine alkanolamine form to iminium form.* Journal of Biomolecular Structure & Dynamics, 2002. **20**(3): p. 455-464.

19. Bajaj, N.P., et al., *Sequence-selective, pH-dependent binding to DNA of benzophenanthridine alkaloids.* J Mol Recognit, 1990. **3**(1): p. 48-54.

20. Adhikari, A., et al., *Energetics of the binding of phototoxic and cytotoxic plant alkaloid sanguinarine to DNA: Isothermal titration calorimetric studies.* Journal of Molecular Structure, 2008. **889**(1-3): p. 54-63.

21. Li, N., et al., *Interaction of anticancer drug mitoxantrone with DNA analyzed by electrochemical and spectroscopic methods.* Biophysical chemistry, 2005. **116**(3): p. 199-205.

22. Kaminskyy, V.O., M.D. Lootsik, and R.S. Stoika, *Correlation of the cytotoxic activity of four different alkaloids, from Chelidonium majus (greater celandine), with their DNA intercalating properties and ability to induce breaks in the DNA of NK/Ly murine lymphoma cells.* Central European Journal of Biology, 2006. **1**(1): p. 2-15.

23. Hammerova, J., et al., *Benzo [c] phenanthridine alkaloids exhibit strong anti-proliferative activity in malignant melanoma cells regardless of their p53 status.* Journal of Dermatological Science, 2011. **62**(1): p. 22-35.

24. Ferguson, L.R. and W.A. Denny, *Genotoxicity of non-covalent interactions: DNA intercalators.* Mutat Res, 2007. **623**(1-2): p. 14-23.

25. Secker-Walker, L., et al., *Secondary acute leukemia and myelodysplastic syndrome with 11q23 abnormalities. EU Concerted Action 11q23 Workshop.* Leukemia, 1998. **12**(5): p. 840-844.

715 26. Felix, C.A., *Leukemias related to treatment with DNA topoisomerase II inhibitors*.*
716 Medical and pediatric oncology, 2001. **36**(5): p. 525-535.

717 27. Maiti, M., R. Nandi, and K. Chaudhuri, *Sanguinarine: a monofunctional intercalating*
718 *alkaloid.* FEBS Lett, 1982. **142**(2): p. 280-4.

719 28. Bai, L.P., et al., *DNA-binding affinities and sequence selectivity of quaternary*
720 *benzophenanthridine alkaloids sanguinarine, chelerythrine, and nitidine.* Bioorg Med
721 Chem, 2006. **14**(16): p. 5439-45.

722 29. Byrn, S.R. and G.D. Dolch, *Analysis of binding of daunorubicin and doxorubicin to DNA*
723 *using computerized curve‐fitting procedures.* Journal of pharmaceutical sciences, 1978.
724 **67**(5): p. 688-693.

725 30. Messori, L., et al., *Solution chemistry and DNA binding properties of MEN 10755, a*
726 *novel disaccharide analogue of doxorubicin.* Bioorganic & medicinal chemistry, 2001.
727 **9**(7): p. 1815-1825.

728 31. Halliwell, B., *Free radicals and antioxidants – quo vadis?* Trends in Pharmacological
729 Sciences, 2011. **32**(3): p. 125-130.

730 32. Kehrer, J.P. and L.-O. Klotz, *Free radicals and related reactive species as mediators of*
731 *tissue injury and disease: implications for Health.* Critical reviews in toxicology, 2015.
732 **45**(9): p. 765-798.

733 33. Klaunig, J.E. and L.M. Kamendulis, *The role of oxidative stress in carcinogenesis.* Annu.
734 Rev. Pharmacol. Toxicol., 2004. **44**: p. 239-267.

735 34. Kvam, E. and R.M. Tyrrell, *Induction of oxidative DNA base damage in human skin cells*
736 *by UV and near visible radiation.* Carcinogenesis, 1997. **18**(12): p. 2379-2384.

737 35. Scharffetter-Kochanek, K., et al., *UV-induced reactive oxygen species in*
738 *photocarcinogenesis and photoaging.* Biological chemistry, 1997. **378**(11): p. 1247.

739 36. de Gruijl, F.R., *Photocarcinogenesis: UVA vs. UVB Radiation.* Skin Pharmacology and
740 Applied Skin Physiology, 2002. **15**(5): p. 316.

741 37. Pallichankandy, S., et al., *ROS-dependent activation of autophagy is a critical*
742 *mechanism for the induction of anti-glioma effect of sanguinarine.* Free Radic Biol Med,
743 2015. **89**: p. 708-20.

744 38. Han, M.H., et al., *Apoptosis induction of human bladder cancer cells by sanguinarine*
745 *through reactive oxygen species-mediated up-regulation of early growth response gene-1.*
746 PLoS One, 2013. **8**(5): p. e63425.

747 39. Wang, Y., et al., *Noninvasive bioluminescence imaging of the dynamics of sanguinarine*

induced apoptosis via activation of reactive oxygen species. Oncotarget, 2016. **7**(16): p. 22355-67.

40. Matkar, S.S., L.A. Wrischnik, and U. Hellmann-Blumberg, *Production of hydrogen peroxide and redox cycling can explain how sanguinarine and chelerythrine induce rapid apoptosis.* Archives of Biochemistry and Biophysics, 2008. **477**(1): p. 43-52.

41. Price, D.A., et al., *Physicochemical drug properties associated with in vivo toxicological outcomes: a review.* Expert Opinion on Drug Metabolism and Toxicology, 2009. **5**(8): p. 921-931.

42. Matkar, S.S., *Mechanism of action of potential anticancer drugs*. 2008, ProQuest Dissertations Publishing.

43. Gu, S., et al., *Sanguinarine-induced apoptosis in lung adenocarcinoma cells is dependent on reactive oxygen species production and endoplasmic reticulum stress.* Oncology Reports, 2015. **34**(2): p. 913-919.

44. Malhotra, J.D. and R.J. Kaufman. *The endoplasmic reticulum and the unfolded protein response*. in *Seminars in cell & developmental biology*. 2007. Elsevier.

45. Malhotra, J.D. and R.J. Kaufman, *ER stress and its functional link to mitochondria: role in cell survival and death.* Cold Spring Harbor perspectives in biology, 2011. **3**(9): p. a004424.

46. Sanchez-Lopez, E., et al., *Choline kinase inhibition induces exacerbated endoplasmic reticulum stress and triggers apoptosis via CHOP in cancer cells.* Cell death & disease, 2013. **4**: p. e933.

47. Malhotra, J.D. and R.J. Kaufman, *Endoplasmic reticulum stress and oxidative stress: a vicious cycle or a double-edged sword?* Antioxidants & redox signaling, 2007. **9**(12): p. 2277-2294.

48. Huh, J., et al., *Cyclooxygenase 2 rescues LNCaP prostate cancer cells from sanguinarine-induced apoptosis by a mechanism involving inhibition of nitric oxide synthase activity.* Cancer Research, 2006. **66**(7): p. 3726-3736.

49. Wang, Q.i., et al., *Expression of inducible nitric oxide synthase in paired neoplastic and non-neoplastic primary prostate cell cultures and prostatectomy specimen.* Urologic Oncology: Seminars and Original Investigations, 2003. **21**(2): p. 117-122.

50. Baudouin, J.E. and P. Tachon, *Constitutive Nitric Oxide Synthase Is Present in Normal Human Keratinocytes.* Journal of Investigative Dermatology, 1996. **106**(3): p. 428-431.

51. Heck, D.E., et al., *Epidermal growth factor suppresses nitric oxide and hydrogen peroxide production by keratinocytes. Potential role for nitric oxide in the regulation of*

wound healing. Journal of Biological Chemistry, 1992. **267**(30): p. 21277.

52. Wang, R., et al., *Human dermal fibroblasts produce nitric oxide and express both constitutive and inducible nitric oxide synthase isoforms.* Journal of investigative dermatology, 1996. **106**(3): p. 419-427.

53. Shindo, Y., et al., *Enzymic and non-enzymic antioxidants in epidermis and dermis of human skin.* Journal of Investigative Dermatology, 1994. **102**(1): p. 122-124.

54. Rhie, G., et al., *Aging- and photoaging-dependent changes of enzymic and nonenzymic antioxidants in the epidermis and dermis of human skin in vivo.* The Journal of investigative dermatology, 2001. **117**(5): p. 1212-1217.

55. Ulrichova, J., et al., *Cytotoxicity of natural compounds in hepatocyte cell culture models. The case of quaternary benzo[c]phenanthridine alkaloids.* Toxicol Lett, 2001. **125**(1-3): p. 125-32.

56. Debiton, E., et al., *Sanguinarine-induced apoptosis is associated with an early and severe cellular glutathione depletion.* Cancer Chemotherapy and Pharmacology, 2003. **51**(6): p. 474-482.

57. Walterova, D., et al., *Inhibition of liver alanine aminotransferase activity by some benzophenanthridine alkaloids.* J Med Chem, 1981. **24**(9): p. 1100-3.

58. Matkar, S.S., L.A. Wrischnik, and U. Hellmann-Blumberg, *Sanguinarine causes DNA damage and p53-independent cell death in human colon cancer cell lines.* Chemico-Biological Interactions, 2008. **172**(1): p. 63-71.

59. Fiers, W., et al., *More than one way to die: apoptosis, necrosis and reactive oxygen damage.* Oncogene, 1999. **18**(54): p. 7719-7730.

60. Marnett, L.J., *Oxyradicals and DNA damage.* Carcinogenesis, 2000. **21**(3): p. 361-370.

61. Cooke, M.S., et al., *Oxidative DNA damage: mechanisms, mutation, and disease.* The FASEB Journal, 2003. **17**(10): p. 1195-1214.

62. Dreher, D. and A.F. Junod, *Role of oxygen free radicals in cancer development.* European Journal of cancer, 1996. **32**(1): p. 30-38.

63. Cerutti, P.A., *Prooxidant states and tumor promotion.* Science, 1985. **227**(4685): p. 375-381.

64. Stiborova, M., et al., *DNA adduct formation from quaternary benzo [c] phenanthridine alkaloids sanguinarine and chelerythrine as revealed by the P-32-postlabeling technique.* Chemico-Biological Interactions, 2002. **140**(3): p. 231-242.

65. Lloyd, D.R., D.H. Phillips, and P.L. Carmichael, *Generation of putative intrastrand cross-links and strand breaks in DNA by transition metal ion-mediated oxygen radical attack.* Chemical research in toxicology, 1997. **10**(4): p. 393-400.

66. Basu, A.K., et al., *Identification of adducts formed by reaction of guanine nucleosides with malondialdehyde and structurally related aldehydes.* Chemical research in toxicology, 1988. **1**(1): p. 53-59.

67. Zdarilova, A., et al., *Investigation of sanguinarine and chelerythrine effects on CYP1A1 expression and activity in human hepatoma cells.* Food and Chemical Toxicology, 2006. **44**(2): p. 242-249.

68. Kosina, P., et al., *Sanguinarine and chelerythrine: assessment of safety on pigs in ninety days feeding experiment.* Food and Chemical Toxicology, 2004. **42**(1): p. 85-91.

69. Jarabek, A.M., et al., *Creating context for the use of DNA adduct data in cancer risk assessment: I. Data organization.* Critical reviews in toxicology, 2009.

70. Himmelstein, M.W., et al., *Creating context for the use of DNA adduct data in cancer risk assessment: II. Overview of methods of identification and quantitation of DNA damage.* Critical reviews in toxicology, 2009. **39**(8): p. 679-694.

71. Swenberg, J.A., et al., *Biomarkers in toxicology and risk assessment: informing critical dose–response relationships.* Chemical research in toxicology, 2007. **21**(1): p. 253-265.

72. Poirier, M.C. and F.A. Beland, *DNA adduct measurements and tumor incidence during chronic carcinogen exposure in animal models: implications for DNA adduct-based human cancer risk assessment.* Chemical research in toxicology, 1992. **5**(6): p. 749-755.

73. Williams, M.K., S. Dalvi, and R.R. Dalvi, *Influence of 3-methylcholanthrene pretreatment on sanguinarine toxicity in mice.* Veterinary and Human Toxicology, 2000. **42**(4): p. 196-198.

74. Pelkonen, O. and D.W. Nebert, *Metabolism of polycyclic aromatic hydrocarbons: etiologic role in carcinogenesis.* Pharmacological Reviews, 1982. **34**(2): p. 189-222.

75. Denison, M.S. and S. Heath-Pagliuso, *The Ah receptor: a regulator of the biochemical and toxicological actions of structurally diverse chemicals.* Bulletin of environmental contamination and toxicology, 1998. **61**(5): p. 557-568.

76. Kovář, J., et al., *Reduction of quaternary benzophenanthridine alkaloids by NADH and NADPH.* Collection of Czechoslovak chemical communications, 1986. **51**(11): p. 2626-2634.

77. Psotova, J., et al., *A liquid chromatographic-mass spectrometric evidence of dihydrosanguinarine as a first metabolite of sanguinarine transformation in rat.* Journal of

Chromatography B-Analytical Technologies in the Biomedical and Life Sciences, 2006. **830**(1): p. 165-172.

78. Kosina, P., et al., *Identification of benzo [c] phenanthridine metabolites in human hepatocytes by liquid chromatography with electrospray ion-trap and quadrupole time-of-flight mass spectrometry.* Journal of Chromatography B-Analytical Technologies in the Biomedical and Life Sciences, 2011. **879**(15-16): p. 1077-1085.

79. Vrublova, E., et al., *The toxicity and pharmacokinetics of dihydrosanguinarine in rat: a pilot study.* Food Chem Toxicol, 2008. **46**(7): p. 2546-53.

80. Sharma, G.G., et al., *hTERT associates with human telomeres and enhances genomic stability and DNA repair.* Oncogene, 2003. **22**(1): p. 131-146.

81. Noureini, S.K. and M. Wink, *Transcriptional down regulation of hTERT and senescence induction in HepG2 cells by chelidonine.* World J Gastroenterol, 2009. **15**(29): p. 3603-3610.

82. Kazemi, N., et al., *Investigation of Telomerase Activity and HTERT Gene Expression in MCF7 Cells Treated with Papaverine.* Journal of Sabzevar University of Medical Sciences, 2013. **20**(2): p. 121-132.

83. Bessi, I., et al., *Spectroscopic, molecular modeling, and NMR-spectroscopic investigation of the binding mode of the natural alkaloids berberine and sanguinarine to human telomeric G-quadruplex DNA.* ACS chemical biology, 2012. **7**(6): p. 1109-1119.

84. Shay, J. and S. Bacchetti, *A survey of telomerase activity in human cancer.* European journal of cancer, 1997. **33**(5): p. 787-791.

85. Jaiswal, M., et al., *Inflammatory cytokines induce DNA damage and inhibit DNA repair in cholangiocarcinoma cells by a nitric oxide-dependent mechanism.* Cancer research, 2000. **60**(1): p. 184-190.

86. Niu, X., et al., *The anti-inflammatory effects of sanguinarine and its modulation of inflammatory mediators from peritoneal macrophages.* Eur J Pharmacol, 2012. **689**(1-3): p. 262-9.

87. Niu, X., et al., *Protective effect of sanguinarine against acetic acid-induced ulcerative colitis in mice.* Toxicol Appl Pharmacol, 2013. **267**(3): p. 256-65.

88. Dvorak, Z., et al., *Differential effects of selected natural compounds with anti-inflammatory activity on the glucocorticoid receptor and NF-kappa B in HeLa cells.* Chemico-Biological Interactions, 2006. **159**(2): p. 117-128.

89. Leecy, T.N., et al., *Histopathological features associated with application of black salve to cutaneous lesions: a series of 16 cases and review of the literature.* Pathology, 2013.

45(7): p. 670-674.

90. Lindelöf, B., et al., *Incidence of skin cancer in 5356 patients following organ transplantation.* British Journal of Dermatology, 2000. **143**(3): p. 513-519.

91. Parrish, J.A., *Immunosuppression, Skin Cancer, and Ultraviolet A Radiation.* The New England Journal of Medicine, 2005. **353**(25): p. 2712-2713.

92. Yoshikawa, T., et al., *Susceptibility to effects of UVB radiation on induction of contact hypersensitivity as a risk factor for skin cancer in humans.* Journal of Investigative Dermatology, 1990. **95**(5): p. 530-536.

93. Czarnecki, D., et al., *Impaired cell‐mediated immunity of apparently normal patients who had multiple skin cancers.* Cancer, 1995. **76**(2): p. 228-231.

94. Prescott, S.M., et al., *Platelet-activating factor and related lipid mediators.* Annual review of biochemistry, 2000. **69**(1): p. 419-445.

95. Lin, L.-L., et al., *cPLA 2 is phosphorylated and activated by MAP kinase.* Cell, 1993. **72**(2): p. 269-278.

96. *EEC Notes for Guidance for the Testing of Medicinal Products for their Mutagenic Potential.* Offi. J. Eur. Commun. , 1987. **L73**.

97. CPMP/ICH, *Note for guidance on genotoxicity: Guidance on Specific Aspects of Regulatory Genotoxicity Test for Pharmaceuticals.* International Conference of Harmonisation, 1995. **Topic S2A CPMP/ICH/141/95**.

98. Frankos, V.H., et al., *Safety of Sanguinaria extract as used in commercial toothpaste and oral rinse products.* J Can Dent Assoc, 1990. **56**(7 Suppl): p. 41-7.

99. Munro, I.C., et al., *Viadent usage and oral leukoplakia: a spurious association.* Regul Toxicol Pharmacol, 1999. **30**(3): p. 182-96.

100. Kevekordes, S., et al., *SOS induction of selected naturally occurring substances in Escherichia coli (SOS chromotest).* Mutation Research-Genetic Toxicology and Environmental Mutagenesis, 1999. **445**(1): p. 81-91.

101. Quillardet, P. and M. Hofnung, *The SOS chromotest: a review*. 1993, Elsevier B.V. p. 235-279.

102. Kaminskyy, V., et al., *Differential effect of sanguinarine, chelerythrine and chelidonine on DNA damage and cell viability in primary mouse spleen cells and mouse leukemic cells.* Cell Biology International, 2008. **32**(2): p. 271-277.

103. Malikova, J., et al., *The effect of chelerythrine on cell growth, apoptosis, and cell*

cycle in human normal and cancer cells in comparison with sanguinarine. Cell Biology and Toxicology, 2006. **22**(6): p. 439-453.

104. Philchenkov, A., et al., *Apoptogenic activity of two benzophenanthridine alkaloids from Chelidonium majus L. does not correlate with their DNA damaging effects.* Toxicol In Vitro, 2008. **22**(2): p. 287-95.

105. Malikova, J., A. Zdarilova, and A. Hlobilkova, *Effects of sanguinarine and chelerythrine on the cell cycle and apoptosis.* Biomed Pap Med Fac Univ Palacky Olomouc Czech Repub, 2006. **150**(1): p. 5-12.

106. Brendler-Schwaab, S., et al., *The in vivo comet assay: use and status in genotoxicity testing.* Mutagenesis, 2005. **20**(4): p. 245-254.

107. Ahmad, N., et al., *Differential antiproliferative and apoptotic response of sanguinarine for cancer cells versus normal cells.* Clinical Cancer Research, 2000. **6**(4): p. 1524-1528.

108. Slaninova, I., et al., *Antitumour activities of sanguinarine and related alkaloids.* Phytochemistry Reviews, 2014. **13**(1): p. 51-68.

109. Williams, G., et al., *Summary report on the performance of the assays for DNA damage.* Progress in Mutation Research, 1985. **5**: p. 59-67.

110. Natarajan, A. and F. Darroudi, *Use of human hepatoma cells for in vitro metabolic activation of chemical mutagens/carcinogens.* Mutagenesis, 1991. **6**(5): p. 399-403.

111. MacGregor, J.T., et al., *IWGT report on quantitative approaches to genotoxicity risk assessment II. Use of point-of-departure (PoD) metrics in defining acceptable exposure limits and assessing human risk.* Mutation Research/Genetic Toxicology and Environmental Mutagenesis, 2015. **783**: p. 66-78.

112. Ku, W.W., et al., *Strategy for genotoxicity testing—metabolic considerations.* Mutation Research/Genetic Toxicology and Environmental Mutagenesis, 2007. **627**(1): p. 59-77.

113. Xu, C., C.Y.-T. Li, and A.-N.T. Kong, *Induction of phase I, II and III drug metabolism/transport by xenobiotics.* Archives of pharmacal research, 2005. **28**(3): p. 249.

114. Kirkland, D., et al., *Evaluation of the ability of a battery of three in vitro genotoxicity tests to discriminate rodent carcinogens and non-carcinogens: I. Sensitivity, specificity and relative predictivity.* Mutation Research/Genetic Toxicology and Environmental Mutagenesis, 2005. **584**(1): p. 1-256.

115. Matthews, E.J., et al., *An analysis of genetic toxicity, reproductive and developmental toxicity, and carcinogenicity data: I. Identification of carcinogens using surrogate endpoints.* Regulatory Toxicology and Pharmacology, 2006. **44**(2): p. 83-96.

116. Matthews, E.J., et al., *An analysis of genetic toxicity, reproductive and developmental toxicity, and carcinogenicity data: II. Identification of genotoxicants, reprotoxicants, and carcinogens using in silico methods.* Regulatory Toxicology and Pharmacology, 2006. **44**(2): p. 97-110.

117. COM (United Kingdom Committee on Mutagenicity of Chemicals in Food, C.P.a.t.E., *Guidance on a Strategy for Testing of Chemicals for Mutagenicity.* 2000.

118. FDA, *Guidance for Industry and Review Staff Recommended Approaches to Integration of Genetic Toxicology Study Results.* 2006.

119. Hartmann, A., et al., *Recommendations for conducting the in vivo alkaline Comet assay.* Mutagenesis, 2003. **18**(1): p. 45-51.

120. Uno, Y., et al., *JaCVAM-organized international validation study of the in vivo rodent alkaline comet assay for detection of genotoxic carcinogens: II. Summary of definitive validation study results.* Mutation Research/Genetic Toxicology and Environmental Mutagenesis, 2015. **786**: p. 45-76.

121. Ansari, K.M., et al., *In vivo DNA damaging potential of sanguinarine alkaloid, isolated from argemone oil, using alkaline Comet assay in mice.* Food and Chemical Toxicology, 2005. **43**(1): p. 147-153.

122. Ansari, K.M., et al., *Unequivocal evidence of genotoxic potential of argemone oil in mice.* Int J Cancer, 2004. **112**(5): p. 890-5.

123. Das, M., et al., *Correlation of DNA damage in epidemic dropsy patients to carcinogenic potential of argemone oil and isolated sanguinarine alkaloid in mice.* International Journal of Cancer, 2005. **117**(5): p. 709-717.

124. Vichkanova, S., et al., *Sanguiritrin-novyi lekarstvenyi preparat protivomikrobnogo deistviya.* Khim-Farm Zh, 1982. **16**: p. 1515-20.

125. Stiborova, M., et al., *Macleaya cordata extract and Sangrovit genotoxicity. Assessment in vivo.* Biomed Pap Med Fac Univ Palacky Olomouc Czech Repub, 2008. **152**(1): p. 35-9.

126. Soeteman-Hernández, L.G., G.E. Johnson, and W. Slob, *Estimating the carcinogenic potency of chemicals from the in vivo micronucleus test.* Mutagenesis, 2015: p. gev043.

127. Krishna, G. and M. Hayashi, *In vivo rodent micronucleus assay: protocol, conduct and data interpretation.* Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis, 2000. **455**(1): p. 155-166.

128. Das, A., A. Mukherjee, and J. Chakrabarti, *Sanguinarine: an evaluation of in vivo cytogenetic activity.* Mutation Research-Genetic Toxicology and Environmental

Mutagenesis, 2004. **563**(1): p. 81-87.

129. Latt, S.A., et al., *Sister-chromatid exchanges: a report of the GENE-TOX program.* Mutation Research/Reviews in Genetic Toxicology, 1981. **87**(1): p. 17-62.

130. Preston, R.J., et al., *Mammalian in vivo and in vitro cytogenetic assays: A report of the US EPA's Gene-Tox Program.* Mutation Research/Reviews in Genetic Toxicology, 1981. **87**(2): p. 143-188.

131. Besaratinia, A. and G.P. Pfeifer, *DNA damage and mutagenesis induced by polycyclic aromatic hydrocarbons.* The Carcinogenic Effects of Polycyclic Aromatic Hydrocarbons, 2005: p. 171-210.

132. Chen, H.-H., et al., *In vivo detection of ribozyme cleavage products and RNA structure by use of terminal transferase-dependent PCR*, in *Ribozymes and siRNA Protocols*. 2004, Springer. p. 109-123.

133. Olivier, M., et al., *TP53 mutation spectra and load: a tool for generating hypotheses on the etiology of cancer.* IARC scientific publications, 2003(157): p. 247-270.

134. Lang, P.L., *Spontaneous neoplastic lesions and selected non-neoplastic lesions in the Crl: CD BR rat.* Charles River Laboratories, 1992: p. 1-36.

135. Mascarenhas, A.K., C.M. Allen, and M.L. Moeschberger, *The association between viadent (R) use and oral leukoplakia - Results of a matched case-control study.* Journal of Public Health Dentistry, 2002. **62**(3): p. 158-162.

136. Karjalainen, K., et al., *Effects of sanguinaria extract on leucocytes and fibroblasts.* Proc Finn Dent Soc, 1988. **84**(3): p. 161-5.

137. Keller, K.A. and D.L. Meyer, *Reproductive and developmental toxicological evaluation of sanguinaria extract.* J Clin Dent, 1989. **1**(3): p. 59-66.

138. Palcanis, K., et al., *Longitudinal evaluation of the effect of sanguinarine on plaque and gingivitis.* General dentistry, 1989. **38**(1): p. 17-19.

139. Waldron, C.A. and W.G. Shafer, *Leukoplakia revisited. A clinicopathologic study 3256 oral leukoplakias.* Cancer, 1975. **36**(4): p. 1386-1392.

140. Weatherell, J.A., C. Robinson, and M. Rathbone, *The flow of saliva and its influence on the movement, deposition and removal of drugs administered to the oral cavity.* Drugs and the pharmaceutical sciences, 1996. **74**: p. 157-189.

141. Eversole, L.R., G.M. Eversole, and J. Kopcik, *Sanguinaria-associated oral leukoplakia: comparison with other benign and dysplastic leukoplakic lesions.* Oral Surg Oral Med Oral Pathol Oral Radiol Endod, 2000. **89**(4): p. 455-64.

142. Allen, C., J. Loudon, and A. Mascarenhas, *Sanguinaria-related leukoplakia: epidemiologic and clinicopathologic features of a recently described entity.* General dentistry, 2000. **49**(6): p. 608-614.

143. Allen, C.M., *Viadent-related leukoplakia--the tip of the iceberg?* Oral Surg Oral Med Oral Pathol Oral Radiol Endod, 1999. **87**(4): p. 393-4.

144. Anderson, K.M., et al., *Immunohistochemical assessment of Viadent-associated leukoplakia.* Oral Oncol, 2005. **41**(2): p. 200-7.

145. Wester, K., et al., *Paraffin section storage and immunohistochemistry: effects of time, temperature, fixation, and retrieval protocol with emphasis on p53 protein and MIB1 antigen.* Applied Immunohistochemistry & Molecular Morphology, 2000. **8**(1): p. 61-70.

146. Nebert, D.W. and D.W. Russell, *Clinical importance of the cytochromes P450.* The Lancet, 2002. **360**(9340): p. 1155-1162.

147. Nebert, D.W., et al., *Role of the aromatic hydrocarbon receptor and [Ah] gene battery in the oxidative stress response, cell cycle control, and apoptosis.* Biochemical pharmacology, 2000. **59**(1): p. 65-85.

148. Karp, J.M., et al., *Sanguinarine activates polycyclic aromatic hydrocarbon associated metabolic pathways in human oral keratinocytes and tissues.* Toxicology Letters, 2005. **158**(1): p. 50-60.

149. Dvorak, Z., et al., *Sanguinarine activates polycyclic aromatic hydrocarbon associated metabolic pathways in human oral keratinocytes and tissues.* Toxicology Letters, 2005. **158**(2): p. 164-165.

150. Dvořák, Z., et al., *Quaternary benzo [c] phenathridine alkaloids sanguinarine and chelerythrine do not affect transcriptional activity of aryl hydrocarbon receptor: Analyses in rat hepatoma cell line H4IIE. luc.* Food and chemical toxicology, 2006. **44**(9): p. 1466-1473.

151. Vrba, J., et al., *Involvement of cytochrome p450 1A in sanguinarine detoxication.* Toxicology Letters, 2004. **151**(2): p. 375-387.

152. Shukla, V., et al., *Primary carcinoma of the gall bladder: A review of a 16 - year period at the university hospital.* Journal of surgical oncology, 1985. **28**(1): p. 32-35.

153. Randi, G., S. Franceschi, and C. La Vecchia, *Gallbladder cancer worldwide: geographical distribution and risk factors.* International journal of cancer, 2006. **118**(7): p. 1591-1602.

154. Serra, I., et al., *Risk factors of gallbladder cancer: An international collaborative case - contol study.* Cancer, 1996. **78**(7): p. 1515-1516.

155. Lowenfels, A., et al., *Gallstone growth, size, and risk of gallbladder cancer: an interracial study.* International journal of epidemiology, 1989. **18**(1): p. 50-54.

156. Diehl, A.K., *Gallstone size and the risk of gallbladder cancer.* Jama, 1983. **250**(17): p. 2323-2326.

157. Albores-Saavedra, J., D.E. Henson, and P. Armed Forces Institute of, *Tumors of the gallbladder and extrahepatic bile ducts*. 1986.

158. Shukla, V.K., et al., *Carcinoma of the gallbladder—is it a sequel of typhoid?* Digestive diseases and sciences, 2000. **45**(5): p. 900-903.

159. Diehl, A.K., *Epidemiology of gallbladder cancer: a synthesis of recent data.* Journal of the National Cancer Institute, 1980. **65**(6): p. 1209-1214.

160. Srivastava, S., et al., *Genotoxic and carcinogenic risks associated with the dietary consumption of repeatedly heated coconut oil.* British journal of nutrition, 2010. **104**(09): p. 1343-1352.

161. Dung, C.-H., S.-C. Wu, and G.-C. Yen, *Genotoxicity and oxidative stress of the mutagenic compounds formed in fumes of heated soybean oil, sunflower oil and lard.* Toxicology in vitro, 2006. **20**(4): p. 439-447.

162. Wu, P.-F., et al., *Nitro-polycyclic aromatic hydrocarbon contents of fumes from heated cooking oils and prevention of mutagenicity by catechin.* Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis, 1998. **403**(1): p. 29-34.

163. Wu, S.-C. and G.-C. Yen, *Effects of cooking oil fumes on the genotoxicity and oxidative stress in human lung carcinoma (A-549) cells.* Toxicology in vitro, 2004. **18**(5): p. 571-580.

164. Sanyal, P., *Argemone and mustard seeds.* Indian medical gazette, 1950. **85**(11): p. 498-500.

165. Das, M. and S.K. Khanna, *Clinicoepidemiological, toxicological, and safety evaluation studies on argemone oil.* Critical Reviews in Toxicology, 1997. **27**(3): p. 273-297.

166. Upreti, K.K., M. Das, and S.K. Khanna, *Biochemical toxicology of argemone oil. I. effect on hepatic cytochrome P‐450 and xenobiotic metabolizing enzymes.* Journal of Applied Toxicology, 1991. **11**(3): p. 203-209.

167. Ghosh, P., M.M.K. Reddy, and R.B. Sashidhar, *Quantitative evaluation of sanguinarine as an index of argemone oil adulteration in edible mustard oil by high performance thin layer chromatography.* Food Chemistry, 2005. **91**(4): p. 757-764.

168. Babu, C.K., S.K. Khanna, and M. Das, *Adulteration of mustard cooking oil with argemone oil: Do Indian food regulatory policies and antioxidant therapy both need revisitation?* Antioxidants & Redox Signaling, 2007. **9**(4): p. 515-525.

169. Gomber, S., et al., *Epidemic dropsy in Trans Yamuna areas of Delhi and UP.* Indian pediatrics, 1994. **31**: p. 671-671.

170. Thatte, U. and S. Dahanukar, *The Mexican poppy poisons the Indian mustard facts and figures.* The Journal of the Association of Physicians of India, 1999. **47**(3): p. 332-335.

171. Mishra, V., et al., *Edible oil adulterants, argemone oil and butter yellow, as aetiological factors for gall bladder cancer.* European Journal of Cancer, 2012. **48**(13): p. 2075-2085.

172. Madle, S., et al., *Recommendations for the performance of UDS tests in vitro and in vivo.* Mutation Research/Environmental Mutagenesis and Related Subjects, 1994. **312**(3): p. 263-285.

173. Dearfield, K.L. and M.M. Moore, *Use of genetic toxicology information for risk assessment.* Environmental and molecular mutagenesis, 2005. **46**(4): p. 236-245.

174. Thybaud, V., et al., *Strategy for genotoxicity testing: hazard identification and risk assessment in relation to in vitro testing.* Mutation Research/Genetic Toxicology and Environmental Mutagenesis, 2007. **627**(1): p. 41-58.

175. Nishikawa, T., et al., *Study of a rat skin in vivo micronucleus test: data generated by mitomycin C and methyl methanesulfonate.* Mutation Research/Genetic Toxicology and Environmental Mutagenesis, 1999. **444**(1): p. 159-166.

176. Nishikawa, T., et al., *Further evaluation of an in vivo micronucleus test on rat and mouse skin: results with five skin carcinogens.* Mutation Research/Genetic Toxicology and Environmental Mutagenesis, 2002. **513**(1): p. 93-102.

177. Gibson, D., L. Krsmanovic, and M. Aardema. *Testing the specificity of the in vivo rodent skin micronucleus assay as developed by Nishikawa et al., for chemicals negative in dermal carcinogenesis assays*. in *Environmental and Molecular Mutagenesis*. 2004. Wiley & Sons Hoboken, NJ, USA.

178. Reus, A.A., et al., *Development and characterisation of an in vitro photomicronucleus test using ex vivo human skin tissue.* Mutagenesis, 2011. **26**(2): p. 261-268.

179. Netzlaff, F., et al., *The human epidermis models EpiSkin®, SkinEthic® and EpiDerm®: an evaluation of morphology and their suitability for testing phototoxicity,*

irritancy, corrosivity, and substance transport. European Journal of Pharmaceutics and Biopharmaceutics, 2005. **60**(2): p. 167-178.

180. Du, L., et al., *Effects of the differentiated keratinocyte phenotype on expression levels of CYP1–4 family genes in human skin cells.* Toxicology and Applied Pharmacology, 2006. **213**(2): p. 135-144.

181. Swanson, H.I., *Cytochrome P450 expression in human keratinocytes: an aryl hydrocarbon receptor perspective.* Chemico-Biological Interactions, 2004. **149**(2): p. 69-79.

182. Merk, H., et al., *Molecular pathways in dermatotoxicology.* Toxicology and applied pharmacology, 2004. **195**(3): p. 267-277.

183. Ioannides, C. and D.V. Parke, *The cytochrome P450 I gene family of microsomal hemoproteins and their role in the metabolic activation of chemicals.* Drug metabolism reviews, 1990. **22**(1): p. 1.

184. Brinkmann, J., et al., *Metabolically competent human skin models: activation and genotoxicity of benzo [a] pyrene.* toxicological sciences, 2012: p. kfs316.

185. Flamand, N., et al., *Development of genotoxicity test procedures with Episkin®, a reconstructed human skin model: Towards new tools for in vitro risk assessment of dermally applied compounds?* Mutation Research/Genetic Toxicology and Environmental Mutagenesis, 2006. **606**(1): p. 39-51.

186. Hu, T., et al., *Xenobiotic metabolism gene expression in the EpiDerm™ in vitro 3D human epidermis model compared to human skin.* Toxicology in Vitro, 2010. **24**(5): p. 1450-1463.

187. Union, E., *EEC on the approximation of the laws of the Members States relating to cosmetic products.* Official J Eur Union, 2003. **L66**: p. 26-35.

188. Curren, R.D., et al., *Development of a method for assessing micronucleus induction in a 3D human skin model (EpiDerm™).* Mutation Research/Genetic Toxicology and Environmental Mutagenesis, 2006. **607**(2): p. 192-204.

189. Reus, A.A., et al., *Comet assay in reconstructed 3D human epidermal skin models—investigation of intra-and inter-laboratory reproducibility with coded chemicals.* Mutagenesis, 2013. **28**(6): p. 709-720.

190. Mun, G.C., et al., *Further development of the EpiDerm™ 3D reconstructed human skin micronucleus (RSMN) assay.* Mutation Research/Genetic Toxicology and Environmental Mutagenesis, 2009. **673**(2): p. 92-99.

191. Yuki, K., et al., *The reconstructed skin micronucleus assay in EpiDerm: reduction of*

false-positive results - a mechanistic study with epigallocatechin gallate. Mutat Res, 2013. **757**(2): p. 148-57.

192. Reus, A.A., M. Usta, and C.A. Krul, *The use of ex vivo human skin tissue for genotoxicity testing.* Toxicology and applied pharmacology, 2012. **261**(2): p. 154-163.

193. Capon, F., et al., *A comparative study of cryogenic lesions in organ-cultured human skin and in reconstituted human skin equivalent.* Cryobiology, 1998. **36**(3): p. 174-183.

194. Dreher, F., et al., *Improvement of the experimental setup to assess cutaneous bioavailability on human skin models: dynamic protocol.* Skin Pharmacology and Physiology, 2002. **15**(Suppl. 1): p. 31-39.

195. Dreher, F., et al., *Comparison of cutaneous bioavailability of cosmetic preparations containing caffeine or α-tocopherol applied on human skin models or human skin ex vivo at finite doses.* Skin Pharmacology and Physiology, 2002. **15**(Suppl. 1): p. 40-58.

196. Osswald, S.S., et al., *Self-treatment of a basal cell carcinoma with "black and yellow salve".* Journal of the American Academy of Dermatology, 2005. **53**(3): p. 509-511.

197. Ouedraogo, M., et al., *Review of current and "omics" methods for assessing the toxicity (genotoxicity, teratogenicity and nephrotoxicity) of herbal medicines and mushrooms.* Journal of ethnopharmacology, 2012. **140**(3): p. 492-512.

198. Walmsley, R.M. and N. Billinton, *How accurate is in vitro prediction of carcinogenicity?* British journal of pharmacology, 2011. **162**(6): p. 1250-1258.

199. Arcos, J.C. and M.F. Argus, *Chemical Induction of Cancer: Structural Bases and Biological Mechanisms*. 2013, Burlington: Elsevier Science.

200. Krivjanský, V., et al., *Induction of respiration-deficient mutants in Saccharomyces cerevisiae by chelerythrine.* FEMS Microbiology Letters, 1994. **120**(1): p. 87-91.

1176 Table 1: *In vitro* Sanguinarine Genotoxicity Results

Test	Description	Result	Ref
Ames: *Salmonella typhimurium*	Genetic mutation enables histidine production and bacterial colony growth	Positive	[98]
SOS Chromotest: *Escherichia coli* PQ37	Assesses lacZ gene damage by measuring its product B-galactosidase	Negative	[100]
Yeast: *Saccharomyces cerevisiae*	Mutations identifiable by colony colour and growth on certain media	Negative	[200]
Comet Assay	DNA strand breaks appear as a comet tail on agarose gel	Positive	[14]
Micronucleus Assay	Micronuclei chromosome fragments develop during mitosis following genotoxin exposure	Negative	[15]
Gamma-H2AX	Marker of DNA damage localizes to DNA strand break sites	Positive	[58]
GADD45a	Gene activated in response to DNA damage	Untested	
UDS Assay	Measures DNA Nucleotide Excision Repair	Untested	
CHO-HPRT Mutation Assay	Exposure to mutagens establishes HPRT negative CHO cell mutants	Equivocal	[99]

1177

1178

1179 Table 2: *In vivo* Sanguinarine Genotoxicity Testing

Test	Result	Dose	Comment	References
Micronucleus Assay			Not Tested	
Comet Assay	Positive	2.7mg/kg IP	SG isol Argemone Oil 88% Purity Murine Lymphocyte/ Bone Marrow Cells Both Positive	[121]
	Negative	367 ppm PO	SG isol *M. cordata* 98.1% Purity 90day ingestion rat lymph/liver neg Plasma SG conc not determined	[125]
CA & SC Exchange Assay	Positive	10mg/kg IP	SG Sigma	[128]

1180 SG= Sanguinarine; CA & SC Exchange Assay= Chromosomal Aberration & Sister Chromatid Exchange Assay

1181