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Induction of DNA strand-breaks in primary rat hepatocytes by ginkgolic acids

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Ginkgolic acids (GA) are 6-alkyl salicylic acids with saturated or unsaturated n-C_{13}- to n-C_{17} alkyl side chains. GA together with closely related 3-alkyl phenols (cardanols) and 5-alkyl resorcinols (cardols) represent major constituents in the lipid fraction of nut shells (sarcotesta) from *Ginkgo biloba L.* These alkylphenols have been demonstrated to be responsible for allergic skin reactions and gastrointestinal disturbances after contact with or consumption of *Ginkgo* fruits. The allergenic potential of GA has been suggested to be due to their amphiphilic nature and their high chemical reactivity. This latter activity of GA also appears to be responsible for the inhibition of many different enzyme systems as well as the observed antimicrobial, antitumor, cytotoxic and molluscicidal effects [1, 2]. In addition, in a number of reports DNA-cleaving properties of 5-alkylresorcinols [3, 4] and 3-alk(en)ylcatechol derivatives [5] have been described. These results suggest that the closely related alkylphenols from *Ginkgo* are likely to cause DNA strand scission as well. Thus, it was of interest to evaluate the DNA-damaging capacity of GA. For this purpose, we used the alkaline single cell gel electrophoresis (comet) assay [6], which is a powerful tool for the detection of DNA-cleaving agents. An advantage of this assay is the observation of genotoxic effects on the single cell level. Furthermore, in combination with primary hepatocytes the assay not only detects directly acting genotoxic agents but also chemicals which exert their DNA-damaging properties only after metabolic activation. The assay has been used to investigate DNA-damage caused by irradiation [7], chemical carcinogens [8] and oxygen radicals [9].

The results of two independent experiments are shown in Table 1. After treatment of primary rat hepatocytes with a mixture of natural occurring GA at concentrations between 0.1–10 μg/ml for 3 hours, DNA strand breaks were observed even at the lowest concentration (i.e., 0.1 μg/ml). In the first experiment the concentration range was between 1 and 30 μg/ml. Control cells treated with only the solvent vehicle DMSO showed little or no damage, whereas extensive DNA cleavage was observed after treatment of cells with 1 μg/ml of GA. Following exposure to 3–10 μg/ml GA, all cells showed massive DNA strand breaks (Type 4–5). Similar results were observed in the second experiment with a dose range between 0.1–3 μg/ml GA. Although the preparation procedure caused some damage in control cells (Type 2–3) in this experiment, a clear and dose dependent increase of DNA-strand breaks was observed after treatment with GA. Only a low level (5%) of apoptotic cells (Type 5b) was observed after incubation of cells with 3 μg/ml GA (i.e., the highest concentration used in this experiment).

The cytotoxicity of GA in primary rat hepatocytes was investigated in two separate experiments using the neutral red incorporation method as described by Borenfreund and Puerner [10] (Table 2). Concentrations below 10 μg/ml GA were non-toxic, whereas toxicity was observable at concentrations $\geq$ 30 μg/ml GA. The absence of apoptotic cell death and the fact that DNA damage occurred after treatment of the cells with concentrations of GA far below cytotoxic levels indicate that these effects are not the result of unspecific toxicity.

Recently we investigated the induction of DNA repair in primary rat hepatocytes by GA. No repair induction could be observed (unpublished results). The discrepancy between the positive results obtained in the comet assay and the negative results observed in the DNA-repair assay suggest that the DNA lesions induced by GA do not elicit long patch repair. An attack by radicals, such as reactive oxygen species is more likely. Because oxidative stress can contribute to processes leading to cancer, these results raise the suspicion that GA might be tumor promoting agents. This possibility, therefore, should be investigated in more detail.

GA are of particular toxicological interest as they may be present in *Ginkgo* leaf extracts, which are extensively used to treat peripheral and cerebral vascular disorders, memory

Table 2: Cytotoxicity of ginkgolic acids in primary rat hepatocytes

Treatment	Concentration	Viability (%)	
	(μg/ml)	1. Exp	2. Exp.
Control		100.0	100.0
GA	1	100.0	100.0
GA	3	97.6	100.0
GA	10	100.0	100.0
GA	30	16.0	67.0
GA	100	1.9	5.5

Table 1: DNA-strand breaks induced by ginkgolic acids in primary rat hepatocytes as determined by the single cell gel electrophoresis assay (comet assay)

Treatment	Type 1 No migration		Type 2 Short migration		Type 3 Medium migration		Type 4 Long migration		Type 5 Complete migration		Type 5b Apoptosis (short patches)	
	1. Exp.	2. Exp.	1. Exp.	2.Exp	1. Exp.	2. Exp.	1. Exp.	2. Exp.	1. Exp.	2. Exp.	1. Exp.	2. Exp.
Control	60	0	32	23	8	77	0	0	0	0	0	0
GA 0.1 μg/ml	–	0	–	0	–	42	–	58	–	0	–	0
GA 0.3 μg/ml	–	0	–	0	–	9	–	91	–	0	–	0
GA 1 μg/ml	0	0	0	0	36	0	64	50	0	50	0	0
GA 3 μg/ml	0	0	0	0	7	4	88	42	5	47	0	5
GA 10 μg/ml	0	–	0	–	0	–	94	–	6	–	0	–
GA 30 μg/ml	0	–	0	–	0	–	92	–	8	–	0	–

Given are the percentage of cells displaying different degrees of DNA-damage as indicated by the electrophoretic mobility of DNA

loss, age-related cognitive impairment and dementia of the vascular or Alzheimer's type. For GA no contribution to the therapeutic efficacy of *Ginkgo* extracts has been demonstrated. On the contrary, GA have been shown to provoke allergic and cytotoxic reactions. Because of their close chemical relationship to urushiols form Anacardiaceae, immunological cross-reactivity between *Ginkgo* fruits or leaves and poison ivy and other members of this plant family has been observed [1, 2, 11]. It has, therefore, been a major goal to develop manufacturing processes, which guarantee the completest possible removal of these constituents. Thus, a maximal concentration of 5 ppm GA has been included in the monographs of the commission E of the former German Federal Health Authority and the WHO [1]. The limitation of GA to 5 ppm is justified by the detection limit of the analytical method and the level achievable by the manufacturing process. This is in accordance with the ICH guideline Q3A [12]. The present investigation strongly indicates that GA may also possess mutagenic and carcinogenic properties, giving further support to the appropriateness of this limit value.

Experimental

1. Chemistry

The GA mixture (Lot-No. Bz 163) investigated in this study was provided by Dr. Willmar Schwabe GmbH, Arzneimittel, Karlsruhe, Germany. It was obtained from the heptane soluble fraction of a 60% w/w acetone extract by a series of column chromatographic procedures on Sephadex LH 20, DIAION HP 20 and silica. Mass spectroscopic analysis revealed the following composition of the mixture of 6-alkyl salicylic acids: 3% C13 : 0, 3% C15 : 0, 47% C15 : 1, 43% C17 : 1 and 3% C17 : 2. The amount of ginkgolic acids was 99%.

2. Comet assay

The comet assay was performed according to the technique described by Singh et al. [13]. In this study we employed a standard protocol as reported by Tice [14].

3. Neutral red assay

The cytotoxicity of GA was monitored by the neutral red accumulation method, described by Borenfreund and Puerner [10].

4. Preparation of primary rat hepatocytes

Male Wistar rats with a weight of 180–200 g were used for the preparation of liver cells as described in detail by Westendorf et al. [15] and Butterworth et al. [16].

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