Evaluation of the anti-tumour action and acute toxicity of kosins from *Hagenia abyssinica*

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Abstract: The kosins are phloroglucinol derivatives isolated from female flowers of *Hagenia abyssinica* (Rosaceae) and were tested for possible cytotoxic activity *in vitro* and *in vivo* against a panel of three transplantable murine adenocarcinomas of the colon of varying growth characteristics and morphology (MAC system). Significant reductions in colony formation were observed *in vitro* in MAC 15A tumour following 1, 3, 6 and 24 h exposure to all kosins (α-kosin, kosotoxin and protokosin). The kosins (kosotoxin and protokosin) were also found to be cytotoxic against MAC tumour cells *in vivo* in some cases. Kosotoxin was subjected to preliminary toxicity studies in mice. It showed no observable toxicity up to 200 mg kg^{-1} orally and was found to be toxic at doses in excess of 50 mg kg^{-1} (i.p.). A single dose of 100 mg kg^{-1} (i.p.) was lethal for 100% of the animals.

Keywords: Hagenia abyssinica; Rosaceae; phloroglucinols; anthelmintics; chemosensitivity; toxicology.

Introduction

A decoction of the female flower of *Hagenia abyssinica* (Kosso) has been effectively used in Ethiopia over many centuries as an anthelmintic against tapeworm [1].

The phloroglucinols α-kosin, kosotoxin and protokosin (Scheme 1) have been reported as constituting *ca* 3% w/w in the female flower of Kosso [2, 3]. Analytical data are available on the purity and identity of the kosins used in these studies [4].

However, the toxicity of this decoction is not fully established, although some preliminary work points to the adverse effects of Kosso [5]. These include occasional visual problems, tachycardia, hypotension and enlarged liver. Kosotoxin is believed to be the anthelmintic and toxic principle [6].

These studies clearly demonstrate that kosins do have pharmacological activity. In relation to malignant disease there is no evidence within the literature to suggest that kosins possess anti-cancer activity. The evaluation of these compounds for anti-cancer activity is warranted for two reasons: first, the kosins are novel chemical structures and secondly, plant material has proved a valuable source of new anti-cancer drugs in the past (e.g. vincristine and vinblastine from *Catharanthus roseus*; camptothecin from *Camptotheca acuminata*) [7]. The aim of this study is to assess whether or not kosins induce cytoxicity in a panel of murine adenocarcinoma of the colon (MAC) cell lines *in vitro* and *in vivo*. These tumours have been extensively characterized and are similar in terms of cell kinetics, histology and chemosensitivity to tumours of the human colon [8]. In addition, kosotoxin was subjected to acute toxicity studies in mice by oral and intraperitoneal administration.
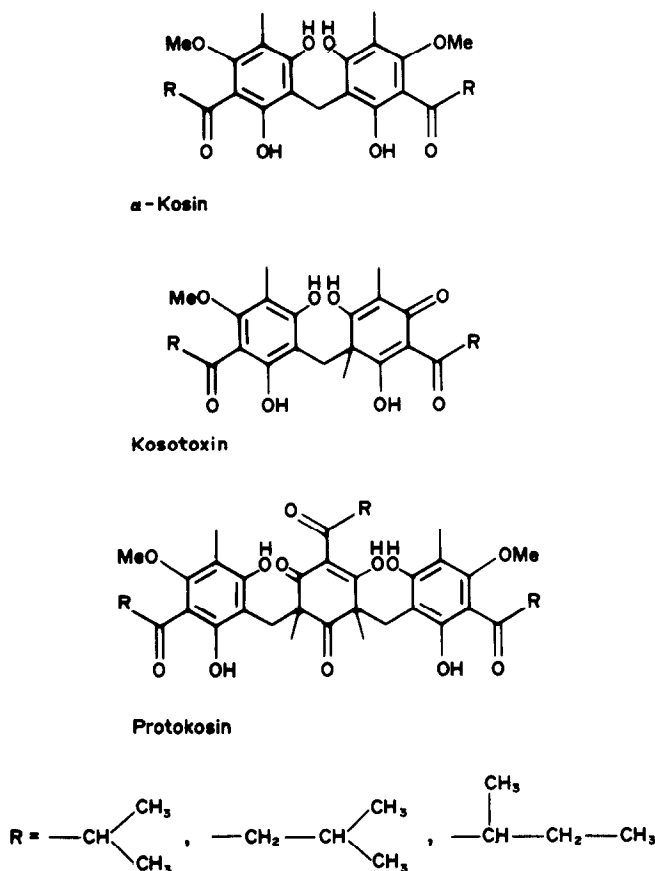
An *in vitro* study to examine whether kosotoxin could be metabolized by the drug-metabolizing enzyme glutathione transferase was also performed.

Materials and Methods

Test compounds

Kosins were isolated from female flowers of *Hagenia abyssinica* as described by Lounasmaa *et al.* [2]. A 300 g quantity of dried female flower was soaked in diethylether for 3 days. The diethylether was removed *in vacuo* leaving a dark green oily residue (Extract 1). The crude extract (Extract 1) was mixed with

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a - Kosin

Kosotoxin

Protokosin

$$R = -CH\begin{smallmatrix}CH_3\\CH_3\end{smallmatrix} \quad , \quad -CH_2-CH\begin{smallmatrix}CH_3\\CH_3\end{smallmatrix} \quad , \quad -\overset{\overset{\displaystyle CH_3}{|}}{CH}-CH_2-CH_3$$

Scheme 1
Structure of the kosins.

200 ml saturated barium hydroxide. The mixture was acidified with 150 ml, 25% acetic acid to give the crude kosins (Extract 2) as an amorphous pale yellow solid. The crude kosins were dried and chromatographed on silicagel column to isolate the three kosins — kosin, kosotoxin and protokosin. Identity and purity were controlled by means of UV, IR, MS, ^{1}H NMR, TLC, and HPLC–diode array detection. All kosins were dissolved initially in 1 M NaOH and diluted with phosphate buffer solution (PBS) (pH 7.0).

Chemosensitivity studies

In vitro studies. Cell lines were derived by mechanical disaggregation of the solid tumours and were routinely maintained as monolayer cultures in RPMI 1640 tissue culture medium, supplemented with foetal calf serum (10%), sodium pyruvate (1 mM), a mixture of penicillin and streptomycin (50 IU ml^{-1} and 50 μg ml^{-1}, respectively) and buffered with HEPES (25 mM). Chemosensitivity studies were

restricted to cultures of less than 10 passages in age.

Clonogenic assay. Chemosensitivity was assessed using a modified clonogenic assay, the details of which have been published elsewhere [9]. Single cell suspensions were obtained from monolayer cultures by trypsinization. All drug solutions were dissolved in 30 μl 1 M NaOH and filled to 1 ml with normal saline and finally the cell line suspension (MAC 15A) was added to bring the volume to 10 ml. A range of drug concentrations (0.01–10 μM) was prepared by serial dilution. Solvent controls were used throughout. Cells and drug mixtures were shaken and incubated at 37°C for various time intervals (1, 3, 6 and 24 h).

Following drug exposure, the cells were washed twice in 20 ml Hanks Balanced Salt Solution (HBSS) by repeated centrifugation (2000 rpm × 3 min) and resuspension of the pellet. After washing the pellet was resuspended in complete medium and the concen-

tration of cells determined using a haemo-cytometer. Approximately $1\text{--}2 \times 10^4$ cells were plated into six well dishes, containing 3 ml of complete RPMI 1640. After 5–7 days' incubation at 37°C, colonies of 50 cells or greater were counted using an inverted micro-scope and plating efficiencies were calculated for each drug concentration.

Triplicate samples for each assay were per-formed and dose–response curves were drawn by plotting the mean survival (%) of colony-forming units against drug concentration.

In vivo *studies*. Pure strain 6–8 week-old NMRI mice from the inbred colony at the Clinical Oncology Unit, University of Brad-ford, were housed in cages in an air-con-ditioned room where regular, alternate 12-h cycles of light and darkness were maintained. Animals were fed with a pellet diet (CRM Labsure, Croydon, UK) and water *ad libitum*.

The well-differentiated MAC 26 tumours were transplanted into male mice by the subcutaneous (s.c.) implantation of tumour fragments ($\approx 1 \times 2$ mm) in the flank. MAC 15A ascites tumour cells [10] were transplanted into male mice by the i.p. inoculation of 1×10^6 tumour cells. In addition, MAC 15A was implanted by the s.c. injection of 2×10^6 tumour cells in 0.2 ml physiological saline. MAC 13 tumours [11] were transplanted into female mice by s.c. implantation of tumour fragments ($\approx 1 \times 2$ mm) in the flank.

Tumour-bearing animals were allocated by restricted randomization into groups of five. Appropriate concentrations of test compound were used for a desired dose to be given in 0.1 ml per 10 g body weight. Control exper-iments were performed with tumour-bearing animals, injected with the drug-free vehicle. With the more rapidly growing MAC 13 and MAC 15A tumours, chemotherapy com-menced 2 days after implantation. MAC 13 tumours are palpable at this stage and anti-tumour responses were assessed 14 days later by recording tumour weights.

Similarly, MAC 15A tumours grown in the s.c. site were weighed and the effects of chemotherapy estimated from the percentage of weight inhibition of tumours 14 days after implantation. As MAC 26 is relatively slow-growing, chemotherapy began when the tumours had reached a size that could be measured accurately (tumour volume ≈ 40 mm^3), viz., approximately 18 days after

implantation. Therapeutic effects were assessed by twice-weekly, two-dimensional caliper measurements of the tumour. Tumour volume was calculated from the formula ($a^2 \times b/2$), where a is the smaller diameter and b is the larger [12]. Tumour volumes were normal-ized with respect to starting volumes and graphs of the relative tumour volume against time were plotted on semi-log graph paper.

For MAC 15A tumour cells implanted via the i.p. route, anti-tumour activity was deter-mined by comparison of the life-span in treated and control groups. Deaths were recorded and the median survival time (MST) determined. The anti-tumour effects of kosins were investi-gated following the i.p. administration of a single (50 mg kg^{-1}) and split dose schedules — 12.5 mg kg^{-1} in four divided daily doses at their maximum tolerated dose.

Acute toxicity

Oral administration in mice. NMRI male mice (20–25 g) in groups of five for each dose level were used for the tests. The mice received a single dose of kosotoxin by gavage in the form of solution initially dissolved in 1 M NaOH and diluted with phosphate buffer solution (PBS) with a control group receiving the vehicle.

The mice received the test drug in escalating levels from 50 to 200 mg kg^{-1}. Thereafter all the animals were observed carefully for 7 days. They were closely observed for gross be-havioural change and other signs of toxicity in the first 24 h.

Intraperitoneal administration. Groups of mice received single doses of kosins as a solution in 1 M NaOH and PBS to give a final concentration of 1% w/v, with a control group receiving the vehicle, in escalating dose levels of 25–100 mg kg^{-1} by intraperitoneal in-jection. All were observed for signs of toxicity and the mortality rate was noted as before.

In vitro *metabolic study*

The non-enzymatic and possible enzymatic reaction of kosotoxin were examined using a Perkin–Elmer Lambda-5 UV–vis spectro-photometer (Beaconsfield, Bucks, UK) as follows:

(1) 0.3 mM kosotoxin was assayed with glutathione (5.0 mM) (in absence of rat liver cytosol preparation) in 3 ml phosphate buffer

(pH 6.5), at ambient temperature; detection at 280 nm.

(2) 0.3 mM kosotoxin was assayed at ambient temperature with 5.0 mM glutathione in 3.0 ml phosphate buffer (pH 6.5) plus 50 μl rat liver cytosol preparation (obtained by a standard procedure from adult male Sprague–Dawley rats, University of Bradford) to test for evidence of any enzymatic reaction; detection at 286 nm.

(3) Tests for non-enzymatic and (4) enzymatic reactions were performed at pH 7.5, using the same assay conditions as in steps (1) and (2) above, respectively; detection at 286 nm.

(5) Furthermore 0.3 mM kosotoxin was assayed with 5.0 mM glutathione in 3.0 ml phosphate buffer (pH 7.5) but at 337 nm at ambient temperature.

Results and Discussion

Chemosensitivity

Preliminary screening studies clearly demonstrated that all three kosins (α-kosin, kosotoxin and protokosin) are cytotoxic towards MAC 15A cells *in vitro* following continuous exposure (96 h) to a broad range of drug concentrations (Table 1). All three compounds were equally cytotoxic with IC_{50} values (concentration required to reduce colony formation by 50%) ranging from 3 to 5 μM. In the case of kosotoxin, increasing both the parameters of drug concentration and the duration of drug exposure significantly increases clonogenic cell kill (Fig. 1). Whilst the mechanism of the action of these compounds is not known, the shape of the dose response curves (i.e. exponential) suggests that if DNA is the principal target, these compounds are cell cycle specific rather than cell cycle phase specific drugs [13]. Furthermore, these results suggest
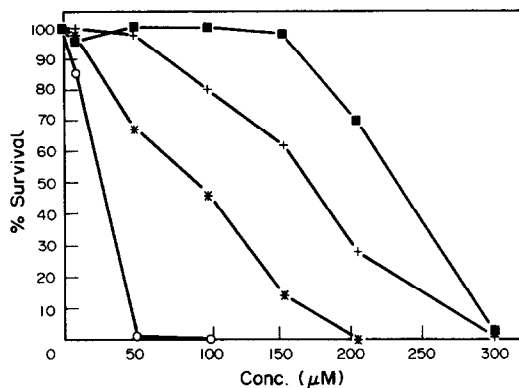

Figure 1
The response of MAC 15A cells *in vitro* following exposure to kosotoxin for 1 h (■–■), 3 h (+ − +), 6 h (*−*) and 24 h (O–O).

that kosotoxin is relatively stable under cell culture conditions for at least 24 h.

The anti-tumour activity of kosins against MAC 15A (i.p. and s.c.), MAC 13 and MAC 26 tumours *in vivo* is presented in Table 2. Intraperitoneal administration of kosotoxin at 100 mg kg^{-1} was toxic (5/5 deaths). A single dose of 50 mg kg^{-1} i.p. was well tolerated in all cases although no activity was observed against any of the tumour lines studied. When a split schedule (12.5 mg kg^{-1} Q1d × 4) was employed however, both kosotoxin and protokosin induced moderate extensions in survival time against MAC 15A ascites tumours. α-kosin, however, was not active against MAC 15A ascites tumours by either the single or split dose schedules. The reasons for the schedule dependency of these compounds are not known although differences in drug exposure parameters (i.e. areas under the drug clearance curves) may be highly significant. Chromatographic techniques to quantify kosins have been published [3] and subsequent pharmacokinetic studies may explain these observations. Nevertheless, the response of ascitic MAC 15A tumours to kosotoxin and protokosin suggests that these compounds should be examined for possible activity against some forms of human malignancy, e.g. ascites tumours derived from ovarian carcinomas to assess whether the activity demonstrated here is worth further study.

Acute toxicity

Oral administration of kosotoxin in doses of 50, 100, 150 and 200 mg kg^{-1} did not show signs of toxicity. A single dose of 50 mg kg^{-1} (i.p.) kosotoxin was found to be the maximum

Table 1
The response of MAC 15A cells *in vitro* following continuous exposure to kosins

Conc. (μM)	α-Kosin	% Survival Kosotoxin	Protokosin
0.001	100	100	100
0.01	100	98	100
0.1	100	95	98
1.0	93	82	88
10.0	4	none	2

$n = 3$; RSD = 1.23.

Table 2
Anti-tumour activity of kosins *in vivo*

Drug	Tumour	Dose*	MST†	T/C‡
kosotoxin	MAC 15A (i.p.)	control (PBS/NaOH)	8	—
		25 mg kg^{-1}	7	—
		50 mg kg^{-1}	7	—
		100 mg kg^{-1} (5/5 deaths)	toxic	—
kosotoxin	MAC 15A	control	8	—
split schedule	(i.p.)	12.5 mg kg^{-1} × 4	14	175%§
protokosin	MAC 15A	control	6	—
	(i.p.)	50 mg kg^{-1}	7	117%
protokosin	MAC 15A	control	8	—
split schedule	(i.p.)	12.5 mg kg^{-1} × 4	15	187%§
α-kosin	MAC 15A	control	8	—
	(i.p.)	50 mg kg^{-1}	7	—
α-kosin	MAC 15A	control	8	—
split schedule	(i.p.)	12.5 mg kg^{-1} × 4	9	112%
kosotoxin	MAC 15A (s.c.)	control	No tumour inhibition	
		25 mg kg^{-1}		
		37.5 mg kg^{-1}		
		50 mg kg^{-1}		
kosotoxin split schedule	MAC 15A (s.c.)	12.5 mg kg^{-1} × 4	No tumour inhibition	

* All drugs administered by intraperitoneal injection.
† Median Survival Time (days).
‡ T/C = MST treated mice/MST control × 100.
§ Significant difference between control and treated mice ($P = 0.05$, Mann–Whitney U test).

tolerable dose (MTD). A single dose of 100 mg kg^{-1} (i.p.) kosotoxin was lethal for 100% of the animals (Table 3). Mice were stiff (similar to rigor), the heart continued to beat for some time and mice eventually died. The results show that route of administration of kosotoxin is important for toxicity. Doses as large as 200 mg kg^{-1} produced no observable toxicity by the oral route suggesting that bioavailability from the gastrointestinal tract is significantly less than the bioavailability of kosotoxin from the peritoneal cavity. Similar studies in other

laboratories also suggest that Kosso is poorly absorbed into the systemic circulation following oral administration [5]. Alternatively, these compounds may be metabolized to inactive products by bacteria within the gut lumen or as a result of 'first pass' metabolism by the liver. These observations indicate that, although kosotoxin is believed to be the toxic principle, some contributing components may well exist in the crude water extract of Kosso and influence its toxicity. Whilst the poor bioavailability of kosins from the GI tract would obviously have major limitations in the treatment of systemic disease, kosins may be of use in the treatment of a limited number of tumour types. In the treatment of superficial bladder carcinoma for example, instillations of kosins may provide high local concentrations of drug with low levels of systemically circulating drug concentrations.

Kloos [14] has reported the mean weight of a single dose of dried kosso flowers to be 23.6 grams (range 15–49.6 g). The dried flower is ground into powder, mixed with cold water to make a suspension, and left to macerate overnight. In the morning the preparation is strained by means of a gauze cloth and taken orally on an empty stomach.

Table 3
Acute toxicity data of kosotoxin

Drug	Dose (mg kg^{-1})	Route	Survivors
control (saline)	—	oral/i.p.	5/5
control (PBS/NaOH)	—	oral/i.p.	5/5
kosotoxin	50	oral	5/5
	100	oral	5/5
	150	oral	5/5
	200	oral	5/5
kosotoxin	12.5	i.p.	5/5
	50	i.p.	5/5
	100	i.p.	0/5

n = five animals.

In accordance with traditional methods of use [14], an aqueous extract (24 g in 400 ml water) of powdered female flowers of Kosso was prepared, allowed to stand for 16 h and filtered. The resulting aqueous extract was chromatographed by LC [4]. The total kosins found in this extract was 208 μg ml^{-1}. From the result obtained in this work, assuming a dose volume of 350 ml, the total amount of anthelmintic taken would be *ca* 73 mg of kosins. This small amount of kosins administered indicates the need for further studies on the anthelmintic activity and toxicity of kosins. Indeed, the phloroglucinol-free part of the extract, the whole of the crude extract and their crude kosins should be examined for any toxicity, individually.

In vitro *metabolic study*

Very slow enzymic and non-enzymic reactions were observed (*ca* 6 × 10^{-4} absorbance unit change per min at 286 nm and also at 337 nm), when kosotoxin was assayed at pH 6.5 and 7.5 with glutathione alone, or in the presence of glutathione transferase. No significant increase in the rate of change of absorbance was observed.

It was concluded from these brief studies, that kosotoxin did not appear to be attacked by the thiol group of glutathione under these physiological conditions, and that any such reaction could not be catalysed by the enzyme glutathione transferase. Unsaturated aldehydes and ketones containing α,β-unsaturated conjugated carbonyl groups sometimes serve as substrates for glutathione transferase, as they react by Michael addition with glutathione [15]; and the non-aromatic 3,3,5-trialkylphloracetophenone moiety of kosotoxin could have been a site for such glutathione attack. However, this was found not to be the case under the physiological conditions studied here.

In conclusion, the results of this study demonstrate that kosins are cytotoxic towards MAC tumour cells *in vitro* and that this activity does translate into anti-tumour activity *in vivo* in some cases. The anti-tumour activity *in vivo* is dependent upon both the site of tumour implantation and the schedule of drug adminis-

tration with responses (moderate) seen only against MAC 15A i.p. tumours treated with kosotoxin or protokosin administered intraperitoneally using a 'split schedule'. These results together with the fact that bioavailability from the GI tract is poor suggests that kosins may have some value in the treatment of a limited number of malignancies. Further studies including evaluation against a broad panel of cell lines *in vitro*, *in vivo* chemosensitivity studies with the aim of optimizing routes of administration/schedule and pharmacokinetic studies are warranted on the basis of these preliminary studies in this novel class of compounds.

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