while the kinin released by plasma kallikrein is bradykinin[15, 16] and that MLB was shown to be 2–5 times less active than bradykinin on the guinea pig ileum[17] (the synthetic MLB we used in the experiments here described was five times less active than our synthetic bradykinin), the kinin released by plasmin seems to account for most of the total kinin that is released by the endogenous kininogenase.

The findings described in the present paper suggest that in the experiments of Elliott and Lewis[17] probably plasmin was the endogenous kininogenase responsible for the release of MLB in their serum incubates. This assumption is supported by the fact that the treatment to which serum was submitted in their experiments does not remove pre-plasmin or plasmin, since pre-plasmin is also precipitated between 0·25 and 0·45 saturation with ammonium sulphate;[18] it is very stable at low pH[19] and is rapidly activated in slightly alkaline medium at 37°.[19]

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Biochemical Pharmacology, Vol. 18, pp. 1790–1793. Pergamon Press. 1969. Printed in Great Britain

Selective inhibition of thymidine incorporation into lymphocytes by cucurbitacins B and D

(*Received* 19 *October* 1968; *accepted* 31 *January* 1969)

THE CUCURBITACINS (elatericins) are tetracyclic triterpenoid bitter principles which have been isolated from *Cucurbitaceae*[1] and other sources, including begonias.[2] Cucurbitacins B and D are quite toxic

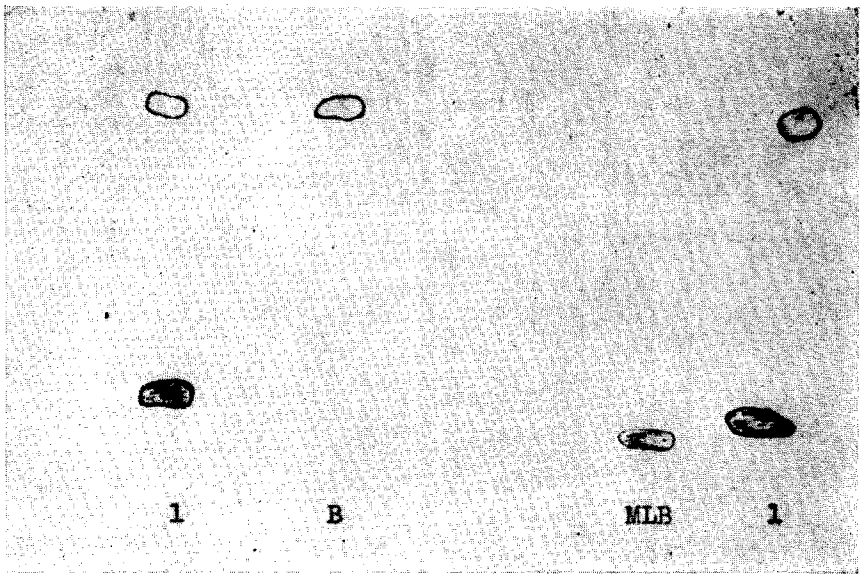

FIG. 1. Paper chromatography in butanol-acetic acid–water (63:27:10) of the spot with a mobility similar to that of methionyl-lysyl-bradykinin and bradykinin obtained after electrophoresis in 3M acetic acid (25v/cm,75 min) of the peptides released in the incubate of kininogen II + plasmin. Staining with ninhydrin. B: syntheric bradykinin; MLB: synthetic methionyl-lysyl-bradykinin; 1: spot described above with a mobility similar to that of MLB and B at electrophoresis.

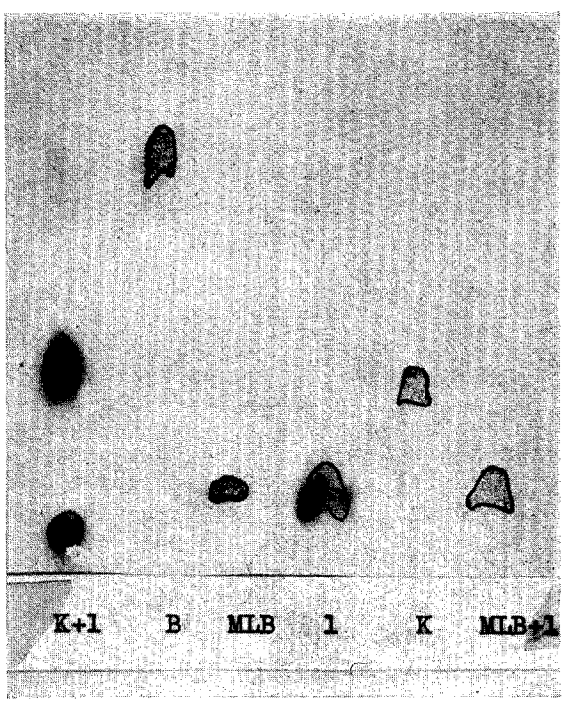

FIG. 2. Rechromatography (same conditions as in Fig. 1) of an eluate of the spot corresponding to MLB separately and in a mixture with synthetic kallidin; B: synthetic bradykinin; MLB: synthetic methionyl-lysyl-bradykinin; 1: eluate of the spot corresponding to MLB from an unstained chromatogram.

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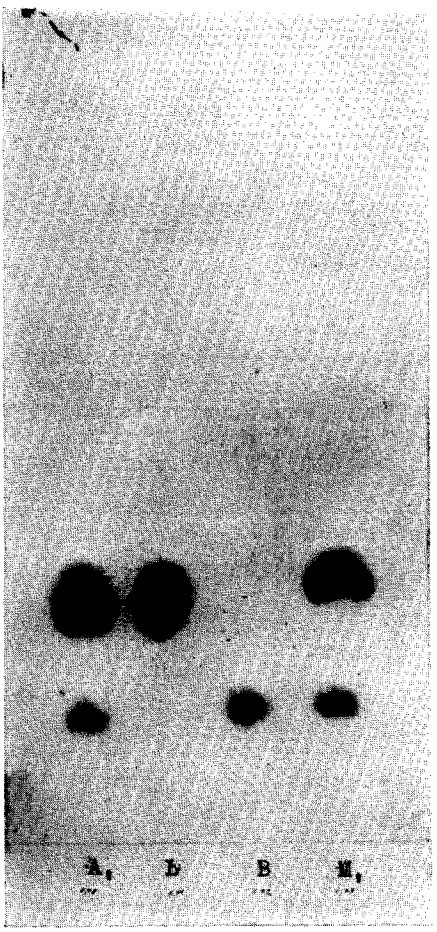
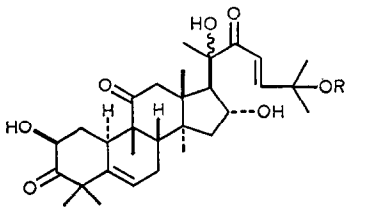


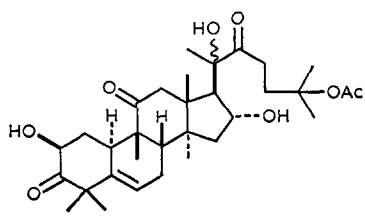
FIG. 4. Paper electrophoresis of the tryptic digests described in Fig. 3 (A_1 and M_1), lysine (L) and bradykinin (B). 25 v/cm in 3M acetic acid. Staining with ninhydrin.

and inhibit growth of some tumors (Sarcoma 180, Ehrlich ascites carcinoma)[3] and Eagle's KB cells.* Cucurbitacin D, also known as elatericin A, (2β,16α,20,25-tetrahydroxy-9-methyl-19-nor-9β,10α-Lanosta-5,23-diene-3,11,22-trione; Fig. 1, III) has an unsubstituted tertiary alcoholic group in the side chain which is acetylated in cucurbitacin B (Fig. 1, I). The conjugated ene–one system in the side chain is readily reduced with hydrogen over palladium yielding the much less toxic[3] dihydro compounds (ketones). Simultaneously, the tertiary acetoxy group in cucurbitacin B may be reductively eliminated, yielding a 25-desoxy derivative. We have now found that dihydro-cucurbitacin B (Fig. 1, II) and the parent cucurbitacins are potent drugs *in vitro* with a moderately selective effect on DNA synthesis.



I R = Ac

III R = H



II

FIG. 1. Structural formulae for I, cucurbitacin B; II, dihydrocucurbitacin B; and III, cucurbitacin D

On incubating these compounds for 30 min at 37° with lymphocyte suspensions and appropriate labelled precursors, the cucurbitacins rapidly inhibited thymidine incorporation, but did not affect the incorporation of amino acids into materials insoluble in 6% (w/v) trichloroacetic acid. The "lymphocytes" were obtained either from the bursa of Fabricius of 8 to 12-week-old White Leghorn chickens or from the thymus of 8 to 12-week-old New Zealand white rabbits, by gently rubbing these tissues over a stainless steel wire mesh in Hank's medium. The cells were then isolated by slow centrifugation, washed once in chilled Hank's medium and finally resuspended in a mixture of four parts Hank's medium and one part 0·1 M sodium phosphate buffer, pH 7·4. Each incubation tube contained 0·8 to 1·2 × 10^8 cells, 0·2 μc tritiated nucleoside or 0·02 μc amino acids-^{14}C (algal protein hydrolysate, Radio-Chemical Centre, Amersham) in a volume of 2 ml to which drugs were normally added in 10 or 20 μl tetrahydrofuran (except the glycyrrhetic acid hemisuccinates which were added in water).

Table 1 shows the relatively selective effect of the cucurbitacins in primarily inhibiting thymidine incorporation, in this respect mimicking the action of 5 mM hydroxyurea and 50 μM cytosine-β-D-arabinofuranoside (Cytarabine, kindly donated by Dr. E. M. Glenn, Upjohn Co., Kalamazoo) on the same cells. That this effect was not due primarily to the unsaturated ketone (thiol-neutralizing pharmacophore) in the side chain was indicated by (a) similar drug action of the dihydro derivative *in vitro*; (b) the nonspecific effect on polymer biosynthesis of some glycyrrhetic acid derivatives (kindly donated by Dr. S. Gottfried, Biorex Laboratories, London), which are also lipophilic unsaturated

* R. W. Doskotch, unpublished findings.

triterpenoid ketones but uncouple oxidative phosphorylation;[4] and by (c) preincubating the cells and drugs with 2 mM mercaptoethanol or glutathione for 10 min before adding the radioactive thymidine, which abolished the effect of the parent cucurbitacins on thymidine incorporation but did not affect this drug action *in vitro* of dihydrocucurbitacin B or glycyrrhetic acid. These latter findings also suggest that an unhindered α-ketol group in the side chain may be required for activity. This may explain why ecdysterone (Mann Laboratories, New York), the insect metamorphosis hormone which structurally resembles the cucurbitacins in many respects but lacks this 22-oxo group (and also lacks the carbonyl groups in rings A and C), had no effect on these lymphocytes under the conditions described.

TABLE 1. INHIBITION OF AMINO ACID-^{14}C AND NUCLEOSIDE-^{3}H INCORPORATION INTO CHICKEN BURSA LYMPHOCYTES BY SOME NATURALLY OCCURRING TRITERPENOIDS AND THEIR DERIVATIVES*

Compound	Concn (μM)	Percentage incorporation† of		
		Amino acids-^{14}C	Uridine-^{3}H	Thymidine-^{3}H
Cucurbitacin B, m.p. 178–180°	50	95	85	40
Dihydrocucurbitacin B, m.p. 159–161°	25	100	85	35
Cucurbitacin D, m.p. 150–151°	50	100	90	50
Ecdysterone, m.p. 243°	100	92		95
18β-Glycyrrhetic acid (G.A.)	50	40	45	40
18β-G.A.-3-*O*-hemisuccinate (Na salt)	100	25		30
18α-G.A.-3-*O*-hemisuccinate (Na salt)	100	10		15
11-Desoxo-18β-G.A.	100	95		95

* Essentially similar results were obtained using rabbit thymocytes. Data are the mean (±5 per cent) of two separate experiments, in both of which each incubation was duplicated. The concentrations of the radioactive precursors added to the incubation medium were : amino acids = 0·008 μg/ml; uridine = thymidine = 0·25 μM. The radioactivity measured in the end products in the drug free controls was of the order of 10^4 dpm of ^{14}C (protein) or 10^5 dpm of ^{3}H (nucleic acids).

† Drug-free controls equal 100 per cent.

After preincubating cells with the cucurbitacins (50 μM) and then transferring the cells to a fresh drug-free medium for incubation with thymidine-^{3}H, the inhibitory effect of the dihydro compound was almost completely abolished by this washing procedure, but the activity of the parent cucurbitacins was still manifested. These findings, together with the fact that the parent cucurbitacins lost their drug activity on preincubation with thiols whereas the dihydro compound did not, suggests that the unsaturated ketone (Δ^{23}-22-one) grouping in the cucurbitacin side chain may bind irreversibly to cellular thiol groups. While this may explain the greater toxicity of the parent cucurbitacins (compared with their dihydro derivatives), they probably inhibit thymidine incorporation through some other, more selective, process than just indiscriminate binding to cellular thiols, since the dihydro compound is likewise effective, and other thiol-blocking agents tend to inhibit protein and RNA synthesis in lymphocytes just as readily as they inhibit DNA synthesis.[5]

It is concluded that cucurbitacins may have useful pharmacological activity (possibly as carcinostatic or immunosuppressive agents) through blocking DNA synthesis by mechanisms other than simply combining with essential thiols. The lower toxicity of dihydrocucurbitacin B and ready reversibility of its action *in vitro* suggest that saturation of the double bond at C-23 in the naturally occurring cucurbitacins may improve their potential therapeutic usefulness.

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