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CLAVINES

NEW ANTIBIOTICS WITH CYTOSTATIC ACTIVITY

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Abstract—The cytostatic potentials of ten ergolines were determined in the L5178y mouse lymphoma cell system; six of them belong to the clavines (agroclavine, 1-propyl-agroclavine, 1-propyl-festuclavine, 1-allyl-festuclavine, 6-cyano-6-nor-festuclavine and 1-hydroxymethyl-festuclavine) and four to the lysergic acid derivatives (methylergometrine, lysergic acid amide, isolysergic acid amide and lysergic acid diethylamide). It is shown that agroclavine (ED_{50}: 3.9 μM), 1-propyl-agroclavine (3.5 μM), 1-propyl-festuclavine (4.3 μM) and 1-allyl-festuclavine (4.3 μM) are potent cytostatic agents. Up to $2 \times ED_{50}$ concentration the inhibitory effect was completely reversible. Incorporation studies suggested that the compounds inhibit DNA synthesis; this assumption was also supported by the findings which revealed that after incubation with these clavines, the cells showed slight 'unbalanced growth'. 6-Cyano-6-nor-festuclavine was less inhibitory (ED_{50} 11.8 μM). 1-Hydroxymethyl-festuclavine and all lysergic acid derivatives tested were without any detectable activity.

Clavines are alkaloids of the ergoline type which have not yet been used therapeutically. They are produced by fungi of the genus *Claviceps* [1] and by dicotyledons of the family Convolvulaceae [2]. The first members of this class of alkaloids, agroclavine and elymoclavine, were discovered at the beginning of the fifties by Abe [3–6]. In contrast to the therapeutically used ergot alkaloids (e.g. ergotamine) [7, 8], the C-atom 17 in the clavines has a lower oxidation stage (Fig. 1). Furthermore not all clavines have the 9,10-double bond in their structure; in agroclavine and elymoclavine it is shifted to position 8,9 and in festuclavine (6,8β-dimethylergoline) it is even lacking in this region.

In spite of differences in the chemical structure, the clavines interact—like the lysergic acid amides—with dopamine and 5-hydroxytryptamine receptors and with a lower affinity even with α-adrenoceptors. Pharmacologically, the clavines cause apathy, drowsiness and sedation in several mammalian systems. Moreover they have hypotensive and oxytocic properties and inhibit prolactin secretion [9–14]. Recently we reported that agroclavine and elymoclavine possess antibiotic activity against a series of pathogen and apathogen bacteria [15], which can be augmented by partial synthetic transformation of agroclavine and festuclavine [16]; ergometrine and ergotamine were inactive in this system. The mode of action of the clavines in the bacteria is not known.

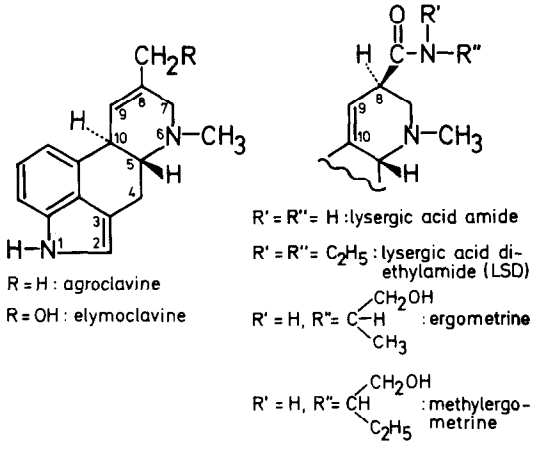

Fig. 1. Chemical structures of the ergoline alkaloids used in the present study.

In the present contribution the influence of clavines on proliferation of mammalian cells has been studied. Using L5178y mouse lymphoma cells, the activity of some clavines to act cytostatically has been demonstrated. In a first approach it is shown that one of the active derivatives, 1-propyl-festuclavine, inhibits incorporation of thymidine into DNA.

MATERIALS AND METHODS

Compounds. The following materials were obtained: [methyl-^{3}H]thymidine (specific activity 12 Ci/mmole), [^{3}H]uridine (generally labeled; sp. act. 7.6 Ci/mmole) and [^{3}H]phenylalanine (sp. act. 9.3 Ci/mmole) from The Radiochemical Centre, Amersham (England); lysergic acid diethylamide and methylergometrine-maleate (Methergin®) from Sandoz AG, Basel (Switzerland).

Lysergic acid amide and isolysergic acid amide were isolated from submerged cultures of *Claviceps paspali*, strain P3, originally collected near Rome (Italy) [17]. Agroclavine was isolated from surface cultures of Claviceps strain 47a by a phase separation procedure (Eich; to be published).

The following compounds were synthesized: Festuclavine was obtained by hydrogenation of agroclavine with Raney-nickel in methanol [18]. The agro- and festuclavine derivatives, substituted at N-1, were synthesized from the natural alkaloids by alkylation of the indole nitrogen via a reaction with potassium and the corresponding alkyl iodide in ammonia [19, 20]. These compounds were prepared as hydrogentartrates. 1-Hydroxymethyl-festuclavine was obtained from festuclavine after treatment with formaldehyde [21] and 6-cyano-6-nor-festuclavine after reaction with cyanogen bromide [22]. The purity of the compounds was greater than 98%, as determined by chromatographical methods as well as by elementary analysis.

All ergoline compounds, with the exception of methylergometrine-maleate, were present as tartrates in aqueous solutions. Immediately before testing for biological activity, the solutions were neutralized with 0.1 N NaOH.

Cell culture. L5178y mouse lymphoma cells were maintained in suspension culture as described previously [23]. For the dose–response experiments the cultures (5 ml) were initiated by inoculation of 4×10^3 cells/ml and incubated at 37° in roller tubes for 72 hr; the controls reached cell concentrations of 5.2×10^5/ml. In parallel experiments, 80 ml spinner cultures [24] were inoculated with 10^5 cells/ml and incubated for 24 hr; controls: 4.8×10^5 cells/ml. Equimolar concentrations of tartrate or maleate were taken as controls. In our test system, these two compounds were inhibitory only at high concentrations; 35 mM of tartrate reduced cell growth by 8% and 45 mM maleate by 32%.

Cell concentrations and volume distributions were determined with a Model B Coulter counter with a 32-channel size-distribution plotter [24].

Statistical methods. The ED_{50} was estimated by logit regression [25]. The slope of the dose–response curve at the ED_{50} was calculated [25]. The size distribution curves are described by the following parameters as reported earlier [24]; mean value: the mean of the distribution, skewness: deviation of the distribution from symmetrical normal (=0; negative: deviation to the right; positive: deviation to the left), kurtosis: extent of curvature of the distribution with respect to normal (=0; negative: broader than normal; positive: smaller than normal). After calibration with paper mulberry pollen (diameter 12–13 μm), the mean values were converted from channel numbers to the absolute cell volumes (given in μm^3).

Incorporation of nucleic acid- and protein precursors. For the determination of DNA-, RNA- and protein synthesis, 5-ml suspensions of exponentially growing cells at 100,000 cells/ml were supplemented with the compound 24 or 2 hr prior to the addition of the labeled precursors 10 μCi [^{3}H]dThd, 10 μCi [^{3}H]Urd or 10 μCi [^{3}H]phenylalanine. The incubation was continued for 2 hr. Samples of 1 ml were analyzed for cell concentration and for acid-insoluble radioactivity [26].

RESULTS

Cytostatic activity of the individual agents. Among the different agroclavine- and festuclavine derivatives tested, 1-propyl-agroclavine, 1-propyl-festuclavine and 1-allyl-festuclavine strongly inhibited L5178y cell proliferation. The ED_{50} concentrations of these compounds in the roller tube assays (inoculation: 4000 cells/ml; incubation period: 72 hr) were determined to range from 3.5 to 4.3 μM (Table 1; Fig. 2). 6-Cyano-6-nor-festuclavine was 3-fold less active. Using the higher inoculation concentration of 100,000 cells/ml 4-fold higher ED_{50} concentrations were determined (Table 1). The slopes of the dose-response curves are steep. In the experiments with 1-propyl-festuclavine, the concentration range 1–10 μM included the complete transition from non-inhibition to complete inhibition of growth of L5178y cells (Fig. 2). One festuclavine derivative, 1-hydroxymethyl-festuclavine, and the four lysergic acid amide derivatives (methylergometrine, lysergic acid amide, isolysergic acid amide and lysergic acid diethylamide) were determined to cause no cytostatic effect up to 20–30 μM concentrations.

The cytostatically active agroclavine-and festuclavine derivatives caused 'unbalanced growth' [27] of L5178y cells. The mean size (estimated on the basis of 25,000 cells) of untreated cells was determined to be 1526 ± 622 μm^3 (mean value: 14.03 ± 5.72; Table 1), while in the presence of the com-

Table 1. Cytostatic activity of selected agroclavine, festuclavine and lysergic acid derivatives

Compound	ED_{50} conc. (μM) 4000 (cells/ml)	100,000 (cells/ml)	Parameters at the ED_{50} concentration Slope	Mean value	Skewness	Kurtosis
Agroclavine	3.91 ± 0.30	18.4 ± 1.4	−4.19	15.31 ± 6.47	+0.51	−0.25
1-Propyl-agroclavine	3.54 ± 0.28	16.8 ± 1.4	−4.27	15.26 ± 6.27	+0.52	−0.31
1-Propyl-festuclavine	4.25 ± 0.31	20.8 ± 1.4	−4.33	15.72 ± 6.44	+0.43	−0.43
1-Allyl-festuclavine	4.28 ± 0.30	19.7 ± 1.5	−4.29	15.01 ± 6.43	+0.51	−0.27
6-Cyano-6-nor-festuclavine	11.84 ± 1.02	55.4 ± 4.6	−3.42	15.32 ± 6.71	+0.54	−0.37
1-Hydroxymethyl-festuclavine	>20	>50	—	14.07 ± 5.83	+0.65	−0.19
Methylergometrine	>30	>50	—	14.21 + 5.86	+0.58	−0.15
Lysergic acid amide	>30	>50	—	14.74 ± 5.17	+0.61	−0.24
Isolysergic acid amide	>30	>50	—	14.11 ± 5.91	+0.57	−0.19
Lysergic acid diethylamide	>30	>50	—	14.18 ± 5.25	+0.73	−0.27
Control	—	—	—	14.03 ± 5.72	+0.75	−0.22

The ED_{50} concentrations were determined under the following two conditions; firstly, inoculation 4000 cells/ml and incubation period 72 hr and secondly, inoculation 100,000 cells/ml and incubation period 24 hr. The different parameters, determined at the ED_{50} concentration under incubation condition 1, are given.

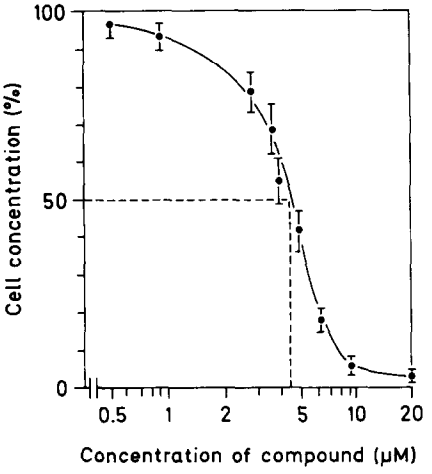

Fig. 2. Effect of 1-propyl-festuclavine on cell proliferation of L5178y cells. Each value (mean ± S.D.) comes from 10 parallel assays. The solid curve was estimated by semi-logarithmic regression; ---, ED_{50} value; x-axis, final compound concentration; y-axis, cell concentration (%) after an incubation period of 72 hr (controls 100%). The assay conditions are described under Materials and Methods.

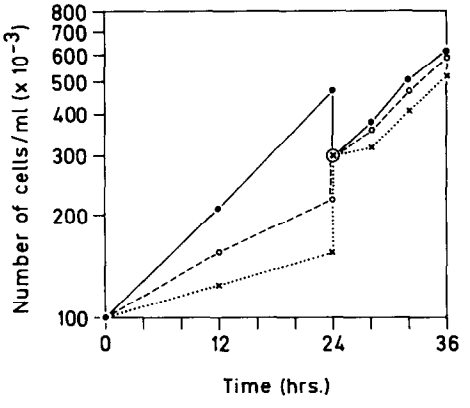

Fig. 3. Reversibility of the effect of 1-propyl-festuclavine on cell proliferation. The experiments were performed in spinner cultures (inoculation 100,000 cells/ml). Three parallel cultures were supplemented with 0 μM (●—●), 20 μM (○--○) or 50 μM of 1-propyl-festuclavine (×·····×) and incubated for 24 hr. Then the compound was washed out and the cells were transferred into fresh medium/serum; the concentration was adjusted to 300,000 cells/ml and incubation in the absence of the compound proceeded for 12 hr.

pounds the mean size of the cells increased to 1665 μm^3 (15.3). Moreover, the distribution curves of treated cells show a deviation to the right (skewness between +0.43 and +0.54) compared to the controls (skewness +0.75). Parallel with these changes, a shift of the kurtosis of the curves from −0.22 (controls) to values between −0.25 and −0.43 (compound-treated cultures) resulted (Table 1), which means a broader distribution of the cell population recorded from treated cultures. Taken together, the results on the changes of the cell volumes, as well as the shifts of the kurtosis and skewness of the size distribution curves, registered from cells treated with clavines indicate an at least slight 'unbalanced growth'. The same changes of the size distribution curves have been quantified from *in vitro* experiments with bleomycin [24]. The ability of this drug to cause 'unbalanced growth' is well proven [24].

The viability of the cells was only slightly changed when treated for 24 hr with 1-propyl-festuclavine (Fig. 3), at approximately 1 × ED_{50} and 2 × ED_{50} concentrations. After transferring the cells into fresh medium/serum, the growth kinetics of the treated cultures show the same slope as the controls. Only a slight lag-phase of approximately 4 hr was determined for the assays with 2 × ED_{50}.

Influence on synthesis of macromolecules in vivo. In a further approach to elucidate the mode of action of agroclavine- and festuclavine derivatives, incorporation studies with the radioactively labeled precursors thymidine, uridine and phenylalanine were performed (Table 2). The experiments with 1-propyl-festuclavine showed that after pretreatment of the cells for 24 hr with up to twice the ED_{50} concentration, the compound selectively inhibited the incorporation rate of thymidine into DNA, whereas the rates for the incorporation of uridine into RNA and phenylalanine into protein were determined not to be significantly changed. Experiments under shorter preincubation conditions (2 hr) resulted in a nonsignificant change of the incorporation rates; controls: [^{3}H]thymidine incorporation rate 84,300 dpm/100,000 cells × 2 hr, [^{3}H]uridine incorporation 15,350 dpm, and [^{3}H]phenylalanine incorporation 14,800 dpm; cultures treated with 20.8 μM of 1-propyl-festuclavine: [^{3}H]thymidine incorporation 82,450 dpm, [^{3}H]uridine incorporation

Table 2. Influence of 1-propyl-festuclavine on the synthesis of macromolecules in exponentially growing cells

Compound conc.		Incorporation into macromolecules/100,000 cells [^{3}H]Thymidine		[^{3}H]Uridine		[^{3}H]Phenylalanine	
(μM)	(ED_{50})	(dpm)	(%)	(dpm)	(%)	(dpm)	(%)
0	0	63,400	100	14,700	100	13,200	100
10.4	0.5	37,800	60	15,200	103	12,900	98
20.8	1	22,200	35	15,600	106	13,100	99
41.6	2	14,900	24	13,900	95	12,400	94

The procedures were as described in Materials and Methods. The values represent means of 5 parallel experiments. S.D. does not exceed 10%. The cells have been pretreated with the compound for 24 hr.

15,700 dpm and [^{3}H]phenylalanine incorporation 13,750 dpm (3 parallel experiments; S.D. does not exceed 12%).

DISCUSSION

In the present study the cytostatic potential of ten ergolines has been examined; among them six belong to the clavines and four are derivatives of lysergic acid amides. With the exception of 1-hydroxymethyl-festuclavine, all other five clavines tested show an inhibitory influence on proliferation of L5178y cells; the ED_{50} concentrations (inoculation 4000 cells/ml) were determined to range from 3.5 to 11.8 μM. The four derivatives of lysergic acid amides were inactive. The cytostatic potential of the active clavines is comparatively high; under otherwise identical conditions the therapeutically used cytostatics bleomycin (28) and 9-β-D-arabinofuranosyl adenine [29] reduce cell proliferation by 50% at 1 and 2.9 μM respectively. In a first approach to determine the mode of action of the cytostatically active clavines, incorporation studies were performed. They revealed that the incorporation of thymidine into DNA is reduced in the presence of 1-propyl-festuclavine; no influence has been determined for uridine- and phenylalanine incorporation. The inhibition of thymidine incorporation rate is only observed after a preincubation period with the compound for 24 hr and is not detectable after the shorter pretreatment period for 2 hr. Knowing that data from thymidine incorporation studies do not reflect absolute values of the extent of DNA synthesis, we still favor the assumption that clavines interfere with DNA replication processes in L5178y cells rather than an unspecific effect of the compound on cell growth. This conclusion is based on the observation, that all active clavines caused 'unbalanced growth' [27], a property which they share with other cytostatic agents acting selectively by inhibition of DNA synthesis. Future studies must show, if the clavines are taken up by mammalian cells. The clavine-related lysergoyl derivatives are known to penetrate into splenic lymphocytes [30]. It is interesting to note, that the cytostatically active clavines are also active in bacteria systems [15, 16]. A substitution at C-atom 8 [methyl group (clavines) by carbonyl amide moiety (lysergic acid amide)] abolishes the cytostatic activity of ergot alkaloids.

Already at the present state of knowledge, some further conclusions about the structure–activity relationship can be drawn. 1-Propyl-agroclavine was found to be a little more active than the nonsubstituted natural compound. Hydrogenation of the double bond at position 8,9 slightly diminished the cytostatic potential, whereas the existence of a double bond in the side-chain at N-1 was of no influence. A replacement of the more lipophilic side-chains at N-1 by the 1-hydroxymethyl group resulted in a total abolition of the biological activity of this clavine derivative in the L5178y cell system. In contrast to the findings with mammalian cells, 1-hydroxymethyl-festuclavine was determined to be in bacteria even more active than 1-methyl-festuclavine or agroclavine [16]. 6-Cyano-6-norfestuclavine, which is not chemically classified into the group of alkaloids, because of the presence of the cyano group at N-6, showed only about one third of the cytostatic activity compared to the N-1 substituted genuine clavines. Again, in the bacteria system, this compound was comparatively more active than the other clavine derivatives [16].

With the discovery, that *Claviceps purpurea* and related fungi produce cytostatically active secondary metabolites, the clavines, a further group of alkaloids (besides Camptothecin; ref. 31) is now known which interferes with DNA synthesis in mammalian cells. The ED_{50} concentration of Camptothecin in the L5178y cell system was determined to be 7.2 μM.

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