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THE INFLUENCE OF THE SESQUITERPENE LACTONES FROM *GEIGERIA* ON MAST CELL DEGRANULATION

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Abstract—The sesquiterpene lactones isolated from *Geigeria* were found to be incapable of inducing rat peritoneal mast cell degranulation at levels of 0.3–1.6 mM. The sulphydryl reagent, *N*-ethylmaleimide, too was unable to trigger mast cell secretion. Instead, it was observed that these compounds inhibited the release of histamine induced by Compound 48/80. Pretreatment of the lactones and *N*-ethylmaleimide with the amino acid, L-cysteine, reduced their inhibition ability of histamine release to a considerable extent, but not completely. Geigerin(V), which lacks an α-methylene group and the chemically prepared cysteine-adduct of dihydrogriesenin(I), were also capable of inhibiting mast cell secretion by Compound 48/80, but to a lesser extent.

Sesquiterpene lactones may be grouped to include: antineoplastic agents, insect feeding deterrents, plant growth regulators, antimicrobial agents, schistosomicidal agents and vertebrate poisons [1]. With a few exceptions the active sesquiterpene lactones contain an exocyclic α,β-unsaturated lactone moiety [2]. In general, these compounds are good alkylating agents and are therefore able to combine with sulphydryl groups in key enzymes that control cell division thus interfering with the normal growth pattern of both plants and animals [2, 3–8].

The plant species *Geigeria*, commonly known as vomiting shrub is responsible for vomiting disease in sheep [9]. Previous investigations have shown that the sesquiterpene lactones from *Geigeria* inhibit mitochondrial respiration [10, 11], and recently they were found to irreversibly inhibit the activities of the glycolytic enzymes phosphofructokinase, hexokinase and glyceraldehyde-3-phosphate dehydrogenase [12].

It has been reported that helenalin(VII), hymenovin(VIII), thapsigargine(IX) and thapsigargicine(X) release histamine from mast cells [13–15]. Here evidence is presented that lactones from *Geigeria* do not cause the direct release of histamine from mast cells, but inhibit the release induced by Compound 48/80.

MATERIALS

Materials. Dihydrogriesenin(I), geigerinin(II) and ivalin(III) were isolated from *G. aspera* [16–18]. Geigerin(V) and vermeerin(VI) were gifts from Dr L. A. P. Anderson (Veterinary Research Institute, Onderstepoort). The dihydrogriesenin-cysteine adduct (DHG-cys, IV) was prepared as described elsewhere [19].

Compound 48/80, *N*-ethylmaleimide (NEM), Ruthenium red and o-phtalaldehyde were from the Sigma Chemical Company. Percoll (polyvinylpyrrolidone coated silica particles) was obtained from Pharmacia Fine Chemicals. Other reagents were from Merck Chemicals and were of analytical grade. Solutions were made up in double-distilled water.

Preparation of peritoneal cell suspension. Male Sprague–Dawley rats (usually three) were sacrificed with ether. Mixed peritoneal cells were collected by injecting 10 ml phosphate-free buffered solution (20 mM Hepes, pH 7.5; 137 mM NaCl; 2.7 mM KCl; 1 mM $MgCl_2$; 1.8 mM $CaCl_2$; 10 μM EGTA; 5.6 mM glucose; 1 mg/ml bovine serum albumin) [20] into the abdominal cavity through a small incision. After gentle massage for 2 min, the fluid containing the peritoneal cells was aspirated and centrifuged at 120 *g* for 10 min (Beckman Model J6 Centrifuge) at 20°. The supernatant was filtered through a 0.22 μM Millex-GV filter unit (Millipore Corporation) and the sedimented cells were resuspended in 0.5 ml of the filtrate referred to as preconditioned buffer [21].

Separation of mast cells. The resuspended cell suspension was carefully layered on top of a preformed Percoll continuous gradient [22] and centrifuged at 450 *g* for 15 min at 20°. After eliminating the upper bands containing erythrocytes, leucocytes and macrophages, the mast cells were aspirated and washed twice with 8 ml preconditioned buffer. The cells were collected after each washing by centrifugation at 120 *g* for 5 min at 20°. Following the final wash the cells were resuspended in 1 ml preconditioned buffer and kept at 20°. Cells were prewarmed to 37° for 10–20 min before the incubation procedures. Cells isolated at 4° according to the method in [22] and prewarmed to 37° before incubation were found to be unresponsive to mast cell stimulation by Compound 48/80, while isolation of mast cells at 20° [21] rendered cells that were responsive to mast cell degranulation by Compound 48/80.

Light microscopy. Biological reactivity of separated mast cells was tested by mixing 20 μl of the cell suspension, 20 μl 0.005% Ruthenium red and

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20 μl Compound 48/80 (1 μg/ml) or ivalin (0.4 mM) [22].

Incubation of mast cells with Compound 48/80, the Geigeria *lactones and NEM.* Twenty microlitre aliquots of the mast cell suspension containing 1–2 × 10^4 cells were incubated with 580 μl phosphate-free buffered solution at 37°, containing either Compound 48/80, *Geigeria* lactones or NEM. Spontaneous release was measured by including controls in which these compounds were omitted. Tubes containing Compound 48/80 were incubated for 5 min, while those containing the lactones and NEM were incubated for 30 min after which the tubes were rapidly cooled in ice-water. The cells were sedimented at 250 *g* for 10 min at 0–4°, and were washed once with 600 μl of ice-cold buffer. The supernatants were combined for the analysis of histamine.

Table 1. Effect of the *Geigeria* lactones and NEM on mast cells

Compound	Concentration	% Histamine release‡
Compound 48/80*	1 μg/ml	45 ± 6
Dihydrogriesenin (I)†	0.4 mM	2 ± 3
Geigerinin (II)	0.4 mM	1 ± 6
Ivalin (III)	0.4 mM	1 ± 2
Geigerin (V)	0.4 mM	1 ± 6
Vermeerin (VI)	0.3 mM	5 ± 3
	1.6 mM	6 ± 1
NEM	1.0 mM	2 ± 1

* Mast cells were pre-incubated for 30 min at 37°. Hereafter, Compound 48/80 was added and the cells were incubated for an additional 5 min.

† Mast cells were incubated with the various lactones (100 μg/ml) and NEM (125 μg/ml) for 30 min at 37°.

‡ Values have been corrected for spontaneous release (8 ± 3%, mean ± SEM of nine experiments) and are reported as the mean ± SEM of three different experiments.

In a separate experiment, mast cells were pre-incubated with the sesquiterpene lactones or NEM or their cysteine derivatives for 30 min at 37° in a final volume of 600 μl of buffer. Thereafter, Compound 48/80 was added and the incubation was continued for an additional 5 min. The rest of the procedure was followed as above.

The cysteine derivatives of NEM and the various sesquiterpene lactones were prepared by incubating a mixture of the compound and L-cysteine (0.8 mM) for 30 min at 37°. Appropriate controls to measure spontaneous release in the presence of cysteine alone were also included.

Histamine determination. Histamine was measured fluorometrically by the procedure of Siraganian *et al.* [23]. The method is based on the coupling of histamine with o-phtalaldehyde in strong alkaline solution. The resulting labile fluorescent product is stabilized upon acidification. The fluorescence at 450 nm resulting from excitation at 350 nm was measured in a Farrand spectrofluorometer. Histamine release is expressed as a percentage of the total histamine content. The total content was determined by suitably diluting samples of cell suspensions and heating at 100° for 5 min.

RESULTS

Light microscopy

The biological reactivity of the isolated mast cells towards Compound 48/80 (1 μg/ml) in the presence of Ruthenium red was followed microscopically. The majority of the cells reacted promptly and vigorously on exposure to the reagent, with accompanying extrusion of granules.

The same procedure was carried out for mast cells in the presence of ivalin(III) at 0.4 mM. No degranulation of mast cells was observed and on addition of Compound 48/80 to the ivalin-treated cells; only a few cells were seen to degranulate as opposed to the untreated cells.

Table 2. Inhibition of Compound 48/80 release by the various compounds and their cysteine-derivatives

Compound	% Histamine release by C48/80†	% Inhibition of C48/80 release	% Histamine release by C48/80 (cysteine pretreatment)‡	% Inhibition of C48/80 release (cysteine pretreatment)
Compound 48/80*	45 ± 6	0	45 ± 6	0
Dihydrogriesenin (I)	2 ± 2	96	28 ± 10	38
Geigerinin (II)	14 ± 2	69	31 ± 4	31
Ivalin (III)	9 ± 2	80	31 ± 4	31
Geigerin (V)	40 ± 3	11		
Vermeerin (VI)	32 ± 2	29	39 ± 4	13
DHG-cys (IV)	34 ± 1	24		
NEM	2 ± 2	96	24 ± 4	47

* Mast cells were pre-incubated for 30 min at 37°. Hereafter, Compound 48/80 (1 μg/ml) was added and the cells were further incubated for 5 min. Mean ± SEM of three experiments.

† Mast cells were pre-incubated with the various lactones (100 μg/ml) and NEM (125 μg/ml) for 30 min at 37°. Compound 48/80 (1 μg/ml) was then added and the incubation was continued for 5 min. Mean ± SEM of two different experiments, each done in duplicate.

‡ Various lactones (I–III, VI) and NEM were incubated with L-cysteine (100 μg/ml) for 30 min at 37°. The cells were then added to this mixture and incubated for 30 min. Compound 48/80 (1 μg/ml) was added and the cells were then incubated for an additional 5 min. Mean ± SEM of two different experiments, each done in duplicate.

*†‡ Values have been corrected for spontaneous release (8 + 3%, mean ± SEM of nine experiments).

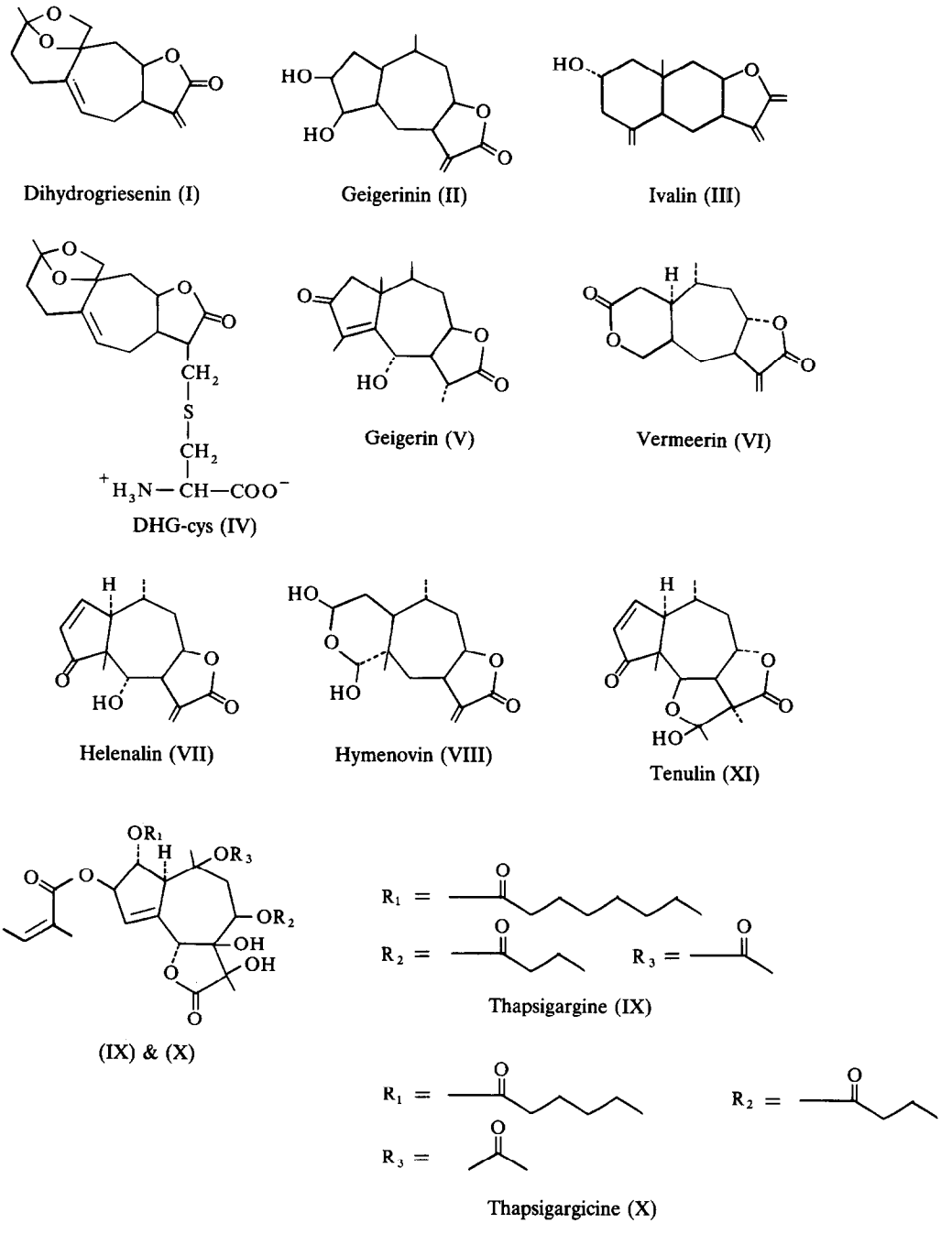

Fig. 1. Structures of the sesquiterpene lactones.

Effect of the Geigeria *lactones and NEM on mast cells*

Compound 48/80, a condensation product of *N*-methyl-*p*-methoxyphenethylamine with formaldehyde is a known histamine releaser [24] and was used as a positive control (see Tables 1 and 2). The results in Table 1 show that histamine release was minimal (1–5%) when mast cells were incubated with the various lactones (I–III, V, VI; see Fig. 1 for structures) at concentrations of 0.3–0.4 mM. At a higher concentration of 1.6 mM, vermeerin(VI) induced a release of about 6%. Similarly, release in the presence of 1.0 mM NEM was found to be minimal (2%).

Effect of pre-incubation of the sesquiterpene lactones and NEM on histamine release induced by Compound 48/80

Table 2 summarizes the results of experiments in which mast cells were pre-incubated with the different compounds prior to mast cell stimulation by Compound 48/80. The results indicate that the lac-

tones (I–III, V, VI) and NEM all inhibited histamine release by Compound 48/80. Both dihydrogriesenin-(I) and NEM at concentrations of 0.4 mM and 1.0 mM respectively, caused 96% inhibition of histamine release, while the DHG-cys(IV) adduct inhibited release only by 24%. Pretreatment of cells with geigerinin(II) and ivalin(III) caused an inhibition of 69 and 80%, respectively. Vermeerin(VI) inhibited the release by 29%, while geigerin(V) which lacks an α-methylene group, inhibited by 11%.

Effect of the alkylation of the lactones and NEM with L-cysteine prior to mast cell stimulation by Compound 48/80

Addition of cysteine to four of the lactones (I–III, VI) and NEM reduced their inhibitory effect on histamine release induced by Compound 48/80 (Table 2). Alkylation of dihydrogriesenin(I), ivalin(III) and NEM reduced their inhibition between 49–58%, while alkylation of geigerinin(II) and vermeerin(VI) diminished their inhibition by 38% and 16%, respectively.

DISCUSSION

Previous investigations [13–15], showed that the sesquiterpene lactones helenalin(VII), hymenovin(VIII), thapsigargine(IX) and thapsigargicine(X) stimulated mast cell secretion. At concentrations of 100 μg/ml (0.4 mM), helenalin(VII) released approximately 35% histamine, while hymenovin(VIII) induced a release of about 65% [13]. Tenulin(XI) which lacks an α-methylene group and at a concentration of 100 μg/ml (0.3 mM) was ineffective in causing mast cell secretion, in contrast to helenalin(VII) and hymenovin which contain α-methylene-γ-lactone groups. Furthermore, alkylation of helenalin(VII) and hymenovin(VIII) with cysteine, significantly reduced their ability to degranulate mast cells. Thapsigargine(IX), which contains an α,β-unsaturated carbonyl moiety in the angeloyl group instead of an α-methylene-γ-lactone group, released approximately 50% histamine at a concentration of 0.05 μg/ml (0.08 μM) [14]. Contrary to these findings, the *Geigeria* lactones, which have structures similar to lactones VII and VIII, caused no significant histamine release (Table 1). Similarly, the thiol-alkylating agent, NEM, was found to be ineffective. Further confirmation of these results was obtained from the qualitative light microscopy studies in which cells in the presence of ivalin(III) were seen not to degranulate as opposed to the almost total degranulation observed in the presence of Compound 48/80.

Ethacrynic acid, which contains a α,β-unsaturated ketone and NEM are both known alkylating agents of sulphydryl-catalyzed enzyme systems [25, 26]. It has been reported that these two compounds completely blocked the release of histamine induced by Compound 48/80 and the calcium ionophore, A23187 [25]. During the preparation of this manuscript it was reported [27] that an extract of the plant *Centipeda minima* showed potent inhibition of histamine release from rat peritoneal mast cells induced by Compound 48/80 or Concanavalin A. The isolated α,β-unsaturated-γ-sesquiterpene lactones arnicolode and 6-0-senesioylphenolin from this plant, were found to inhibit histamine release by Concanavalin A [28]. Accordingly, it was found that the lactones (I–III, V, VI), DHG-cys(IV) and NEM inhibited histamine release by Compound 48/80 (Table 2). Various degrees of inhibition were observed for the different compounds. These results are corroborated by the observation that ivalin-treated cells are almost unresponsive towards stimulation by Compound 48/80.

In order to investigate whether the inhibition observed by the lactones involved their reaction with thiol-containing components, mast cells were challenged with the cysteine-treated lactones. Alkylation of the various lactones significantly diminished but did not completely eliminate their inhibitory effect on histamine release (Table 2). The remaining ability of the cysteine-treated lactones to inhibit mast cell degranulation may be due to the incomplete alkylation of all the available α-methylene-γ-lactone groups. However, it has been shown that these lactones react rapidly with cysteine and that the reaction is completed within 20 min at room temperature [19]. Since it was found that the controls in which cells were incubated in the presence of L-cysteine (0.8 mM) did not differ from those where cells were incubated alone (8 ± 3% spontaneous release) it was decided that removal of the unreacted cysteine was unnecessary. Nevertheless, the findings that geigerin(V), which lacks an α-methylene group, and the DHG-cys(IV) adduct also inhibit histamine release suggest that although the α-methylene-γ-lactone moiety may be the principal prerequisite for inhibition, there may be other structural requirements on the molecule that contribute to the inhibition.

The reason why helenalin(VII) and hymenovin (VIII) release histamine and the *Geigeria* lactones do not, is not obvious. A possible reason why thapsigargine(IX) and thapsigargicine(X) release histamine in contrast to the *Geigeria* lactones is the fact that the former compounds contain no α,β-unsaturated-γ-lactone group. Furthermore, it was reported [29] that when the hydroxyls on positions 7 and 11 were replaced with an epoxide in thapsigargine(IX) and thapsigargicine(X), no histamine was released. It appears then that the possible alkylation of sulphydryl groups by the α,β-unsaturated carbonylmoiety in the angeloyl groups of IX and X plays a minor role, if any, in the histamine releasing properties of these compounds. The *Geigeria* lactones do not contain hydroxyl groups in these positions.

Since the sesquiterpene lactones from *Geigeria* were unable to degranulate mast cells, it appears that direct histamine release plays a minor or no role in vomiting disease.

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REFERENCES

1. E. Rodgrigues, G. H. N. Towers and J. C. Mitchell, *Phytochemistry* **15**, 1573 (1976).

2. K. L. Stevens, in *Isopentenoids in Plants: Biochemistry and Function* (Eds. W. D. Nes *et al.*), p. 65. Marcel Dekker, New York and Basel (1984).
3. I. H. Hall, K. H. Lee, M. Eng and C. O. Starnes, *J. med. Chem.* **20**, 333 (1977).
4. K. H. Lee, I. H. Hall, E. Mar, C. O. Starnes and S. A. Eigebaly, *Science* **196**, 533 (1977).
5. C. H. Smith, J. Larner, A. M. Thomas and S. M. Kupchan, *Biochim. biophys. Acta* **276**, 94 (1972).
6. S. M. Kupchan, D. C. Tessler, M. A. Eakin and T. J. Giacobbe, *Science* **168**, 376 (1970).
7. R. L. Hanson, H. A. Lardy and S. M. Kupchan, *Science* **168**, 378 (1970).
8. I. H. Hall, K. H. Lee and S. A. Eigebaly, *J. Pharm. Sci.* **1968**, 522 (1978).
9. J. F. W. Grosskopf, Tech. Comm. No. 21, 1, Dept. Agric. Techn. Services, Pretoria (1964).
10. C. H. van Aswegen, D. J. J. Potgieter and N. M. J. Vermeulen, *S. Afr. J. Sci.* **75**, 84 (1979).
11. C. H. van Aswegen, D. J. J. Potgieter and N. M. J. Vermeulen, *S. Afr. J. Sci.* **78**, 125 (1982).
12. A. R. M. D. Gaspar, D. J. J. Potgieter and N. M. J. Vermeulen, *Biochem. Pharmac.* **35**, 493 (1986).
13. M. H. Elissalde, G. W. Ivie, L. D. Rowe and G. S. Elissalde, *Am. J. vet. Res.* **44**, 1894 (1983).
14. U. Rasmussen and S. B. Christensen, *Acta Pharm. suec.* **15**, 133 (1978).
15. S. A. Patkar, V. Rasmussen and B. Diamant, *Agents and Actions* **9**, 53 (1979).
16. J. A. Hamilton, A. T. McPhail and G. A. Sim, *J. Chem. Soc.* **708** (1962).
17. N. M. J. Vermeulen, M. E. Vogelzang and D. J. J. Potgieter, *Agrochemophysica* **10**, 1 (1978).
18. M. E. Vogelzang, N. M. J. Vermeulen, D. J. J. Potgieter and H. Strauss, *Phytochemistry* **17**, 2030 (1978).
19. J. A. Verschoor, L. Bornman and N. M. J. Vermeulen, *Onderstepoort J. vet. Res.* in press
20. S. Cockcroft and B. D. Gomperts, *Biochem. J.* **178**, 681 (1979).
21. L. Thilo, *Proc. natn. Acad. Sci. U.S.A.* **82**, 1711 (1985).
22. A. Nemeth and P. Röhlich, *Eur. J. cell. Biol.* **20**, 272 (1980).
23. R. P. Siraganian, in *Manual of Clinical Immunology* (Eds. N. R. Rose *et al.*), p. 603. American Society of Microbiology, Washington, DC (1976).
24. F. C. McIntosh and W. D. Paton, *J. Physiol., Lond.* **109**, 190 (1949).
25. N. K. Chakravarty, in *The Mast Cell, Its Role in Health and Disease* (Eds. J. Pepys *et al.*), p. 38. Pitman Medical, London (1979).
26. K. H. Beyer, J. E. Baer, J. K. Michaelson and H. F. Russo, *J. Pharmac. exp. Ther.* **147**, 1 (1965).
27. Y. Hirai, H. Takase, H. Kobayashi, M. Yamamoto, N. Tujioka, H. Kohda, K. Yamasaki, T. Yasvhara and T. Nakamima, *Shoyakugaku Zasshi* **37**, 374 (1983).
28. J. Wu, Y. Chun, Y. Ebizuka and V. Sankawa, *Chem. pharm. Bull., Tokyo* **33**, 4091 (1985).
29. S. B. Christensen, I. K. Larsen and U. Rasmussen, *J. org. Chem.* **47**, 649 (1982).