cene makes it difficult to explain the cosmopolitan nature of the Indian terrestrial faunas during this period. These findings suggest the likelihood of a migration from eastern Africa sometime during the Upper Cretaceous (about 80 million years ago) as India drifted close to the east coast of Africa and Madagascar. A possible filter corridor from Africa may be found in two aseismic ridges, the Mascarene Plateau and the Chagos-Laccadive Ridge. Deep-Sea Drilling Project (DSDP) site 237 bottoms in shallow-water Paleocene sediments and has a bathymetry (less than 100 m) similar to that estimated for Paleocene sediments at DSDP site 219 on the Chagos-Maldive Ridge. Although there are no data for the Cretaceous, it is plausible that both aseismic ridges may have provided the necessary dispersal corridor, as these elements are considered to have been joined together before initial spreading along a transform fault (*20*). The Maldive Ridge probably fractured away from the west coast of India before the Paleocene (*21*), and would have formed a suitable extension of the same dispersal route.

ASHOK SAHNI

Professor in Geology (Paleobiology), Centre of Advanced Study in Geology, Panjab University, Chandigarh 160014, India

References and Notes

1. A. G. Smith, A. M. Hurley, J. C. Briden, *Cambridge Earth Sci. Ser. 1* (1981).
2. E. J. Barron *et al.*, *Eclogae. Geol. Helv.* **74**, 443 (1981).
3. A. Sahni *et al.*, *Bull. Geol. Soc. France* **24**, 1093 (1982).
4. A. Sahni, paper presented at the Asia Symposium, Paris, 1983.
5. D. M. Mohabey, *Geol. Surv. India West. Reg. News* **1**, 5 (1982).
6. H. Capetta, *Palaeovert. Montpellier* **5**, 179 (1972).
7. F. DeBroin *et al. C.R. Acad. Sci.* **279**, 469 (1974).
8. S. L. Jain, *J. Palaeontol. Soc. India* **20**, 360 (1977).
9. E. H. Colbert, paper presented at the Fourth International Symposium on Gondwana, Calcutta, 1977.
10. A. F. Lapparent, *J. Palaeont. Soc. India* **2**, 109 (1957).
11. H. Wensink *et al.*, paper presented at the Fourth International Symposium on Gondwana, Calcutta, 1979.
12. P. O. Alexander, *Mem. Geol. Soc. India* **3**, 244 (1981).
13. R. Lydekker, *Recent Geol. Surv. India* **23**, 22 (1890).
14. M. B. Bande and U. Prakash, *Geophytology* **12**, 152 (1982).
15. S. B. Bhatia and M. S. Mannikeri, *ibid.* **6**, 75 (1976).
16. L. Van Valen and R. E. Sloan, *Nature (London)* **270** (5633), 193 (1977).
17. A. Sahni and V. J. Gupta, *Bull. Indian Geol. Assoc.* **15**, 85 (1982).
18. R. S. Rana, thesis, Panjab University (1984).
19. S. L. Jain and A. Sahni, paper presented at the Cretaceous of India Symposium, Lucknow, 1983.
20. R. Schlich, in *The Ocean Basin and Margins*, A. E. M. Nairn, F. G. Stehli, M. Churkin, Eds. (Plenum, New York, 1982), vol. 6, p. 51.
21. Z. B. Avraham and E. T. Bunce, *J. Geophys. Res.* **82**, 1295 (1977).

8 May 1984; accepted 17 July 1984

A Conformationally Constrained Vasopressin Analog with Antidiuretic Antagonistic Activity

Abstract. *Application of information derived from a three-dimensional model of vasopressin bound to its antidiuretic receptor resulted in the design and synthesis of a bicyclic vasopressin analog, [5,8-*cyclo*(1-β-mercaptopropionic acid,2-phenylalanine,5-aspartic acid,8-lysine)]vasopressin. The analog acts as an antagonist of the antidiuretic activity of vasopressin.*

Through integration of data from structure-activity and conformation studies of peptide hormones, models of the biologically active conformation of a peptide while bound to a receptor responsible for a specific activity can be developed. Such models may be helpful in designing peptide analogs with high and specific biological activity, and, with an adequate hypothesis of the interaction of the receptor with the "binding elements and active elements" (*1*) present on the peptide, it may also be possible to design competitive inhibitors. In the study reported here, a working model of the biologically active conformation of the neurohypophyseal hormones arginine vasopressin and lysine vasopressin (*2, 3*),

$$\overline{Cys^1\text{-}Tyr^2\text{-}Phe^3\text{-}Gln^4\text{-}Asn^5\text{-}Cys^6}\text{-}$$
$$Pro^7\text{-}(Arg^8 \text{ or } Lys^8)\text{-}Gly^9\text{-}NH_2$$

was used to design a bicyclic vasopressin analog, [5,8-*cyclo*-(1-β-mercaptopropionic acid,2-phenylalanine,5-aspartic acid,8-lysine)]vasopressin (bicyclic MPA-LVP) (Fig. 1).

The hypothetical biologically active conformation of vasopressin responsible for the antidiuretic activity of the hormone was described previously (*2*). The utility of this model has been demonstrated clearly by the design and synthesis of vasopressin analogs with extraordinarily high and specific antidiuretic agonistic potency (*4, 5*). This model suggested that the carboxamide group of asparagine in position 5 was a key active element in determining intrinsic activity or efficacy. Furthermore, it suggested that the basic moiety on the side chain of the residue in position 8 (lysine or arginine), in close proximity to the asparagine carboxamide, formed another active element. These two elements act cooperatively for maximum efficacy (Fig. 2). Modifications that perturb the orientation and cooperativity of these elements might be expected to lead to antidiuretic inhibitors, provided that the perturbations do not also reduce binding. Joining the side chains of the residues at positions 5 and 8 could theoretically eliminate cooperativity while maintaining necessary side-chain orientations for binding.

Synthesis of the protected peptide intermediate was accomplished on a poly-*N*-acrylylpyrrolidine resin (*6*). *N-tert*-butyloxycarbonylglycyl-(4-oxymethyl)benzoic acid (*7*) was quantitatively attached to the resin through a norleucine residue that had been introduced as an internal

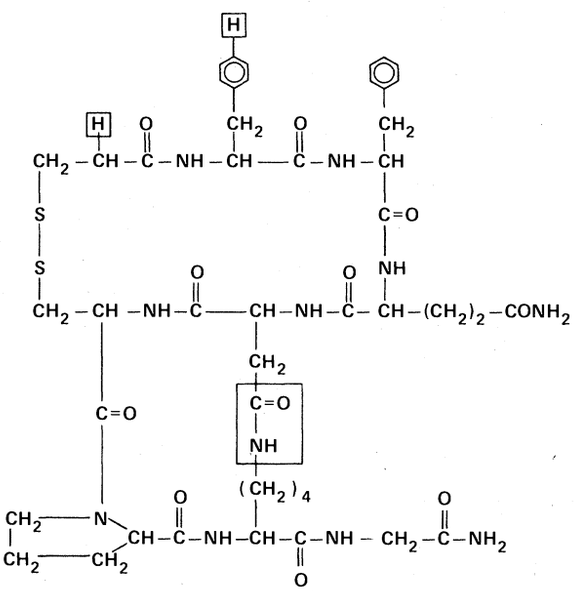

Fig. 1. Structure of bicyclic MPA-LVP with the modifications that make it different from lysine vasopressin highlighted by boxes.

standard. After the *N-tert*-butyloxycarbonyl (BOC) protecting group was removed with 1*M* HCl in acetic acid (*7*), the peptide was extended in a stepwise manner with N^{α}-fluorenylmethyloxycarbonyl (FMOC)–protected amino acids (*8*). Coupling was effected by prior activation of the protected amino acid with dicyclohexylcarbodiimide mediated with 1-hydroxybenzotriazole (*9*). During each elongation cycle FMOC protection was removed by treatment with piperidine in dimethylformamide (*10*). The side chains of the amino acid residues were protected as follows: lysine with BOC, aspartic acid as the *tert*-butyl ester, and cysteine and β-mercaptopropionic acid as the *tert*-butylthioether (*11*). The *tert*-butyl ester and BOC groups were removed by treating the peptide resin with trifluoroacetic acid containing 10 percent water, and the partially protected peptide, β-Mpa(Bu^t)-Phe-Phe-Gln-Asp-Cys(Bu^t)-Pro-Lys-Gly-NH_2, was cleaved from the resin by ammonolysis in 3,3,3-trifluoroethanol saturated with ammonia (93 percent yield) (*6*). The *tert*-butylthioether groups were displaced by 2-nitrophenylsulfenyl chloride (*11*) and the 2-nitrophenylsulfenyl groups were in turn removed by treatment with dithiothreitol. The cyclic disulfide bond was formed by oxidation in dilute solution with diiodoethane. The MPA-LVP thus formed was purified by gel filtration on Sephadex G-15 in 50 percent aqueous acetic acid and by high-performance reversed-phase chromatography (65 percent yield). MPA-LVP was checked for purity by high-performance liquid chromatography and thin-layer chromatography and its identity was verified by amino acid analysis and fast atom bombardment mass spectrometry. The bond between aspartic acid and lysine was formed by treating MPA-LVP with diphenylphosphorylazide (*12*) in dilute dimethylformamide solution. Bicyclic MPA-LVP was purified by gel filtration in 50 percent aqueous acetic acid on Sephadex G-15 and high-performance reversed phase chromatography (22 percent yield). The purity of bicyclic MPA-LVP was verified by high-performance liquid chromatography and thin-layer chromatography and its identity was confirmed by amino acid analysis and mass spectrometry.

Antidiuretic potency was determined with anesthetized male Sprague-Dawley rats by the method of Jeffers *et al.* (*13*), as modified by Sawyer (*14*). The pressor assay was performed with anesthetized male rats as described in the *United States Pharmacopeia* (*15*). Antidiuretic antagonistic potency was estimated by

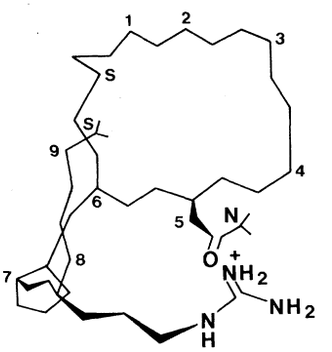

Fig. 2. Active elements (Asn^5,Arg^8) of the biologically active conformation shown in schematic representation of vasopressin. Numbers indicate residue positions.

methods previously described (*16, 17*). The USP posterior pituitary reference standard was used in all assays of agonistic or antagonistic activities.

The bicyclic analog was found to antagonize an antidiuretic response to the pituitary reference standard with an effective dose of 47 ± 4 nmol/kg (pA_2, 6.15) (*17*), while tests with up to 70 nmol/kg revealed only insignificant and transient agonistic activity. In contrast, monocylic MPA-LVP showed the same pattern of antidiuretic activity as the posterior pituitary reference standard and had an agonistic potency of 0.34 ± 0.13 unit/mg. Moreover, the antagonistic activity of bicyclic MPA-LVP in these rats cannot be attributed to diuretic activity per se, since, at the effective dose and at 1.5 times the effective dose, the bicyclic analog was neither diuretic nor natriuretic.

In the pressor assay bicyclic MPA-LVP gave highly variable responses, such that a complete four-point experiment was never achieved. However, pressor activity ranging from 0.03 to 0.5 unit/mg was suggested from five matching points. When tested as an antagonist of the pressor response to the reference standard, bicyclic MPA-LVP continued to give highly variable results. In only one animal did a higher dose of bicyclic MPA-LVP cause greater inhibition than a lower dose. In other experiments inhibition by the higher dose was equal to inhibition by the lower dose or none of the doses inhibited appreciably. However, if inhibitory responses at each dose for all animals are averaged, an effective inhibitory dose of approximately 100 nmol/kg is suggested. Therefore, the results indicate only partial agonistic and antagonistic activity of bicyclic MPA-LVP in the pressor assay. These results are not surprising, since bicyclic MPA-LVP was designed with modifications (β-mercaptopropionic acid in position 1 combined with phenylalanine in position 2) that reduce interaction with vascular vasopressin receptors (*5*).

While bicyclic MPA-LVP is less potent than other analogs with antidiuretic antagonistic activity (*16, 20*), it is a unique structure that may be more selective for the antidiuretic receptor. Previously reported analogs with such activity are potent antagonists of the pressor activity of vasopressin as well (*16, 20*). More important, bicyclic MPA-LVP was designed from conformational considerations to be an antagonist of the antidiuretic activity of vasopressin, and may be the first example of predictive design of an inhibitory peptide analog based on information derived from a hypothetical biologically active conformation. These results demonstrate that, when there is an adequate hypothesis for the relation between conformation and biological activity of a peptide, inhibitory analogs as well as highly potent and specific agonists can be designed. In this example, the enormous effort required for the empirical structure-activity approach, which finally led to antagonists of the antidiuretic activity of vasopressin (*16*), was reduced to the synthesis of one unique analog. This approach is relevant to the synthesis of other peptide hormone analogs and should reduce the work necessary to discover peptide hormone antagonists.

GERALD SKALA
CLARK W. SMITH
CATHERINE J. TAYLOR
JAMES H. LUDENS

Upjohn Company,
Kalamazoo, Michigan 49001

References and Notes

1. R. Walter, *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **36**, 1872 (1977).
2. _____ *et al.*, in *Disturbances in Body Fluid Osmolality*, T. E. Andreoli, J. Grantham, F. C. Rector, Jr., Eds. (American Physiological Society, Bethesda, Md., 1977), p. 1.
3. Abbreviations of amino acids follow the Tentative Rules on Biochemical Nomenclature of the International Union of Pure and Applied Chemistry and the International Union of Biochemistry [*J. Biol. Chem.* **247**, 577 (1972)]. Optically active amino acids are of the L configuration. β-Mpa is the abbreviation for β-mercaptopropionic acid.
4. C. W. Smith and R. Walter, *Science* **199**, 297 (1978).
5. C. R. Botos, C. W. Smith, Y.-L. Chan, R. Walter, *J. Med. Chem.* **22**, 926 (1979).
6. C. W. Smith, G. L. Stahl, R. Walter, *Int. J. Pept. Protein Res.* **13**, 109 (1979); G. L. Stahl, R. Walter, C. W. Smith, *J. Am. Chem. Soc.* **101**, 5383 (1979).
7. C. W. Smith, G. Skala, J. W. Wilks, *Int. J. Pept. Protein Res.* **21**, 127 (1983).
8. L. A. Carpino and G. Y. Han, *J. Org. Chem.* **37**, 3404 (1972).
9. G. Jager, W. Konig, H. Wissman, R. Geiger, *Chem. Ber.* **107**, 215 (1974).
10. E. Atherton, M. J. Gait, R. C. Sheppard, B. J. Williams, *Bioorg. Chem.* **8**, 351 (1979); E. Atherton, C. J. Logan, R. C. Sheppard, *J. Chem. Soc. Perkin Trans. 1*, 538 (1981).
11. J. J. Pastusazak and A. Chimiak, *J. Org. Chem.* **46**, 1868 (1981).
12. S. F. Brady *et al.*, *ibid.* **44**, 3101 (1979).
13. W. A. Jeffers, M. M. Livezey, J. H. Aus-

tin, *Proc. Soc. Exp. Biol. Med.* **50**, 184 (1942).
14. W. H. Sawyer, *Endocrinology* **63**, 694 (1958).
15. *Pharmacopeia of the United States of America* (Mack, Easton, Pa., 1970), p. 771.
16. W. H. Sawyer *et al.*, *Science* **212**, 49 (1981).
17. Antagonistic potency was determined and expressed as the effective dose and as a pA_2 value [H. O. Schild, *Br. J. Pharmacol.* **2**, 189 (1947)]. The effective dose is the dose (in nanomoles per kilogram) that reduces the response obtained with $2x$ U of agonist to the response with x U of agonist. Estimated pA_2 in vivo is the negative logarithm of the effective dose divided by the estimated volume of distribution (67 ml/kg) [D. F. Dyckes, J. J. Nestor, Jr., M. F. Ferger, V. du Vigneaud, *J. Med. Chem.* **17**, 250 (1974)]. In the antagonist assay the dose of agonist was administered 5 to 10 minutes after the test material.
18. M. Manning, B. Lammek, A. M. Kolodziejczyk, J. Seto, W. H. Sawyer, *J. Med. Chem.* **24**, 701 (1981); M. Manning *et al.*, *ibid.* **25**, 45 (1982); M. Manning, W. A. Klis, A. Olma, J. Seto, W. H. Sawyer, *ibid.*, p. 414; M. Manning, A. Olma, W. A. Klis, J. Seto, W. H. Sawyer, *ibid.* **26**, 1607 (1983); M. Manning, *ibid.* **27**, 423 (1984).

18 May 1984; accepted 23 July 1984

Estrogen-Dependent Leydig Cell Protein Recognized by Monoclonal Antibody to MCF-7 Cell Line

Abstract. *A protein (27,000 molecular weight) was previously found in rat Leydig cells after treatment with estradiol (E_2) and human chorionic gonadotropin (hCG) in vitro. The effect of hCG occurred through increased E_2 production. This hormone-regulated rat testicular protein was compared to an estrogen-regulated protein of similar physical characteristics isolated from a human mammary cancer cell line (MCF-7) and present in normal human estrogen target organs. The Leydig cells from rat and human tissue showed specific immunofluorescence and immunoperoxidase staining in the cytoplasm upon incubation with a monoclonal antibody (C11) to the estrogen-regulated protein from MCF-7 cells. Leydig cells after exposure to E_2 or hCG showed the highest fluorescence intensity; this intensity was reduced by treatment with Tamoxifen. No reaction was associated with other testicular cells. The estrogen-regulated protein from human cell lines is therefore immunologically similar to that from the rat Leydig cell. The monoclonal antibody should be useful for further characterization of the Leydig cell protein.*

The distribution of an estrogen-regulated protein detected by monoclonal antibodies (*1*) has been described in human estrogen target organs, tumors, and cell lines (*2*). This protein was first found in the MCF-7 human breast cancer cell line (*3*), and its function is not known. The protein is interesting because it is present only in cell lines positive for estrogen and progesterone receptors (*2*) and because there is evidence that it is a marker of hormonal events in the human endometrium (*2*). Also, an estrogen-regulated protein of similar molecular weight (27,000) was found in cultured rat Leydig cells treated with estradiol (E_2) and human chorionic gonadotropin (hCG) (*4*). The effect of hCG was dependent on increased E_2 production derived from androgen that was stimulated by the trophic hormone, and it preceded the appearance of the estrogen-induced distal steroidogenic lesion of the androgen pathway (*4*). It was therefore of interest to explore the cross-immunological identity of these proteins and to determine whether the protein isolated from human cancer cells was also produced by human and rat Leydig cells.

For these studies we used Leydig cells isolated from adult rat testes and from two normal human testes prepared as described (*4*), and small pieces of rat and human testes fixed in Bouin's solution and routinely processed to obtain paraffin sections. Leydig cell preparations were evaluated histochemically for their content of Δ_4-3β-hydroxysteroid dehydrogenase by staining with nitro blue tetrazolium (*4*). The Leydig cells isolated from rat and human tissue showed marked immunofluorescence (indirect technique) (*5*) when incubated with a mouse monoclonal antibody (C11) to the estrogen-regulated protein from MCF-7 human breast cancer cells (*1*). Comparison of the histochemical reaction with the immunocytochemical reaction revealed that the rat Leydig cells were positive for immunofluorescence (Fig. 1, a and b). In some of the Leydig cells the fluorescence was clearly seen in the cytoplasm (Fig. 1c). No reaction was observed when the isolated Leydig cells were incubated with a negative control, that is, with C11 that had been absorbed with the purified protein from MCF-7 cells (Fig. 1d). To establish that the Leydig cell protein (27,000 molecular weight) was the source of the cross-reacting determinant, we absorbed the monoclonal antiserum with ruptured Leydig cells. For this experiment, isolated Leydig cells from adult (60-day-old) and immature (5-day-old) normal rats were used. After being frozen and thawed three times, the cells were resuspended in distilled water (8×10^5 cells per milliliter), and cell rupture was verified under phase-contrast microscopy. The immunostaining was attenuated when C11 was absorbed separately with the supernatant (100,000*g*) and the pellet for 72 hours at 4°C. The final concentration of C11 was 5 μg/ml; this concentration has given specific immunostaining in the avidin-biotin peroxidase complex (ABC) technique (Vector Laboratories) (*2*). No attenuation was observed when C11 was absorbed with the pellet or supernatant of ruptured Leydig cells from immature rats (Fig. 1, e and f).

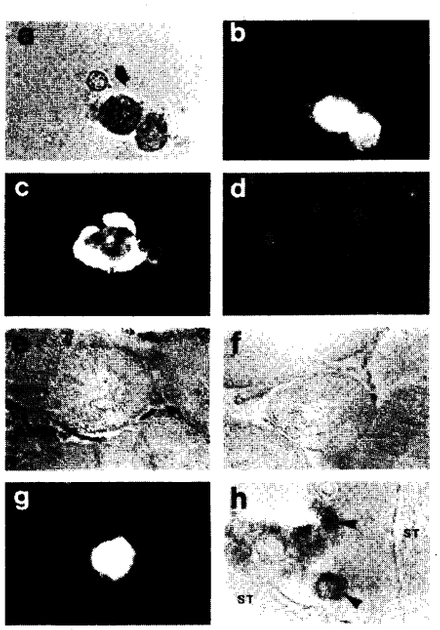

Fig. 1. Phase-contrast micrographs showing the reactivity of the monoclonal antibody (C11) to the estrogen-regulated protein from MCF-7 cells with rat and human Leydig cells. (a) Isolated rat Leydig cells from a normal testis stained with nitro blue tetrazolium (compare to b). The arrow points to a lymphocyte that remained unstained; this cell type is often associated with Leydig cells (*4*). (b) The cells stained with nitro blue tetrazolium showed positive fluorescence (×500). The immunofluorescence staining was strongest in the isolated rat Leydig cells obtained from hCG-treated testes (c); note that the reaction is mainly localized in the cytoplasm of the cell (×600). (d) Staining of the Leydig cells with a negative control (absorbed C11) (×450). Paraffin sections from a normal rat testis processed for the ABC technique (*2*) showed positive staining in the Leydig cells (e) when C11 was absorbed with the pellet of ruptured Leydig cells from immature rats. The adjacent serial section (f) shows attenuated reaction in the Leydig cells when C11 was absorbed with the particulate fraction of Leydig cells from adult rats (×150). (g) Positive fluorescence in the Leydig cells from a normal human testis maintained in short-term culture (*4*) (×500). (h) Paraffin sections from a normal human testis processed for ABC immunostaining (*2*) showing moderate positive reaction in the Leydig cells (arrows); tubule cells did not react (ST, seminiferous tubule) (×400).