preparation. When maintained in tissue culture, a line of cultured tumor cells became attenuated in the sense that, when such cells were injected into C3H mice, the tumor grew but later regressed in all animals. In contrast, uncultured parent tumor cells grew and killed all recipient mice. Mice rejecting the tissue culture line of tumor cells could now reject inoculated parent tumor cells. To raise syngeneic antibody of high titer, C3H mice were inoculated intraperitoneally at weekly intervals with 2×10^7 tumor cells of the tissue culture line in 50 percent saline–complete Freund's adjuvant emulsion. After 4 to 5 weeks, immune ascites developed, and the animals were tapped. After centrifugation to remove cells, the ascites fluids were heated at 56°C for 45 minutes and again centrifuged to remove debris. Tumor suppressive activity resided mainly in the γ1 class of antibody and was not absorbed by normal cells from spleen, thymus, or lymph node. Nonimmune ascites prepared from mice given only the complete Freund's adjuvant emulsion did not have any tumor suppressive activity. The term syngeneic was used only to denote that the tumor arose from a C3H mouse.

5. The term cytostasis was used to mean that cells do not divide but remain viable.
6. The lag period for the tumor cell was determined with a 10^6 cell inoculum; below this level, it is not possible to detect a significant number of tumor cells in histological sections. Twenty male mice were each inoculated with 10^6 tumor cells in the calf muscle. Groups of six mice were given an intraperitoneal injection of 0.25 ml of saline containing 25 μc of tritiated thymidine (20 c/mmole, New England Nuclear) 3 hours, 24 hours, or 5 days after tumor inoculation. Two mice in each group were killed at 3, 6, and 16 hours after the administration of tritiated thymidine. The calf muscles were fixed for 2 days in 10 percent formalin containing 1 percent calcium chloride. Tissue sections (4 μm thick) were processed for autoradiography as described in R. Baserga and D. Malamud [*Autoradiography: Techniques and Application* (Harper & Row, New York, 1969)]. Briefly, slides were dip-coated in Kodak NTB-2 emulsion. After a 20-day exposure they were developed in Kodak Dektol, fixed, and stained with hematoxylin and eosin. Cells with more than five grains over the nuclear area were scored as positive. Two hundred tumor cells were counted per mouse. Mice not given tritiated thymidine served as controls.
7. The time required for a single tumor cell to multiply and increase the leg diameter of a mouse to 8 mm can be determined by a limiting dilution method in which a tumor cell suspension containing a precisely known number of tumor cells is diluted to the point where only some mice receiving such diluted inoculums develop tumors. The tumor cell inoculums, containing an average of about five tumor cells, produced tumors in about 60 percent of recipients. In practice, however, it is not clear whether the inoculum contained the expected number of tumor cells since a loss of a few cells on the wall of a pipette, syringe, or needle would have seriously altered the actual number of tumor cells injected. Therefore, we took the longest time required for the tumor to grow at various limiting dilutions to be that required for a single cell. The longest time observed was 21 days. Another way to determine the value is to extrapolate a growth curve such as that shown in Fig. 1B to the one-cell level. An extrapolation of three such curves gave an average of 19.3 days.
8. G. Pasternack, J. Bukowski, H. S. Shin, *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **35**, 471 (1976).
9. N. Kaliss, *Transplant. Proc.* **2**, 59 (1970); K. Hellström and I. Hellström, *Annu. Rev. Microbiol.* **24**, 373 (1970).
10. H. S. Shin, J. S. Economou, G. Pasternack, R. J. Johnson, *J. Exp. Med.*, in press.
11. R. Snyderman and M. Pike, *Science* **192**, 370 (1976); R. M. Fauve, B. Hevin, H. Jacob, J. Gaillard, F. Jacob, *Proc. Natl. Acad. Sci. U.S.A.* **71**, 4052 (1974); R. J. North, O. Kirstein, R. Tuttle, *J. Exp. Med.* **143**, 559 (1976).
12. M. Segerling, S. Ohanian, T. Borsos, *Science* **188**, 55 (1975); R. D. Rubens and R. Dulbecco, *Nature (London)* **284**, 81 (1974).
13. R. Metzgar, T. Mohanahumar, D. Bolognes, *J. Exp. Med.* **143**, 47 (1976).
14. R. J. Johnson, G. Pasternack, H. S. Shin, in preparation.

15. Supported in part by NIH grant CA-14113, a grant from the Leukemia Research Foundation Inc., and NIH career development award GM-50193 to H.S.S. We thank Drs. M. M. Mayer, G. Santos, H. Kaizer, P. Murphy, and W. Willoughby for constructive criticism.

7 June 1976; revised 13 July 1976

Endocrine-Dependent Rat Mammary Tumor Regression: Use of a Gonadotropin Releasing Hormone Analog

Abstract. *Long-term administration of [D-Leu^6, des-Gly-NH$_2$^{10}, Pro-ethylamide^9]-GnRH, an analog of gonadotropin releasing hormone, caused regression of neoplastic tissue in a rat bearing a spontaneous mammary adenocarcinoma and in rats in which tumors had been induced by treatment with dimethylbenzanthracene (DMBA). During two separate treatment periods with the analog, the tumor in the single animal regressed although it had previously grown spontaneously. After a third period of growth, ovariectomy also induced regression, suggesting endocrine dependency of the tumor. These observations were confirmed in the DMBA-induced tumor system, where tumor regression in the analog-treated rats was comparable to that observed in the ovariectomized rats, and in both cases the tumor regression was significant when compared to untreated controls.*

We have shown (*1*) that twice-daily injections of microgram quantities of the gonadotropin releasing hormone analog, [D-Leu^6, des-Gly-NH$_2$^{10}, Pro-ethylamide^9]-GnRH (A-43818) (analog **1**) (*2*, *3*), into female rats inhibits normal reproductive processes. When given to immature animals analog **1** postponed maturation of the reproductive organs, while in the mature animal analog **1** caused cessation of cycling and atrophy of the ovary and uterus. Since the primary anatomical findings in these animals were atrophy of the ovary and of the uterus, we speculated that the initial endocrinological effect of the analog is interference with the biosynthesis or the utilization of the ovarian steroid hormones. This view is in accord with the effects on reproductive function and upon reproductive tissues.

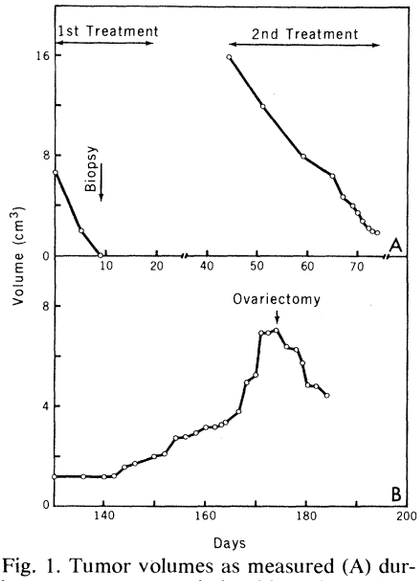

Fig. 1. Tumor volumes as measured (A) during two treatment periods with analog **1** (5 μg of analog **1** was injected subcutaneously twice a day), and (B) before and after ovariectomy.

We proposed that analog **1** might not only have important implications in reproduction control, but might also be applicable to the treatment of steroid-dependent neoplasms of the reproductive organs, especially the mammary gland. We tested this theory in a female rat bearing a large spontaneous tumor in the breast region, and in rats in which tumors had been induced by dimethylbenzanthracene (DMBA) (*4*).

The single animal bearing a spontaneous mammary tumor was given two periods of treatment with analog **1**. As shown in Fig. 1, the tumor volume was reduced in both treatment cycles. A biopsy specimen taken after the first treatment cycle indicated that the tumor was a mammary adenocarcinoma (*5*). Af-

Table 1. Comparison of DMBA-induced tumor responses in rats treated with analog **1**, ovariectomized rats, controls. Analog **1** [D-Leu^6, des-Gly-NH$_2$^{10}, Pro-ethylamide^9]-GnRH was administered subcutaneously (5 μg) twice a day. Ovariectomy was performed on the same day that the treatment with analog **1** was started. The ovariectomized and control animals received saline. The results are given as the numbers of animals showing tumor regression (R) or no regression (NR); R and NR were judged on the basis of the change in the total volume of the tumors on each animal. (*P*, probability; NS, not significant.)

Treatment	7 days			14 days			21 days			30 days		
	R	NR	*P**	R	NR	*P*	R	NR	*P*	R	NR	*P*
Control	0	6	NS	0	6	.001	0	6	.001	1	5	.01
Ovariectomized	2	4		6	0		6	0		6	0	
Control	0	6	.015	0	6	.002	0	6	.002	1	5	.01
Analog **1**	4	1		5	0		5	0		5	0	
Ovariectomized	2	4	NS	6	0	NS	6	0	NS	6	0	NS
Analog **1**	4	1		5	0		5	0		5	0	

*Calculated from Fisher's exact probability for 2 × 2 contingency tables (*8*).

ter the third appearance of the tumor, bilateral ovariectomy also resulted in regression, suggesting that the tumor was endocrine dependent (*6*).

The observations made on the single animal appeared to justify our initial assumption that analog **1** acted through a suppression of ovarian steroid function. We therefore sought confirmatory evidence through the use of the DMBA-induced rat mammary tumor system (*7*). In this experiment we administered the carcinogen as described (*7*). However, all animals were started on treatment on the same day, 5 months after a single administration of DMBA. At this point all animals bearing tumors were stratified according to three criteria: body weight, time after the appearance of the first tumor, and total tumor volume per animal. Allocation of animals into groups was done on the basis of random selection from this stratification. Results of the experiment are shown in Table 1. It is evident that both the group treated with analog **1** and the group subjected to ovariectomy showed significant tumor regression when compared to controls.

E. S. JOHNSON, J. H. SEELY
W. F. WHITE
Division of Antibiotics and Natural Products, Abbott Laboratories, North Chicago, Illinois 60064
E. R. DESOMBRE
University of Chicago, Ben May Laboratory for Cancer Research, Chicago, Illinois 60637

References and Notes

1. E. Johnson, R. Gendrich, W. White, *Fertil. Steril.* **27**, 853 (1976).
2. The structure of analog **1** is as follows: pGlu-His-Trp-Ser-Tyr-D-Leu-Leu-Arg-Pro-NHC_2H_5. Thus the -Gly-NH_2[10] in gonadotropin releasing hormone has been replaced by -NHC_2H_5. The abbreviations for the amino acid residues are as follows: pGlu, pyroglutamic acid; His, histidine; Trp, tryptophan; Ser, serine; Tyr, tyrosine; Leu, leucine; Arg, arginine; and Pro, proline.
3. M. Fujino, T. Fukuda, S. Shinagawa, S. Kobayashi, I. Yamazaki, R. Nakayama, J. Seely, W. White, R. Rippel, *Biochem. Biophys. Res. Commun.* **60**, 406 (1974).
4. D. Sinha, D. Cooper, T. D. Dao, *Cancer Res.* **33**, 411 (1973).
5. Histological examination of tumor tissue led to a diagnosis of mammary adenocarcinoma based upon modified cellular morphology and anaplasia.
6. Estrogen receptor assay [E. R. DeSombre, G. Kledzik, S. Marshall, J. Meites, *Cancer Res.* **36**, 354 (1969)] of the cytosol of the regressing tumor 10 days after ovariectomy showed specific estrogen receptor in low amounts (205 fmole per gram of tumor). Since it is known that tumor estrogen receptor content decreases after ovariectomy, and since a recent report [F. Vignon and H. Rochefort, *Endocrinology* **98**, 722 (1976)] estimates a 90 percent reduction 10 days after ovariectomy, this result is not inconsistent with the proposal that the spontaneous tumor is hormone dependent.
7. C. Huggins, L. C. Grand, F. P. Brillantes, *Nature (London)* **189**, 204 (1961).
8. R. A Fisher, *Statistical Methods for Research Workers* (Oliver & Boyd, Edinburgh, ed. 13, 1958).
9. We thank J. W. Kesterson of the Abbott Laboratories for diagnosis of the biopsy specimen.

10 May 1976; revised 14 June 1976

[D-Ala^2]-Met-Enkephalinamide: A Potent, Long-Lasting Synthetic Pentapeptide Analgesic

Abstract. *[D-Ala^2]-Met-enkephalinamide (DALA), a synthetic enkephalin analog designed by in vitro analysis, binds to opiate receptors almost as tightly as methionine-enkephalin. Since it is not susceptible to degradation by brain enzymes, low doses (5 to 10 micrograms) cause profound, long-lasting, morphine-like analgesia when microinjected into rat brain.*

Opiate receptors are stereospecific components of vertebrate synaptic membranes distributed heterogeneously in the mammalian nervous system. Morphine and other opiates initiate their pharmacological effects by complexing reversibly with these receptors, which have been indentified (*1*) and studied extensively with isotopically labeled opiates (*2*). A number of endogenous peptides, which appear to serve as natural ligands for opiate receptors, have been shown to exist in brain (*3*), pituitary (*4*), human cerebrospinal fluid (*5*), and human blood (*6*). The first of these to be identified, enkephalin, is a mixture of two pentapeptides (Tyr-Gly-Gly-Phe-Met and Tyr-Gly-Gly-Phe-Leu) (*7*) extracted from porcine brain by Hughes and Kosterlitz and their colleagues (*8*), and also from calf brain (*9*). While enkephalin has potent and readily demonstrable morphine-like effects on the guinea pig ileum and mouse vas deferens (*8*), extremely high quantities (120 to 200 μg) are required to elicit analgesia when microinjected into rat cerebral ventricles (*10*) and brain (*11*). Thus, Met-enkephalin is at least 50-fold weaker than morphine as an analgesic, despite the fact that it has about half of the affinity of morphine for rat brain opiate receptors (*11*). Indeed, Met-enkephalin's mild analgesia dissipates completely in several minutes (*11*), even when injected directly into active brain sites where morphine induces analgesia which persists for several hours. This apparent discrepancy is explained by the finding that enkephalin's affinity for opiate receptors is rapidly and efficiently destroyed by brain enzymatic activity (*11*).

We now report the synthesis of a novel pentapeptide, designed and detected by in vitro analysis, which elicits a potent, long-lasting analgesia.

Affinity for opiate receptors was assessed by the inhibition of stereospecific [^{3}H]naloxone binding to rat brain membranes, as originally described (*12*), with minor modifications designed to avoid the enzymatic destruction of peptides which occurs at warm temperatures (*11*). Analgesia was assessed by the "tail-flick" procedure (*13*), the "flinch-jump" task (*14*), and by subjectively evaluating the animals' reactions to pinches of the limbs with forceps. In the "tail-flick" task, a high-intensity light was focused on a rat's tail, which had been blackened with a felt-tip pen, and the time that it took an animal to remove its tail from this radiant heat was measured to the nearest 0.5 second. In the "flinch-jump" task, the shock intensity at which an animal jumped and squealed was quantified 15 minutes after injection (immediately after testing the tail-flick). Morphine and peptides were dissolved in pyrogen-free water and microinjected in a volume of 1 μl through permanent indwelling cannulae located in the periaqueductal gray region of the midbrain (*11*). This brain region mediates the analgesic actions of opiates in rodents (*15*) and primates (*16*). Peptides were synthesized by the solid phase method with the use of a Beckman 990 synthesizer, and peptide content was determined by amino acid analysis after demonstration of purity by thin-layer chromatography in three solvent systems and electrophoresis (*17*).

We have demonstrated earlier that several substitutions of enkephalin's amino terminal tyrosine residue (Tyr^1) result in severe losses of opiate receptor affinity of two to three orders of magnitude (*11*). We reasoned that cleavage of the Tyr^1-Gly^2 peptide bond would thus be a likely mechanism for rapid enzymatic inactivation of enkephalin, so that enzyme-resistant analogs must contain substitutions that protect this bond. Substitution of Met-enkephalinamide at the 2-position with D-alanine results in a peptide which almost completely retains its affinity for opiate receptors ($K_D = 4 \times 10^{-7}$ to $5 \times 10^{-7} M$) (Fig. 1A). Substitution in the same position with the "natural" enantiomer, L-alanine, results in about a tenfold weaker receptor affinity. These values reflect actual affinity for receptors since enzymatic degradation is avoided by the low temperature employed (*11*). The slope of the line describing inhibition of [^{3}H]naloxone binding as a function of peptide concentration is the same as that characteristic of morphine, the benzomorphans, and all other opiates previously examined (*12*, *18*). A drug's affinity for opiate receptors in vitro is closely predictive of its analgesic po-