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Stimulation of Murine Interferon by a Substituted Pyrimidine

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2-Amino-5-bromo-6-methyl-4-pyrimidinol (U-25,166) induced high levels of circulating interferon in mice when administered either parenterally or orally. Peak titers of interferon were found in the serum between 6 and 12 h after inoculation of the drug. Lower but significant levels of interferon were found in rat serum after administration of U-25,166 by either the intraperitoneal or oral route, and good levels of circulating interferon were observed in cats after oral treatment. Repeated intraperitoneal doses (50 mg/kg) of U-25,166 protected mice against intranasal encephalomyocarditis virus challenge. The minimal effective acute oral dose for antiviral activity was approximately 250 mg/kg. This was also the minimal dose that produced detectable levels of interferon. Maximum tolerated doses in mice were four to six times the minimal effective doses. A single oral treatment was protective in mice against challenge virus inoculated 24 h later. The compound protected mice from challenge with high levels of encephalomyocarditis virus, up to 20,000 mean lethal doses. Antiviral activity in mice was retained when certain minor substitutions were made in the U-25,166 structure.

Practical use of interferon in human viral disease may ultimately be achieved, either by administering exogenous interferon or by stimulating production of endogenous interferon. Several chemically defined, nonpolymeric, relatively low-molecular-weight compounds have been reported to stimulate interferon production in laboratory models (4, 5, 7, 8, 10). These interferon inducers represent a diversity of chemical structures, and all differ structurally from an inducer discovered in our laboratory. This report describes the antiviral activity and interferon-stimulating properties of a substituted pyrimidine, 2-amino-5-bromo-6-methyl-4-pyrimidinol (U-25,166) (see Fig. 1).

MATERIALS AND METHODS

Animals. (i) **Mice.** Mice were of random sex and weighed 16 ± 2 g. Upj:TUC(ICR)spf and Upj:TUC(CF-1)spf strains were provided by the Upjohn Animal Rearing and Procurement Unit. The CF-W mice were purchased from Carworth Laboratories (Kalamazoo, Mich.); S/W mice were from Camm Research (Wayne, N.J.); and the C57/Bl/6J mice, an inbred strain, were from Jackson Memorial Laboratories (Bar Harbor, Me.). All mouse studies were done with ICR mice unless otherwise indicated.

(ii) **Rats.** Female Sprague-Dawley rats weighed approximately 110 g each and were provided by the Upjohn Animal Rearing and Procurement Unit.

(iii) **Cats.** Mixed-breed barn cats, weighing from 1.7 to 3.3 kg each, were obtained from area farmers.

Drug. U-25,166 was purchased from Aldrich Chemical Co. (Milwaukee, Wis.). The compound is only slightly soluble in water (approximately 0.3 mg/ml at 25 C); therefore, it was finely ground in a glass tissue grinder (Ace Glass, Inc., Vineland, N.J.) and suspended in sterile aqueous 1% carboxymethylcellulose (CMC) for treating animals.

Drug administration and blood collection. (i) **Mice.** Drug was administered by various routes by standard procedures. Groups of 15 mice were bled by decapitation, the pooled blood was stored overnight at 4 C, and the serum was collected.

(ii) **Rats.** Drug was administered by standard procedures. Rats were bled by orbital puncture with a glass capillary. Serum for each sample was collected from the pooled bleedings of five rats. The same rats were bled repeatedly throughout an experiment.

(iii) **Cats.** Before all treatments and bleedings, cats were inoculated intramuscularly with Vetalar (ketamine-hydrochloride) at a concentration of 11 mg/kg. This produced good restraint in about 3 min, and the animals appeared normal within 30 min post-treatment. U-25,166 was administrated orally by intubation through a no. 10 nelaton catheter purchased from Daval, Inc. (Providence, R.I.). A volume of 20 to 40 ml was administered, depending on the weight of the cat. Repeated serum samples were collected from each cat by bleeding from the jugular vein.

Serum interferon assays. Mouse sera were assayed on L929 mouse embryo fibroblast cultures, obtained originally from Flow Laboratories (Rock-

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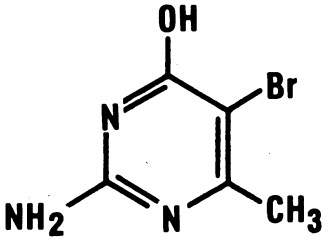


FIG. 1. *Structure of 2-amino-5-bromo-6-methyl-4-pyrimidinol (U-25,166).*

ville, Md.). Monolayers were maintained in continuous culture using Eagle minimal essential medium supplemented with 10% fetal calf serum, 100 μg of streptomycin per ml, 100 U of penicillin per ml, and 2 mM glutamine (E-MEM-10). Confluent monolayers of L929 cells, grown in 60-mm tissue culture plates, were treated overnight with 3 ml of an appropriate twofold dilution of the test sample. Fluid was then removed, and the cells were washed once with 5 ml of warm E-MEM-10. Each monolayer was then inoculated with 0.5 ml of medium containing 75 to 100 plaque-forming units of vesicular stomatitis virus. After 1 h at 37 C to allow for adsorption of virus, plates were overlayed with 5 ml of medium 199 containing 1% agar, 5% fetal calf serum, and standard antibiotics. Average plaque numbers on duplicate plates were recorded 72 h later. Interferon titers are expressed as the reciprocal of the highest dilution of sample reducing the number of plaques by 50% as compared to the appropriate control. A standard interferon preparation was included in each experiment to insure validity of the assay. The NIH mouse interferon standard (G002-904-511), with a label value of 6,500 U/ml, titered 6,400 in our assay system.

The same general procedure was followed in assaying serum samples from other species: rat sera on primary rat embryo cells and cat sera on primary cat kidney cells, with L cell-propagated vesicular stomatitis virus as the challenge virus in each system.

Mouse infection with EMC virus. Encephalomyocarditis (EMC) virus was obtained from the American Type Culture Collection. This virus had been through five serial mouse passages in our laboratory, inoculating the virus intraperitoneally (i.p.) and harvesting the brains for subsequent passage. The 10% mouse brain suspension used in the current studies had a titer of $10^{7.4}$ mean lethal doses (LD_{50})/0.05 ml when administered intranasally (i.n.). The standard challenge 0.05-ml inoculum, administered i.n. to mice under light ether anesthesia, was equivalent to 10 to 30 LD_{50}. Deaths were recorded daily, and experiments were terminated 6 days post EMC infection. Deaths occurring within 48 h postinfection were attributed to trauma or drug toxicity and were not included in calculations of antiviral activity. Statistical significance (*P* values) of difference in numbers of survivors between drug-treated and placebo-treated groups was calculated by the chi-square test with one degree of freedom.

Testing of U-25,166 congeners. Test compounds were administered i.p. at a dose of 50 mg/kg to groups of 20 ICR mice. The animals were treated at −44, −26, −19, −3, and +4 h. Chemicals which were toxic at a dose of 50 mg/kg were retested at lower doses. All compounds were prepared in CMC. Mice were infected i.n. with 10 to 30 LD_{50} of EMC virus at 0 h. Compounds were declared active if they increased survival of the treated group over the CMC control group with $P < 0.05$. Limited supplies were available with many of the compounds, precluding exhaustive animal studies. Results, therefore, are reported only as active or inactive without further attempt to quantitate the level of antiviral activity.

RESULTS

The bioactivity of U-25,166 was first detected in a mouse screen designed to detect endogenous interferon inducers. Multiple i.p. injections of U-25,166 protected mice from i.n. EMC infection. All mice survived when treated with five doses of 200 mg/kg, whereas minimum activity was obtained at a dose of 50 mg/kg (Table 1).

A substance inhibitory for vesicular stomatitis virus replication on L929 cells could be recovered from the serum of mice treated with U-25,166. This virus inhibitory substance was characterized as an interferon based on the following criteria: (i) it was not sedimented by centrifugation at 100,000 × *g* for 2 h; (ii) the inhibitor was stable at pH 2 for at least 4 days at 4 C; (iii) it did not inactivate vesicular stomatitis virus on direct contact, and the protective effect was not reversed by washing cells previously exposed to the inhibitor; (iv) the inhibitor found in mouse serum was effective as an antiviral on mouse cells but not on rabbit or chick cells; (v) the mouse serum inhibitor was inactivated by trypsin; (vi) the inhibitor was not dialyzable; (vii) its in vitro action was suppressed by pretreatment of L929 cell cultures with 1 μg of actinomycin D per ml.

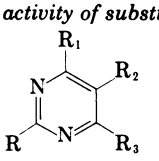
After discovery of the antiviral and interferon-inducing properties of U-25,166, some 75 pyrimidines, many of which differed in structure only slightly from U-25,166, were tested in EMC-infected mice. A summary of results ob-

TABLE 1. *Effect of multiple U-25,166 treatment on survival of EMC-infected mice*[a]

Dose[b] (mg/kg)	Dead/total	*P*[c]
200	0/18	<0.001
100	1/18	<0.001
50	6/19	0.006
0	16/20	

[a] Mice were infected i.n. at 0 h with EMC virus.
[b] U-25,166 was administered i.p. at −44, −26, −19, −3, and + 4 h.
[c] χ^2 test with 1 degree of freedom.

TABLE 2. *Antiviral activity of substituted pyrimidines*



No.	U no.	R	R_1	R_2	R_3	Activity[a]
1	25,166	NH_2	OH	Br	CH_3	+
2	30,042	H	OH	Br	CH_3	−
3	29,736	$NHCH_3$	OH	Br	CH_3	−
4	11,465	NH_2	CH_3	Br	CH_3	−
5	31,314	NH_2	NH_2	Br	CH_3	−
6	31,825	NH_2	OH	H	CH_3	−
7	28,717	NH_2	OH	Cl	CH_3	−
8	30,056	NH_2	OH	I	CH_3	+
9	30,377	NH_2	OH	Br	H	−
10	33,059	NH_2	OH	Br	Cl	−
11	10,512	NH_2	OH	Br	NH_2	−
12	31,672	NH_2	OH	Br	CH_2CH_3	+
13	31,989	NH_2	OH	Br	$CH_2CH_2CH_3$	−
14	31,784	NH_2	OH	Br	$CH(CH_3)_2$	−
15	33,138	NH_2	OH	Br	$CH_2C_6H_5$	−
16	32,465	NH_2	OH	Br	CF_3	−
17	5,554	NH_2	OH	H	H	−
18	32,334	NH_2	OH	H	Cl	−
19	29,668	NH_2	OH	Cl	H	−
20	30,387	NH_2	OH	I	H	−
21	30,063	NH_2	OH	I	Cl	+
22	30,340	NH_2	OH	I	CH_2CH_3	+
23	32,228	NH_2	OH	I	$CH_2CH_2CH_3$	+
24	32,504	NH_2	OH	I	$CH(CH_3)_2$	−
25	32,229	NH_2	OH	I	$CH_2C_6H_5$	+
26	32,467	NH_2	OH	I	CF_3	−
27	32,314	NH_2	OH	I	COOH	−

[a] Compounds were administered at a dose of 50 mg/kg at −44, −26, −19, −3, and +4 h to groups of 20 mice. Animals were infected i.n. at 0 h with EMC virus. Values are recorded as active (+) if percent survivors in treated group minus percent survivors in virus control group was ≥25 at 6 days after infection. Inactive (−).

TABLE 3. *Kinetics of interferon appearance in serum of mice treated with U-25,166*

Route	Dose[a] (mg/kg)	Interferon titer at (h):					
		3	6	9	12	24	48
i.p.	1,000	80	1,280	2,560	2,560	320	0
s.c.	2,000	10	1,280	2,560	2,560	80	40
p.o.	1,000	80	5,120	2,560	2,560	80	0

[a] Single dose of U-25,166 at 0 h.

tained with 27 of the most closely related structural analogues, including all compounds found active, is shown in Table 2. As indicated, removal of the 2-amino or its replacement with a methylamino group resulted in loss of antiviral activity. Replacing the 4-hydroxyl with either methyl or amino also gave inactive compounds. Activity was lost when the 6-methyl was removed or replaced with chloro or amino. The 6-ethyl analogue of U-25,166 was active, but the 6-propyl, -isopropyl, and -benzyl compounds were all inactive. If antiviral activity were to be retained, the 5-bromo in U-25,166 could be replaced by iodine but not by chlorine; in fact, iodo at the 5-position appeared to permit more diversity at position 6, with 5-iodo-6-chloro, 5-iodo-6-propyl, and 5-iodo-6-benzyl all showing antiviral activity. In these screening tests, none of the compounds was appreciably more active than U-25,166. This compound, therefore, was further evaluated to better define its interferon-inducing and antiviral potential.

High doses of U-25,166, determined in preliminary experiments to be well tolerated, were administered by various routes to ICR mice. At intervals thereafter, serum was collected for determination of interferon content. It can be seen (Table 3) that a good interferon response in mice resulted from single doses of U-25,166 given i.p., subcutaneously (s.c.), or orally (p.o.). Peak titers in each case occurred 6 to 12 h after dosing. Yields of circulating interferon were comparable by the various routes of drug administration.

Rats were treated with U-25,166 by the same routes and relative dosages as were used in the mouse study. Circulating interferon was present in the rats after i.p. or p.o. drug treatment (Table 4), but titers were much lower than those found in mice. Only trace levels of interferon were detected in rats treated s.c. with U-25,166. Again, as with the mice, highest levels of circulating interferon were apparently present within the first 12 h after drug treatment.

U-25,166 induced interferon in cats also (Table 5). Five cats were each treated by oral intubation with 1,000 mg of U-25,166 per kg, and repeated serum samples were collected. Four of the five cats showed good levels of interferon in their sera, whereas cat no. 3 was marginal. Time of peak titer was variable: cat no. 1 showed a late peak compared to results obtained with mice and rats. Time of maximum interferon titer in cats no. 2, 4, and 5, however, appeared similar to the rodent results. Cat no. 2 died prior to the 24-h bleeding—all of the other cats survived in apparent good health.

Best yields of U-25,166-induced interferon were obtained in mice and, as indicated above, the p.o. route of drug administration appeared at least as effective as parenteral treatment. An experiment was run to determine the minimal acute p.o. dose necessary for inducing detectable levels of interferon in mouse serum. Single twofold decreasing doses of U-25,166 were given to separate groups of mice, and serum was harvested from all groups 12 h later. The minimal p.o. dose of U-25,166 that yielded detectable serum interferon was 250 mg/kg (Table 6).

It was of interest that this was the same as the minimal effective antiviral dose when mice were challenged i.n. with EMC virus (Table 7). Also shown in this table are approximate acute LD_{50} values for U-25,166 in noninfected 16-g mice. In another tolerance study, 12- to 13-g ICR mice were treated by various routes twice daily for 5 consecutive days with incremental levels of U-25,166 (Table 8). These data indicate that the drug is well tolerated in mice, both parenterally and p.o.

As noted in Table 3, a single oral treatment of mice with U-25,166 produced high levels of circulating interferon through 12 h, lower levels at 24 h, and no detectable serum interferon at 48 h. An experiment was run to determine whether this pattern was consistent with duration of antiviral activity. At daily intervals, groups of 20 mice were treated orally with a single 1,000 mg/kg dose of U-25,166 in CMC. Control groups were treated with CMC only. All mice were challenged i.n. at zero time with

TABLE 4. *Kinetics of interferon appearance in serum of rats treated with U-25,166*

Route	Dose[a] (mg/kg)	Interferon titer at (h):			
		8	12	24	48
i.p.	1,000	60	40	10	0
s.c.	2,000	10	10	10	0
p.o.	1,000	40	60	0	0

[a] Single dose of U-25,166 at 0 h.

TABLE 6. *Minimum interferon-inducing dose of U-25,166 in mice*

Dose[a] (mg/kg)	Serum interferon titer
1,000	2,560
500	640
250	10
125	0
62	0

[a] Single oral dose of U-25,166 at 12 h prebleeding.

TABLE 5. *Kinetics of interferon appearance in serum of cats treated with U-25,166*[a]

Cat no.	Interferon titer at (h):						
	0[b]	3	6	12	24	30	48
1	0	0	0	10	160	80	
2	0		80	320			
3	0		0	10	0		0
4	10		>160	80	10		0
5	0		20	80	10		10

[a] Single oral dose of 1,000 mg of U-25,166 per kg at 0 h.
[b] Zero-hour blood samples were collected prior to drug treatment.

30 LD_{50} of EMC virus (Table 9). No protection was found in mice treated 48 h prior to infection, nor in those treated 24 h after infection. Consistent with the circulating interferon results, however, drug treatment was effective when given 24 h prior to infection. This strain of EMC was rapidly lethal for mice, usually producing 100% fatalities within 5 days at the 30 LD_{50} virus level.

An experiment was run to determine whether U-25,166 was effective in mice against higher challenge levels of EMC virus. Groups of mice were treated orally with a single 800 mg/kg dose of the compound in CMC. Twelve hours later, when high circulating interferon levels would be present, paired groups of drug-treated and CMC-treated animals were challenged i.n. with 20, 200, 2,000, or 20,000 LD_{50} of EMC virus (Table 10). U-25,166 showed significant antiviral activity in all groups, with somewhat less protection noted as the level of challenge virus was increased.

Because of the variable interferon response elicited by U-25,166 in different species of animals, the compound was tested for antiviral activity in several strains of mice. Groups of mice were treated orally with a single 1,000 mg/kg dose of the drug in CMC and were challenged i.n. 12 h later with approximately 30 LD_{50} of EMC virus. The viral inoculum for each strain was based on previous titrations conducted in nontreated mice of the same strain. U-25,166 showed significant antiviral activity in all mouse strains tested (Table 11).

TABLE 7. *Minimal effective dose (MED) of U-25,166 versus EMC in mice*[a]

Route	MED (mg/kg)	LD_{50}[b] (mg/kg)
i.p.	250	1,500
s.c.	500	>2,500
p.o.	250	4,000

[a] Single dose of U-25,166 at 9 h prior to i.n. infection with EMC virus.
[b] Approximate acute LD_{50} of U-25,166 for 16-g ICR mice.

DISCUSSION

Interferon appears to possess many of the properties associated with the ideal antiviral agent, i.e., it is active against a broad spectrum of both ribonucleic acid and deoxyribonucleic acid viruses, it is apparently well tolerated in animals and man, it shows little antigenicity, and it is a natural substance produced physiologically, particularly as a result of viral infec-

TABLE 9. *Effect of time of U-25,166 treatment on antiviral activity in mice*

Treatment[a]	Time (h)[b]	Dead/total	P[c]
U-25,166	−48	20/20	
U-25,166	−24	8/19	0.001
CMC	−24	20/20	
U-25,166	−3	4/19	<0.001
CMC	−3	20/20	
U-25,166	+24	20/20	

[a] U-25,166 was inoculated p.o. at 1,000 mg/kg.
[b] Mice were infected i.n. at 0 h with EMC virus.
[c] See Table 1.

TABLE 8. *Chronic toxicity of U-25,166 for mice*

Dose[a] (mg/kg/day)	Route	Average mouse weight (g) on day:						No. dead[b]
		0	2	4	7	9	11	
800	i.p.	11.9	10.6	9.7	11.0	12.8	14.2	6
600	i.p.	11.9	10.8	9.8	11.1	13.1	15.4	3
400	i.p.	12.0	11.6	10.9	11.8	14.0	15.2	2
300	i.p.	12.3	12.3	12.0	14.0	16.7	18.4	0
200	i.p.	12.3	12.4	13.0	15.2	18.0	20.9	0
100	i.p.	12.7	13.0	14.4	16.4	18.6	20.7	1
1,000	p.o.	12.9	12.9	12.4	14.8	17.2	19.3	1
800	p.o.	13.0	12.8	12.1	15.0	17.4	18.8	1
600	p.o.	12.1	12.1	12.1	14.8	16.9	19.2	0
400	p.o.	12.6	13.1	13.1	15.5	18.5	20.2	0
800	s.c.	12.4	12.9	13.4	16.0	18.3	19.2	1
600	s.c.	12.1	13.3	13.2	15.7	17.6	18.1	0
400	s.c.	12.3	13.9	15.1	17.2	18.4	19.1	0
200	s.c.	12.4	14.9	16.6	17.6	19.5	20.8	0
0[c]	s.c.	12.6	14.8	16.7	17.9	19.7	20.9	0

[a] Treated twice daily from day 0 to day 4.
[b] Ten mice/group.
[c] CMC.

TABLE 10. *Activity of U-25,166 in mice versus increasing levels of EMC virus*

Virus level (LD_{50})	Dead/total		P[b]
	U-25,166[a]	CMC	
20	0/19	19/20	<0.001
200	1/16	20/20	<0.001
2,000	4/17	20/20	<0.001
20,000	6/14	20/20	0.001

[a] Single oral dose of 800 mg of U-25,166 per kg at 12 h prior to i.n. infection with EMC virus.
[b] See Table 1.

TABLE 11. *Activity of U-25,166 versus EMC virus in various mouse strains*

Mouse strain	Dead/total		P[b]
	U-25,166[a]	CMC	
ICR	2/16	20/20	<0.001
CF-W	6/16	17/19	0.004
CF-1	4/18	19/20	<0.001
S/W	0/13	13/20	<0.001
C/57/Bl/6J	4/15	17/17	<0.001

[a] Single oral dose of 1,000 mg of U-25,166 per kg at 12 h prior to i.n. infection with 30 LD_{50} of EMC virus.
[b] See Table 1.

tion. There is good evidence that interferon represents one of the major host defense mechanisms for limiting the spread of viral infections.

Despite these desirable attributes of interferon as an antiviral agent, progress in the practical application of exogenous interferon has been slow. A major reason for this is the fact that interferon is essentially species specific, so that interferon for use in man must be produced in human cells. This requirement, and the difficulty of concentrating and purifying interferon, has resulted in a limited supply of human interferon for clinical studies. This limitation is likely to continue for the forseeable future.

Efforts have been directed, therefore, toward the discovery of agents which can be administered to the animal host, particularly to man, and will lead to the production of endogenous interferon. The rationale is that such agents could be given early in disease to shorten the course of clinical signs and symptoms or, if sufficiently well tolerated, could be given prophylactically to prevent viral infection. A variety of nonviral interferon inducers, which are effective in animals, have been discovered. Some of these are high-molecular-weight substances, such as pyran co-polymers and double-stranded polyribonucleotides. So far, experience with the polymers has indicated that they are probably too toxic for widespread human use.

A limited number of relatively low-molecular-weight compounds have also been reported to induce interferon in animals. Among the first of these was tilorone hydrochloride, a fluorenone derivative (7, 8). This compound is active p.o. and induces high levels of interferon in mice. It has been tested clinically but may have limited potential due to toxicity and lack of interferon induction in man (6). Two other tricyclic ring compounds, quinacrine and acranil, which are used clinically as antiparasitic drugs, were also found to induce an interferon-like substance in mice (4). Siminoff et al. reported (10) that a substituted pyrazoloquinoline induced interferon and displayed antiviral activity in mice. Apparently the drug has not been evaluated clinically. A substituted propanediamine, CP-20,961, induced interferon and showed antiviral activity in mice, but only when given parenterally (5); there was no evidence of serum interferon in dogs, cats, rabbits, monkeys, or pigs after intramuscular administration of the compound (3). Nevertheless, CP-20,961, given intranasally to volunteers, induced measurable interferon (2, 3) in nasal secretions and showed significant protection against an experimental rhinovirus challenge (9).

U-25,166, the substituted pyrimidine discussed in this report, represents another unique chemical structure which induces interferon in animals. Peak interferon titers in mouse sera occurred 6 to 12 h after treatment with U-25,166. By contrast, peak titers with the other low-molecular-weight inducers discussed above were reported to occur 18 to 24 h after drug treatment. This difference in response time may indicate a different mechanism of interferon induction or perhaps a different population of cells that is stimulated to produce interferon.

There is no apparent structural similarity among these several low-molecular-weight compounds now reported to be effective interferon inducers in mice. Despite this diversity of chemical inducers, there is apparently a rather strict limitation on the substitutions possible in a given compound if the property of interferon induction is to be retained (1). This is further illustrated in our experiments concerning the antiviral activity of various congeners of U-25,166. The structural specificity requirements, as illustrated in Table 2, indicate that receptor sites on susceptible cells may be quite specific with respect to a given type of compound, even though a variety of parent compounds appar-

ently are capable of stimulating interferon production. As the list of active types of compounds is enlarged, and the limitations on substitution with each compound type is defined, it may be possible to approach more rationally the synthesis of effective, well tolerated interferon inducers.

The potential practical utility of U-25,166 cannot be realistically evaluated as yet. An apparent disadvantage is the fact that the minimal effective dose in mice is relatively high. The compound does, however, appear to be well tolerated and induces good levels of interferon when administered p.o. as well as parenterally. Despite its discovery in a mouse screen, U-25,166 also induces interferon in a non-rodent, i.e., the cat. This observation is of possible practical importance because of the need for effective chemotherapeutic agents to treat a variety of feline viral diseases.

We are further evaluating U-25,166 as an interferon inducer and antiviral agent in other species. In addition, studies are under way to determine the cell types responsible for interferon production after treatment with U-25,166. These, as well as toxicology and pharmacology studies in animals, must be completed before assessing the clinical potential of U-25,166 in man.

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