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POTENT SYNERGISTIC INHIBITION OF HERPES SIMPLEX VIRUS-2 BY 9-[(1, 3-DIHYDROXY-2-PROPOXY)METHYL]GUANINE IN COMBINATION WITH RECOMBINANT INTERFERONS

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DHPG, an acyclic guanine nucleoside with the structure 9-[(1,3-dihydroxy-2-propoxy)methyl]-guanine], showed potent synergism with recombinant α or β interferons and modest synergism with γ interferon in inhibiting the replication of herpes simplex virus type 2 *in vitro*. The most potent direct anti-herpes viral synergism was obtained by combination of DHPG and recombinant human interferon-β-ser_{17}; when combined, doses of each near their separate effective $dose_{50}$'s resulted in almost complete elimination of production of infectious virus within a single viral replication cycle. The anti-herpes viral activity of DHPG-interferon combinations was significantly greater than that obtained with acyclovir-interferon combinations.

The acyclic guanine nucleoside analog 9-[(1,3-dihydroxy-2-propoxy)methyl]guanine (DHPG)[a] has been shown to inhibit herpes simplex viruses *in vitro* (1-4) and *in vivo* (2,3). Although the HSV 50% inhibitory doses obtained with DHPG *in vitro* are comparable to those obtained with acyclovir, DHPG is significantly (up to 80 times) more potent when given orally than is acyclovir in protecting laboratory animals from HSV infections (2,3).

The mechanism of action of both DHPG and acyclovir against herpes simplex viruses involves their selective initial phosphorylation to the monophosphate derivative by the HSV-coded thymidine kinase, and then their subsequent phosphorylation by cellular enzymes to the 5'-triphosphate derivative which is the active species (2,3,5). DHPG is mono-phosphorylated by the viral thymidine kinase much more efficiently than is acyclovir (ACV); whereas, initial phosphorylation by the cellular thymidine kinase is even lower for DHPG than for ACV. The subsequent phosphorylations of the nucleoside monophosphate to the triphosphate, catalyzed by cellular kinases, is much more rapid for DHPG than for ACV (2). These events result in selective and higher accumulation of DHPG triphosphate in virally-infected cells, which accounts for its efficacy and low toxicity. These combined properties make DHPG a promising anti-herpes agent.

We were interested in studying the combination of DHPG with various interferons *in vitro* for three reasons. Firstly, as a prelude to *in vivo* studies, we wanted to determine if there existed a synergy between DHPG and various interferons towards inhibition of multiplication of HSV-2 during a single viral replication cycle which would be separate from (but in addition to) any potential

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[a] ABBREVIATIONS: ACV, acyclovir; DHPG, 9-[(1,3-dihydroxy-2-propoxy)methyl]guanine; ED_{50}, effective dose for inhibiting by 50%; IFN, interferon; MOI, multiplicity of infection; PFU, plaque forming units.

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synergistic effects obtained *in vivo* due to interferons' immunomodulatory effects. Secondly, we wanted to compare any direct antiviral synergism obtained by the combination of DHPG and interferon with that obtained by combining acyclovir and interferon. It has previously been reported that low doses of human leukocyte interferon (6) or human fibroblast interferon (7,8) acted synergistically with acyclovir in inhibiting replication of herpes simplex viruses *in vitro*. If a significantly greater synergistic inhibition of HSV replication was obtained by DHPG as compared to ACV in combination with low-dose interferon, this could in part explain the significantly increased efficacy of DHPG over ACV *in vivo*. Lastly, we wanted to determine if the three different major classes of interferons (α, β and γ), both alone as well as when combined with DHPG, exerted similar or different antiviral effects *in vitro* against HSV-2.

Our results now show that there is a marked synergistic inhibition of replication of HSV-2 *in vitro* by combinations of DHPG with recombinant human α or β interferons. Anti-herpes synergism was also obtained by combination of rHuIFN-γ with DHPG, although the effect *in vitro* was significantly less than that obtained by combination of DHPG with the α or β interferons. Finally, we show that the synergism of these interferons with DHPG is significantly greater than that obtained by similar combination with ACV.

MATERIALS AND METHODS

Chemicals: EMEM was from Irvine Scientific; calf and fetal calf serum were from Sterile Systems, Logan, Utah; Sea plaque agarose was from Marine Colloid Division, FMC Corp.; penicillin (10,000 U/ml)-streptomycin (10,000 U/ml) was from GIBCO; neutral red was from Sigma; DHPG and ACV were synthesized at Syntex Research, Institute of Bio-Organic Chemistry (9); deoxyguanosine was from Pharma-Waldof, Düsseldorf, West Germany; rHuIFN-α_1 (Lot #14566-060-1, 7 x 10^6 IU/mg) and rHuIFN-α_2 (Lot #13837-122, 1.5 x 10^8 IU/mg) were from Schering Corp., Bloomfield, N.J.; rHuIFN-β-ser_{17} (Lot #PR8B125AB, 5 x 10^7 IU/mg) was from Cetus Corp., Berkeley, CA; and rHuIFN-γ (Lot #D0007 SF-A; 1.2 x 10^7 U/mg) was from Genentech, Inc., S. San Francisco, CA.

Cells and Viruses: Herpes simplex virus type 2, strain G [HSV-2(G)], and Vero and HeLa cells were from American Type Culture Collection, Rockville, Md. Human embryonic tonsil (HET) cells were from Flow Laboratories, Rockville, Md. Encephalomyocarditis virus (EMCV), MDBK, and WISH cells were obtained from the Sloan-Kettering Institute, Interferon Laboratory, New York, NY. GM-2767 cells were from Mammalian Mutant Cell Repository, Camden, NJ. A549 cells were from Dr. G. Todaro, NCI, Frederick, MD. Vesicular stomatitis virus (VSV), Indiana strain, was obtained from Dr. C. Samuel, University of California, Santa Barbara, CA.

Treatments and Virus-Yield Determinations: Confluent monolayers of HET cells plated in Costar 24-well dishes (10^5 cells/well, incubated 24 hr before use), with or without 24 hr interferon treatment, were washed, and then infected with HSV-2, strain G, at a multiplicity of infection (MOI) of 5 PFU per cell. After 1 hr adsorption at 37°C in 5% CO_2 atmosphere, virus inocula were aspirated off, cells were washed, and interferons, DHPG and ACV were added as indicated to triplicate wells. Virus yield was determined 1 day after virus adsorption, by plaque assay in Vero cells.

Interferon Assays: Antiviral titers of the various interferons tested were determined by a microtiter inhibition-of-cytopathic-effect assay against VSV or EMCV (10). Titers of rHuIFN-α_1 and rHuIFN-α_2 were determined against VSV using GM-2767, MDBK and WISH cells, based on NIH HuIFN-α reference reagent, G023-901-527; activity of rHuIFN-β-ser_{17} was determined with GM-2767 and WISH cells, based on NIH HuIFN-β reference reagent, G023-902-527; and the titer of rHuIFN-γ was determined in A549 cells against EMCV, based on an in-house HuIFN-γ standard.

RESULTS

DHPG-Interferon Synergism

A very marked synergistic inhibition of replication of HSV-2 was obtained by *in vitro* combinations of DHPG with rHuIFN-α_1, α_2, and β-ser_{17}, (Table 1, A-C, synergism Combination Indexes (C.I.'s) range from 1.3 to 6.0). A more modest anti-herpes viral synergism was obtained by the *in vitro* combination of rHuIFN-γ with DHPG; this synergism was significantly less than that obtained with α or β interferons ($P < 0.05$, students t test with unequal variance). The most striking

TABLE 1: INHIBITION OF REPLICATION OF HSV-2 BY DHPG IN COMBINATION WITH RECOMBINANT HUMAN INTERFERONS ALPHA-1 AND 2, BETA-SER$_{17}$, and GAMMA[a]

	DHPG (μM)	rHuIFN[b] (IU/ml)	rHuIFN[b] (nM)	Virus Yield (PFU/ml ± S.E.)	Ratio of Virus Yield Untreated / Treated	Combination[c] Index	Combined S.E. x
A. rHuIFN-α1	0	0	0	$(1.45 \pm 0.2) \times 10^5$	≡1	---[d]	---
	0.3	0	0	$(2.0 \pm 0.2) \times 10^3$	73	---	---
	0.1	0	0	$(2.1 \pm 0.1) \times 10^4$	7.0	---	---
	0.03	0	0	$(1.1 \pm 0.06) \times 10^5$	1.4	---	---
	0	1000	7.0	$(6.5 \pm 0.5) \times 10^4$	2.2	---	---
	0	300	2.1	$(8.2 \pm 0.9) \times 10^4$	1.8	---	---
	0	100	0.7	$(1.4 \pm 0.1) \times 10^5$	1.0	---	---
	0.03	200	1.4	$(5.8 \pm 0.5) \times 10^2$	249	4.96*	0.18
	0.07	100	0.7	$(4.8 \pm 0.5) \times 10^2$	300	4.89*	0.19
	0.1	10	0.07	$(8.8 \pm 1.2) \times 10^2$	164	3.14*	0.22
	0.1	1	0.007	$(9.0 \pm 0.6) \times 10^3$	16	0.82*	0.18
	0.03	10	0.07	$(3.9 \pm 0.7) \times 10^3$	37	3.31*	0.25
	0.03	1	0.007	$(3.0 \pm 0.3) \times 10^4$	4.8	1.27*	0.21
B. rHuIFN-α2	0	0	0	$(7.7 \pm 0.8) \times 10^4$	≡1	---	---
	0.3	0	0	$(3.1 \pm 0.3) \times 10^3$	25	---	---
	0.1	0	0	$(1.7 \pm 0.06) \times 10^4$	4.5	---	---
	0.03	0	0	$(4.6 \pm 0.5) \times 10^4$	1.7	---	---
	0	1000	0.3	$(7.3 \pm 0.3) \times 10^4$	1.1	---	---
	0	300	0.1	$(8.2 \pm 0.2) \times 10^4$	0.9	---	---
	0	100	0.03	$(8.7 \pm 0.5) \times 10^4$	0.9	---	---
	0.03	200	0.07	$(8.0 \pm 0.3) \times 10^2$	96	4.14*	0.15
	0.07	100	0.03	$(6.5 \pm 0.6) \times 10^2$	118	4.00*	0.20
	0.1	10	0.003	$(5.0 \pm 1.0) \times 10^2$	154	3.65*	0.24
	0.1	1	0.0003	$(6.2 \pm 1.4) \times 10^3$	12	1.13*	0.26
C. rHuIFN-β-ser17	0	0	0	$(1.45 \pm 0.06) \times 10^5$	≡1	---	---
	0.3	0	0	$(2.4 \pm 0.1) \times 10^3$	60	---	---
	0.1	0	0	$(8.2 \pm 1.2) \times 10^3$	18	---	---
	0.03	0	0	$(8.7 \pm 0.6) \times 10^4$	1.7	---	---
	0	1000	1.0	$(3.0 \pm 0.2) \times 10^4$	4.8	---	---
	0	300	0.3	$(4.4 \pm 0.3) \times 10^4$	3.3	---	---
	0	100	0.1	$(7.2 \pm 0.2) \times 10^4$	2.0	---	---
	0.03	200	0.2	$(8.3 \pm 1.7) \times 10^1$	1750	6.04*	0.22
	0.07	100	0.1	$(6.7 \pm 1.7) \times 10^1$	2160	5.87*	0.28
	0.1	10	0.01	$(3.8 \pm 0.5) \times 10^2$	379	3.07*	0.20
	0.1	1	0.001	$(6.9 \pm 1.2) \times 10^3$	21	0.17	0.23
	0.03	10	0.01	$(2.5 \pm 0.3) \times 10^3$	58	3.55*	0.14
	0.03	1	0.001	$(2.4 \pm 0.3) \times 10^4$	6.2	1.29*	0.15
D. rHuIFN-γ	0	0	0	$(2.6 \pm 0.3) \times 10^5$	≡1	---	---
	0.1	0	0	$(6.8 \pm 0.2) \times 10^4$	3.8	---	---
	0.03	0	0	$(9.0 \pm 0.6) \times 10^4$	2.9	---	---
	0	1000	4.1	$(1.4 \pm 0.1) \times 10^5$	1.8	---	---
	0	200	0.8	$(2.5 \pm 0.1) \times 10^5$	1.0	---	---
	0.03	200	0.8	$(2.5 \pm 0.1) \times 10^4$	10.4	1.24*	0.09
	0.1	10	0.04	$(2.7 \pm 0.2) \times 10^4$	9.6	1.07*	0.10
	0.03	10	0.04	$(5.8 \pm 0.4) \times 10^4$	4.5	0.58*	0.13

[a]Human embryonic tonsil cells (Flow Laboratories) were treated with interferon or media for 24 hr, washed, then infected with HSV-2(G) at 5 PFU/cell. After 1 hr adsorption, the appropriate drugs were added (DHPG, interferon, or media). Cells were harvested after 24 hr, and single-cycle virus yield was determined by plaque titration in Vero cells.

[b]For all interferons as single agents, $\leq$ 10 IU/ml showed no anti-herpes activity in these assays.

herpes viral inhibitory effects were obtained by combinations of rHuIFN-β-ser$_{17}$ and DHPG at doses of each near their ED_{50}'s (100 IU/ml of β-IFN plus 0.07 μM DHPG). This combination resulted in up to a 1000 - 2000-fold reduction in virus yield (C.I.'s = 5.9 - 6.0), with the net effect of almost completely eliminating production of infectious virus within a single cycle of viral replication. The synergistic inhibition of HSV-2 replication by combinations of DHPG and rHuIFN-β-ser$_{17}$ was significantly greater than that obtained with the other interferons tested ($P<0.01$). It is noteworthy that, on a molecule per cell basis, rHuIFN-β-ser$_{17}$ by itself was also the best of any of the interferons tested in this system in directly inhibiting HSV-2 replication *in vitro*. Three independent experiments yielded comparable results. Deoxyguanosine, which alone showed no anti-herpes viral activity at 0.1 μM, was also inactive when combined with 100 IU/ml rHuIFN-α_2 (data not shown). This result substantiates that the synergism observed with DHPG and interferon was indeed a function of the biological anti-herpes activity of DHPG and was not due to some other property of guanine nucleosides in general.

DHPG Compared with ACV

The anti-herpes viral activity of ACV alone and in combination with the different interferons is shown in Table 2. A comparison of the anti-herpes viral activity obtained by combination of DHPG with rHuIFN -α_1, α_2, β-ser$_{17}$, and γ versus that observed with ACV-interferon combinations is shown in Table 3. Although the *in vitro* ED_{50}'s of DHPG and ACV as single antiviral agents are comparable ($P>0.1$), a significantly more potent anti-herpes viral activity was obtained by the synergistic combination of interferon with DHPG than with ACV at all doses tested ($P<0.02$). This was most striking again with the β-interferon. For example, with 200 IU/ml of rHuIFN-β-ser$_{17}$ and 0.03 μM DHPG, a 100-fold further reduction in virus yield was obtained as compared to the interferon-ACV combination ($P<0.01$). Even at the very low interferon doses of 1 or 10 IU/ml, the combination with DHPG still resulted in a further reduction in virus yield as compared to equivalent dose combinations with ACV.

DISCUSSION

At this time, the mechanism(s) involved in the striking *in vitro* anti-herpes viral synergy between DHPG and the interferons has not been determined. It is not yet known at what stage(s) interferons inhibit HSV replication *in vitro*. Inhibition of viral protein synthesis could be involved as is the case with many RNA viruses (Ref. 11 for review), or inhibition of assembly of infectious virions might be important as is the case for some other enveloped viruses such as MuLV (Ref. 12 for review) and VSV (13), or even inhibition of transcription as has been reported to occur for the DNA virus SV-40 (14). Recently, Panet and Falk (15) reported that mouse interferon (α, β mixture) inhibited HSV-1 replication at an early step prior to DNA synthesis. As interferons have not been shown to directly inhibit viral DNA synthesis as DHPG and ACV do

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[c]The Combination Index (C.I.) was calculated as described by Spector et al. (23) from the equation $(IFN)(DHPG)/[(IFN + DHPG)(VC)] = 1$ (assuming additive activity), where IFN and DHPG represent the virus yield after treatment with each drug alone, (IFN + DHPG) is the yield after treatment with the combination, and VC is the yield from untreated virus control cultures. Taking the natural log of the above equation yields the Combination Index. The two drugs are shown to be additive if $C.I. = \ln 1 = 0$ (+ 2 combined S.E.'s). If $C.I. > 0 + 2$ S.E., the interaction between the drugs is synergistic, and if $C.I. < -2$ S.E., drug antagonism exists (23). The * after the C.I. in the table indicates synergism exists.

[d]---, Not applicable.

TABLE 2: INHIBITION OF REPLICATION OF HSV-2 BY ACV IN COMBINATION WITH RECOMBINANT HUMAN INTERFERONS ALPHA-1 and 2, BETA-SER$_{17}$, and GAMMA[a]

	ACV (μM)	rHuIFN[b] (IU/ml)	(nM)	Virus Yield (PFU/ml ± S.E.)	Ratio of Virus Yield Untreated / Treated	Combination[c] Index	Combined S.E. x 2
A. rHuIFN-α1	0	0	0	(1.33 ± 0.1) x 10^5	≡1	---	---
	0.3	0	0	(9.0 ± 0.8) x 10^3	15	---	---
	0.1	0	0	(3.8 ± 0.3) x 10^4	3.5	---	---
	0.03	0	0	(5.8 ± 0.3) x 10^4	2.3	---	---
	0	300	2.1	(7.5 ± 0.8) x 10^4	1.8	---	---
	0	100	0.7	(1.0 ± 0.1) x 10^5	1.0	---	---
	0.03	200	1.4	(2.7 ± 0.2) x 10^4	4.9	0.35*	0.15
	0.07	100	0.7	(7.0 ± 0.3) x 10^3	19	1.64*	0.13
	0.1	10	0.07	(1.6 ± 0.2) x 10^4	8.3	0.58*	0.17
	0.1	1	0.007	(3.2 ± 0.2) x 10^4	4.2	-0.11	0.15
B. rHuIFN-α2	0	0	0	(1.1 ± 0.1) x 10^5	≡1	---	---
	0.3	0	0	(1.3 ± 0.1) x 10^4	8.5	---	---
	0.1	0	0	(4.4 ± 0.4) x 10^4	2.5	---	---
	0.03	0	0	(8.0 ± 0.5) x 10^4	1.4	---	---
	0	300	2.1	(1.2 ± 0.1) x 10^5	0.9	---	---
	0	100	0.7	(1.2 ± 0.1) x 10^5	0.9	---	---
	0.03	200	1.4	(2.1 ± 0.3) x 10^4	5.2	1.43*	0.17
	0.07	100	0.7	(1.2 ± 0.1) x 10^4	9.2	1.73*	0.15
	0.1	10	0.07	(1.9 ± 0.1) x 10^4	5.8	0.93*	0.16
	0.1	1	0.007	(2.3 ± 0.2) x 10^4	4.8	0.74*	0.17
C. rHuIFN-β-ser17	0	0	0	(1.45 ± 0.1) x 10^5	≡1	---	---
	0.3	0	0	(3.4 ± 0.3) x 10^3	43	---	---
	0.1	0	0	(1.1 ± 0.2) x 10^4	13	---	---
	0.03	0	0	(8.7 ± 0.5) x 10^4	1.7	---	---
	0	1000	7.0	(3.0 ± 0.2) x 10^4	4.8	---	---
	0	300	2.1	(4.4 ± 0.3) x 10^4	3.3	---	---
	0	100	0.7	(7.2 ± 0.2) x 10^4	2.0	---	---
	0.03	200	1.4	(9.8 ± 0.9) x 10^3	15	1.27*	0.12
	0.07	100	0.7	(3.1 ± 0.3) x 10^3	47	2.06*	0.15
	0.1	10	0.07	(3.1 ± 0.2) x 10^3	47	1.27*	0.22
	0.1	1	0.007	(1.4 ± 0.2) x 10^4	10	-0.24	0.25
D. rHuIFN-γ	0	0	0	(2.6 ± 0.3) x 10^5	≡1	---	---
	0.1	0	0	(3.1 ± 0.2) x 10^4	8.4	---	---
	0.03	0	0	(1.1 ± 0.1) x 10^5	2.4	---	---
	0	200	0.8	(2.5 ± 0.1) x 10^5	1.0	---	---
	0.03	200	0.8	(7.7 ± 0.3) x 10^4	3.4	0.32*	0.09
	0.1	10	0.04	(3.0 ± 0.1) x 10^4	8.7	0.18	0.10
	0.03	10	0.04	(9.7 ± 0.6) x 10^4	2.7	0.27*	0.11

a,b Footnotes as in Table 1.

c Combination Index was calculated as described in Table 1, except that virus yields after ACV instead of DHPG treatment were used.

TABLE 3: INCREASED INHIBITION OF HSV-2 BY DHPG-INTERFERON TREATMENT AS COMPARED TO ACV-INTERFERON TREATMENT[a]

	Nucleoside[b] (μM)	rHuIFN (IU/ml)	rHuIFN (nM)	Ratio of HSV-2 Yield[c] ACV/DHPG
	0.3			4.9
	0.1			2.0
	0.03			0.6
A. rHuIFN-α_1	0.03	200	1.4	51
	0.07	100	0.7	16
	0.1	10	0.07	20
	0.1	1	0.007	3.8
B. rHuIFN-α_2	0.03	200	0.07	18
	0.07	100	0.03	13
	0.1	10	0.003	27
	0.1	1	0.0003	2.5
C. rHuIFN-β-ser-17	0.03	200	0.2	118
	0.07	100	0.1	46
	0.1	10	0.01	8.2
	0.1	1	0.001	2.0
D. rHuIFN-γ	0.03	200	0.8	3.1
	0.1	10	0.04	1.1
	0.03	10	0.04	1.7

[a] Experimental conditions were as described in Table 1.

[b] DHPG or ACV treatment was at the indicated dose.

[c] Ratio of HSV-2 yield was calculated from the virus yield ratios in Table 1 (untreated/DHPG treated) divided by the virus yield ratios in Table 2 (untreated/ACV treated). Direct comparisons were made between samples tested at the same time. Duplicate experiments yielded the same increased synergy of DHPG-IFN as compared to ACV-IFN combinations.

(2,3,5), part of the synergy observed could be due to the inhibition of two different stages of the viral replication process by the two different drugs, resulting in a cascade-type of ultimate inhibition of viral replication. That DHPG showed much more potent synergism with α, β, and γ interferons than did ACV is intriguing; it may be related to the more efficient inhibition of the viral DNA polymerase by DHPG than by ACV due to the more efficient accumulation of DHPG triphosphate in infected cells. We are currently exploring the possibility that additional mechanisms may be involved for DHPG.

It is noteworthy that a potent direct anti-herpes viral synergism was observed by combination of DHPG with α or β interferons *in vitro*, but the direct antiviral synergism was less marked when γ interferon was used. This is especially interesting in light of the fact that α and β interferons appear to share the same receptor, whereas γ interferon apparently has a separate, unrelated receptor (16,17). Our results now suggest that the mechanisms involved in γ interferon's direct inhibition of herpes viral replication are different than those for α and β interferons. Along these lines, it has previously been shown that, for VSV, a longer time is

required after treatment with γ interferon to establish the antiviral state than is required with α or β interferons (18), implicating mechanistic differences. Zerial et al. (19) showed that a synergistic inhibition of HSV-1 was obtained by combination of murine interferon-γ with murine interferons α or β. Similarly, Czarniecki et al. (17) have shown that rHuIFN-γ acted synergistically with either rHuIFN-β or rHuIFN-αA in inhibiting replication of HSV-1 in vitro, whereas α and β interferon combinations were not synergistic.

In vivo, interferons will be produced by the host in response to the HSV infection (20-22). As DHPG was significantly more synergistic than was ACV with α, β, and γ interferons in inhibiting HSV-2 replication, this may partly explain the increased in vivo efficacy of DHPG over ACV. However, it is likely that additional factors, such as more efficient cellular accumulation of DHPG triphosphate, may contribute to its greater in vivo efficacy (2).

In addition to the synergism existing between DHPG and interferons determined in the present in vitro studies, it is likely that additional factors are important in vivo. The immunomodulatory effects of the interferons, especially of the γ-interferon, may contribute significantly to the overall antiviral effects in vivo. Thus it is conceivable that γ interferon may show greater anti-herpes viral efficacy in vivo than it did in vitro, both alone as well as in combination with DHPG. Preliminary results indicate that as a single systemic agent, rMuIFN-γ does show good anti-herpes viral efficacy in protecting mice from lethal systemic HSV-2 infection. It will be enlightening therefore to determine the in vivo anti-herpes viral efficacy of DHPG in comparative combination with the various interferons α, β, and γ. Such studies are currently in progress; preliminary results indicate that synergy is obtained in vivo by specific DHPG-interferon combinations (Fraser-Smith, E.B., Eppstein, D.A., Marsh, Y.V., and Matthews, T.R., manuscript in preparation).

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