Journal of Natural Products
Vol. 53, No. 5, pp. 1234-1240, Sep-Oct 1990

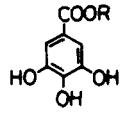
DIFFERENTIAL INHIBITION OF REVERSE TRANSCRIPTASE AND VARIOUS DNA POLYMERASES BY DIGALLIC ACID AND ITS DERIVATIVES

HIDEO NAKANE, MASANORI FUKUSHIMA,[1] and KATSUHIKO ONO*

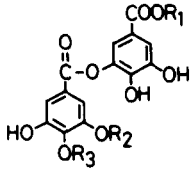
Laboratory of Viral Oncology, Aichi Cancer Center Research Institute, Chikusa-ku, Nagoya 464, Japan

ABSTRACT.—Digallic acid (gallic acid 5,6-dihydroxy-3-carboxyphenyl ester) [4] was found to be a potent inhibitor of the activities of the reverse transcriptases from murine leukemia virus (MLV) and human immunodeficiency virus (HIV). Under the reaction conditions specified for each of MLV and HIV reverse transcriptases, both enzymes were inhibited by approximately 90% in the presence of 0.5 μg/ml digallic acid. Under the same conditions, however, gallic acid had no effect on the reverse transcriptase activity. The mode of the inhibition by digallic acid was partially competitive with respect to the template·primer, $(rA)_n \cdot (dT)_{12-18}$, and noncompetitive to the triphosphate substrate, dTTP. The Ki value of digallic acid for HIV-reverse transcriptase was determined to be 0.58 μM. Examination of several derivatives of digallic acid have shown that all three hydroxyl groups at the 3, 4, and 5 positions seem to be required for the inhibitory activity of these compounds. Besides reverse transcriptase, DNA polymerases α and β were moderately inhibited by digallic acid, whereas DNA polymerase γ, terminal deoxynucleotidyltransferase, and *E. coli* DNA polymerase I were virtually insensitive to inhibition by this compound.

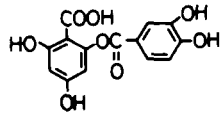
Inhibitors of reverse transcriptase play an important role in the chemotherapeutic approaches to acquired immune deficiency syndrome (AIDS). The replicative cycle of human immunodeficiency virus (HIV), the causative retrovirus of AIDS, is interrupted by a reverse transcriptase inhibitor, leading to the arrest of progeny virus production. In fact, various reverse transcriptase inhibitors have been described to inhibit HIV production in vitro and/or in vivo: i.e., hexasodium *sym*-bis(*m*-aminobenzoyl-*m*-amino-*p*-methylbenzoyl-1-naphthylamino-4,6,8-trisulfonate) carbamide (trivial name: suramin) (1,2), ammonium 21-tungsto-9-antimoniate [heteropolyanion 23 (HPA23)] (3), 3′-azido-3′-deoxythymidine (AZT) (4,5) and various 2′,3′-dideoxynucleosides (6–17). 2′,3′-Dideoxynucleosides should, however, be phosphorylated by host cell kinase system to the active triphosphate form for the inhibition of reverse transcriptase.



1 R=H
2 R=$-CH_2CH_2OCH_2CH_2OCH_3$
3 R=$-CH_2CH_2OCH_2CH_2OCH_2CH_2OCH_3$



4 $R_1=R_2=R_3=H$
5 $R_1=-CH_2CH_2OCH_2CH_2OCH_3$, $R_2=R_3=H$
6 $R_1=-CH_2CH_2OCH_2CH_2OCH_2CH_2OCH_3$, $R_2=R_3=H$
7 $R_1=R_3=H$, $R_2=-CH_2CH_2OCH_2CH_2OCH_3$
8 $R_1=R_2=H$, $R_3=-CH_2CH_2OCH_2CH_2OCH_3$



9

[1]Department of Internal Medicine, Aichi Cancer Center Hospital, Chikusa-ku, Nagoya 464, Japan.

We have been looking for natural substances that have inhibitory activity to the reverse transcriptase, and we found that some plant extracts (18, 19), as well as a flavonoid (20), had strong inhibitory effects on murine leukemia virus (MLV) and HIV reverse transcriptases. Extensive surveys of various kinds of natural compounds have revealed that gallic acid 5,6-dihydroxy-3-carboxyphenyl ester (digallic acid) [**4**] is also a potent inhibitor of HIV reverse transcriptase. Furthermore, several derivatives of digallic acid were examined to discover the structure-activity relationship, and two compounds (methoxyethoxyethyl *m*-digallate [**5**] and methoxyethoxyethoxyethyl *m*-digallate) [**6**] were also shown to be strong inhibitors of the reverse transcriptase. This paper describes the details of these findings.

EXPERIMENTAL

DIGALLIC ACID AND ITS DERIVATIVES.—Gallic acid [**1**], digallic acid [**4**], and their derivatives, i.e., methoxyethoxyethyl gallate [**2**], methoxyethoxyethoxyethyl gallate [**3**], 2,4-dihydroxy-2-(3′,4′-dihydroxybenzoyloxy)-benzoic acid [**9**], methoxyethoxyethyl *m*-digallate [**5**], methoxyethoxyethoxyethyl *m*-digallate [**6**], 3,4-dihydroxy-5-(3′,4′-dihydroxy-5′-methoxyethoxyethoxybenzoyloxy)-benzoic acid [**7**], 3,4-dihydroxy-5-(3′,5′-dihydroxy-4′-methoxyethoxyethoxybenzoyloxy)-benzoic acid [**8**], were the products of Fujirebio, Tokyo, Japan.

OTHER CHEMICALS.—[^{3}H]dTTP and [^{3}H]dGTP were from Amersham Internationl (Amersham, England); unlabeled deoxynucleoside triphosphates, poly(rA), oligo(dA), oligo(dT) from P-L Biochemicals (Milwaukee, Wisconsin); activated calf thymus DNA from Worthington Biochemical (Freehold, New Jersey); and DEAE-cellulose paper disc (DE81, diameter 23 mm) from Whatman (Springfield Mill, Maidstone, Kent, England).

ENZYMES.—Rauscher murine leukemia virus (R-MLV) was collected from the culture medium of an established virus-producing cell line, R-17, and purified by ultracentrifugation in a sucrose gradient as described previously (21). The reverse transcriptase [deoxynucleoside-triphosphate: DNA deoxynucleotidyltransferase (RNA-directed); EC 2.7.7.49] was purified on a DEAE-Sephadex A-50 column, as previously described (21). HIV reverse transcriptase was purified from *Escherichia coli* harboring an expression plasmid for the precise coding sequence of the enzyme. The purified enzyme was a gift from Dr. S.H. Wilson, NIH.

DNA polymerases [deoxynucleoside-triphosphate: DNA deoxynucleotidyltransferase (DNA-directed); EC 2.7.7.7] α, β, and γ were purified from KBIII cells as previously described for DNA polymerases α (22), β (23), and γ (24), with some modifications. Terminal deoxynucleotidyltransferase (EC 2.7.7.31) was purified from calf thymus, as described previously (25). Highly purified preparations of *E. coli* DNA polymerase I were purchased from P-L Biochemicals (Milwaukee, Wisconsin).

ASSAYS FOR REVERSE TRANSCRIPTASE AND VARIOUS DNA POLYMERASES.—Reverse transcriptase and DNA polymerases β and γ were assayed with $(rA)_n \cdot (dT)_{12-18}$ as the template·primer. DNA polymerase α and *E. coli* DNA polymerase I were assayed with activated DNA as the template·primer, and terminal deoxynucleotidyltransferase was assayed with $(dA)_{12-18}$ as the primer. All assay conditions were optimized with respect to the ratios and concentrations of all the template·primers used as well as to pH and divalent and monovalent cation concentrations, as described previously (26). For reverse transcriptase assay, however, the concentration of $(rA)_n \cdot (dT)_{12-18}$ (base ratio, 1:1) was 4 μg/ml. The concentrations of [^{3}H]dNTP or template·primer and digallic acid inhibitors varied in experiments designed to determine the Km and Ki values. All incubations (50 μl) were carried out at 37° for 30 min, and the reaction was stopped by adding 20 μl 0.2 M EDTA and immersing the mixture in ice. Then, 50 μl of the mixture was transferred to a DE81 filter paper disc and processed for measurement of radioactivity as described previously (27).

RESULTS

EFFECTS OF DIGALLIC ACID ON THE ACTIVITIES OF MLV AND HIV REVERSE TRANSCRIPTASES.—The effects of digallic acid [**4**] on the activities of MLV and HIV reverse transcriptases were examined under the conditions described in Materials and Methods. When examined with $(rA)_n \cdot (dT)_{12-18}$ as the template·primer, the activities of MLV and HIV reverse transcriptases were inhibited by more than 70% in the presence of 0.2 μg/ml digallic acid (Figure 1). The inhibition by digallic acid was dose-de-

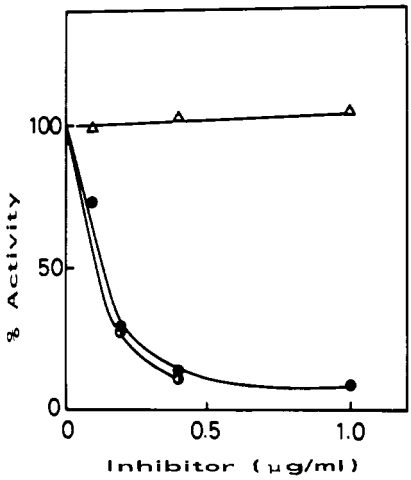


FIGURE 1. Effects of gallic and digallic acids on the activities of MLV and HIV reverse transcriptases. Reverse transcriptase activity was measured under the conditions described in Materials and Methods in the presence of various concentrations of gallic acid [**1**] or digallic acid [**4**] as indicated in the figure, by determining the incorporation of [^{3}H]dTMP (400 cpm/pmol) with $(rA)_n \cdot (dT)_{12-18}$ as the template·primer. The reverse transcriptases tested were from Rauscher murine leukemia virus (●) and human immunodeficiency virus (Δ, ◑). The compounds tested were gallic acid (Δ) and digallic acid (●, ◑). The 100% values (pmol) were 2.5 (●), and 45.0 (Δ, ◑).

pendent, and both enzyme activities were almost completely inhibited at a concentration of 1 μg/ml. On the other hand, gallic acid [**1**] had no inhibitory effect on the enzyme activity at concentrations up to 10 μg/ml.

ANALYSIS OF THE MODE OF INHIBITION AND DETERMINATION OF KINETIC CONSTANTS.—The inhibition was analyzed kinetically by changing the concentration of either the template·primer or the triphosphate substrate with HIV reverse transcriptase. A typical example is shown in Figure 2. Digallic acid inhibited HIV reverse transcriptase activity by partially competing with the template·primer, $(rA)_n \cdot (dT)_{12-18}$ (Figure 2A), for the same binding site, but not with the triphosphate substrate, dTTP (Figure 2B). The Ki value of digallic acid for HIV reverse transcriptase was determined by replotting (Dixon plot) the data in Figure 2A and is described in Table 1 in comparison with other Ki's of digallic acid derivatives.

EFFECTS OF DIGALLIC ACID ON THE ACTIVITIES OF VARIOUS DNA POLYMERASES.—The effects of digallic acid on the activities of various DNA polymerases were examined under the conditions described in Materials and Methods. As shown in Figure 3, digallic acid was found to inhibit differentially all the polymerases tested. Besides the reverse transcriptase, DNA polymerase α was fairly sensitive to inhibition by this compound (more than 90% inhibition was observed in the presence of 1 μg/ml digallic acid). DNA polymerase β was moderately inhibited by this compound, whereas DNA polymerase γ, terminal deoxynucleotidyltransferase, and DNA polymerase I were virtually insensitive to this compound (Figure 3). These results are summarized in Table 2 with the Ki values of DNA polymerases α and β.

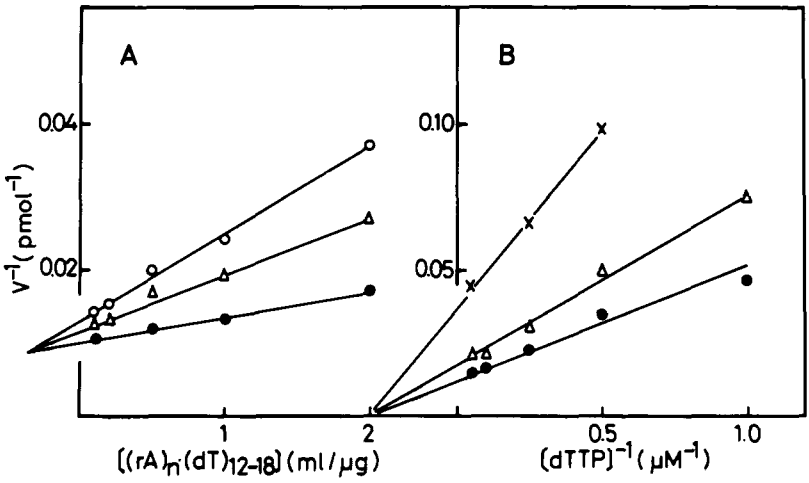


FIGURE 2. Analysis of the inhibition of HIV reverse transcriptase by digallic acid [**4**]. Reactions were carried out under the conditions described in Materials and Methods, except that various concentrations of $(rA)_n \cdot (dT)_{12-18}$ (A) and [^{3}H]dTTP (400 cpm/pmol) (B) were used as the template·primer and the triphosphate substrate, respectively, in the presence of various concentrations of digallic acid as indicated in the figure. Digallic acid concentrations were 0 (●), 0.2 (Δ), 0.4 (○), and 0.6 (×) μg/ml. The figure represents double reciprocal plots.

EFFECTS OF DIGALLIC ACID DERIVATIVES ON THE REVERSE TRANSCRIPTASE ACTIVITY.—Five derivatives **5**–**9** of digallic acid [**4**] and two derivatives **2** and **3** of gallic acid [**1**] were tested for the ability to inhibit the reverse transcriptase activity. Neither gallic acid nor either of its derivatives with modified carboxyl group were inhibitory to the enzyme (data not shown), whereas compounds **5** and **6**, two of the five derivatives of digallic acid, were found to be strongly inhibitory to the enzyme activity (Figure 4). Both of them have the same structure as that of digallic acid, except that the carboxyl group was modified with a methoxyethoxyethyl or a methoxyethoxyethoxyethyl group. Neither **7** nor **8** had inhibitory effect on the enzyme activity, indicating an important role of 4′,5′-hydroxyl and probably also 3′-hydroxyl groups in the inhibition of reverse transcriptase. Also, **9** was ineffective for the inhibition of the reverse transcriptase activity.

DISCUSSION

Some plant extracts and plant-derived compounds have previously been shown in

TABLE 1. Characterization of Inhibition of HIV Reverse Transcriptase by Digallic Acid and its Derivatives.

Compound	Inhibition	
	Mode	Ki (μM)
Digallic acid [**4**]	mixed-type to $(rA)_n \cdot (dT)_{12-18}$ noncompetitive to dTTP	0.58
Methoxyethoxyethyl *m*-digallate [**5**]	competitive to $(rA)_n \cdot (dT)_{12-18}$ noncompetitive to dTTP	0.12
Methoxyethoxyethoxyethyl *m*-digallate [**6**]	mixed-type to $(rA)_n \cdot (dT)_{12-18}$ noncompetitive to dTTP	0.39

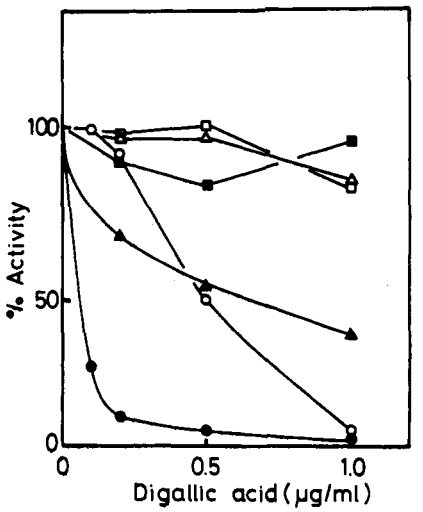


FIGURE 3. Effects of digallic acid [4] on the activities of various DNA polymerases. DNA polymerase activities were measured under the conditions described in Materials and Methods in the presence of various concentrations of digallic acid as indicated in the figure. DNA polymerases α (○), β (▲), and γ (Δ), terminal deoxynucleotidyltransferase (■), *Escherichia coli* DNA polymerase I (□), and Rauscher MLV reverse transcriptase (●). Specific radioactivity (cpm/pmol) of [^{3}H]dTTP was 1000 (○), 400 (▲), 6000 (Δ), 400 (□), and 400 (●), and that of [^{3}H]dGTP was 400 (■). The 100% values (pmol) were 37.5 (○), 6.4 (▲), 3.6 (Δ), 35.0 (■), 190.4 (□), and 16.3 (●).

our laboratories to have inhibitory effects on the activity of reverse transcriptase (18–20). Digallic acid is also a plant-derived compound originally isolated from Aleppo gallotannin and Chinese gallotannin. This compound proved in this study to be effective in inhibiting the activities of the reverse transcriptases from MLV and HIV. It inhibited the enzyme activity by partially competing with the template·primer, $(rA)_n \cdot (dT)_{12-18}$, for the same binding site on the enzyme but not with the triphosphate

TABLE 2. Characterization of Inhibition of various DNA Polymerases by Digallic Acid [4].

DNA polymerase	Inhibition	
	Mode	Ki (μM)
Eukaryotic		
α	competitive to activated DNA noncompetitive to dNTP	0.83
β	competitive to $(rA)_n \cdot (dT)_{12-18}$ noncompetitive to dTTP	0.39
γ	NI[c]	
TdT[a]	NI	
Bacterial		
Pol I[b]	NI	

[a]TdT, terminal deoxynucleotidyltransferase.
[b]Pol I, DNA polymerase I.
[c]NI, no inhibition.

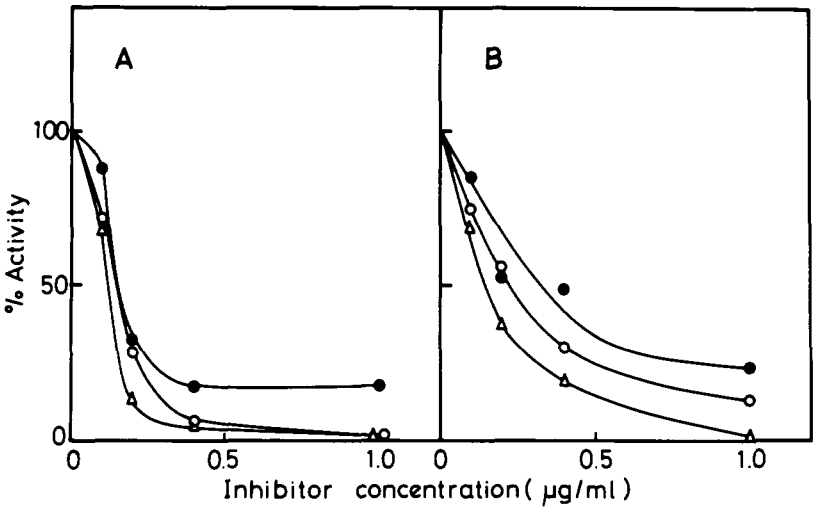


FIGURE 4. Effects of digallic acid derivatives on the reverse transcriptase activity. Reverse transcriptase activity was measured under the same conditions as in Figure 1, in the presence of various concentrations of digallic acid derivatives as indicated in the figure. The reverse transcriptases were from Rauscher MLV (A) and HIV (B). Digallic acid derivatives were methoxyethoxyethyl *m*-digallate [**5**] (Δ) and methoxyethoxyethoxyethyl *m*-digallate [**6**] (○). Digallic acid (●). The 100% values (pmol) were 15.8 (A) and 19.6 (B).

substrate, dTTP (Figure 2). The Ki value was determined to be as low as 0.58 μM, which is in a similar order of magnitude as those of baicalein (20), quercetin, and quercetagetin (28). It was unfortunate, however, that the highly purified reverse transcriptases used in the present study could transcribe only $(rA)_n \cdot (dT)_{12-18}$, and , therefore, we could not test whether digallic acid is also inhibitory with other template·primers.

Several derivatives of digallic acid and gallic acid were examined to study the structure-activity relationship in the inhibition of the reverse transcriptase activity by this class of compounds. As shown in Figure 1, neither gallic acid [**1**] nor its two derivatives **2** and **3** inhibited the enzyme activity, indicating the requirement of the digallic acid structure for the inhibition. On the other hand, the digallic acid derivatives modified on the carboxyl group have been shown to have strong inhibitory activity to the enzyme, indicating that this carboxyl group had no role in inhibiting the enzyme activity (Figure 4). In contrast, methoxyethoxyethylation of the hydroxyl group on either the 4′ or 5′ position greatly reduced the inhibitory potencies of these compounds, suggesting an important contribution of these two hydroxyl groups in the inhibition of the reverse transcriptase by digallic acid.

Digallic acid is also inhibitory to DNA polymerase α. The activity of α-polymerase was almost completely inhibited in the presence of 1 μg/ml digallic acid. On the other hand, DNA polymerase β was moderately inhibited, whereas DNA polymerase γ, terminal deoxynucleotidyltransferase, and DNA polymerase I were not inhibited by this compound at concentrations up to 1 μg/ml. In summary, the fact that the inhibition by digallic acid at its lower concentrations (<0.5 μg/ml) is relatively specific for reverse transcriptase may indicate that the compound is not toxic for the retrovirus-infected host cells at low concentrations. Further testing with HIV-infected cultured cells will answer this question and determine possible use of this compound as an antiretroviral agent.

ACKNOWLEDGMENTS

We are grateful to Mrs. S. Shinmura for the preparation of this manuscript.

LITERATURE CITED

1. H. Mitsuya, M. Popovic, R. Yarchoan, S. Matsushita, R.C. Gallo, and S. Broder, *Science (Washington, D.C., 1883–)*, **226**, 172 (1984).
2. S. Broder, R. Yarchoan, J.M. Collins, H.C. Lane, P.D. Markham, R.W. Klecker, R.R. Redfield, H. Mitsuya, D.F. Hoth, E. Gelmann, J.E. Groopman, L. Resnick, R.C. Gallo, C.E. Myers, and A.S. Fauci, *Lancet*, (II), 627 (1985).
3. W. Rozenbaum, D. Dormont, B. Spire, E. Vilmer, M. Gentilini, C. Griscelli, L. Montagnier, F. Barré-Sinoussi, and J.-C. Chermann, *Lancet*, (I), 450 (1985).
4. H. Mitsuya, K.J. Weinhold, P.A. Furman, M.H. St. Clair, S. Nusinoff-Lehrman, R.C. Gallo, D. Bolognesi, D.W. Barry, and S. Broder, *Proc. Natl. Acad. Sci. USA*, **82**, 7096 (1985).
5. R. Yarchoan, R.W. Klecker, K.J. Weinhold, P.D. Markham, H.K. Lyerly, D.T. Durack, E. Gelmann, S. Nusinoff-Lehrman, R.M. Blum, D.W. Barry, G.M. Shearer, M.A. Fischl, H. Mitsuya, R.C. Gallo, J.M. Collins, D.P. Bolognesi, C.E. Myers, and S. Broder, *Lancet*, (I), 575 (1986).
6. H. Mitsuya and S. Broder, *Proc. Natl. Acad. Sci. USA*, **83**, 1911 (1986).
7. J. Balzarini, R. Pauwels, P. Herdewijn, E. De Clercq, D.A. Cooney, G.-J. Kang, M. Dalal, D.G. Johns, and S. Broder, *Biochem. Biophys. Res. Commun.*, **140**, 735 (1986).
8. T.-S. Lin, R.F. Schinazi, M.S. Chen, E. Kinney-Thomas, and W.H. Prusoff, *Biochem. Pharmacol.*, **36**, 311 (1987).
9. J. Balzarini, G.-J. Kang, M. Dalal, P. Herdewijn. E. De Clercq, S. Broder, and D.G. Johns, *Mol. Pharmacol.*, **32**, 162 (1987).
10. M. Baba, R. Pauwels, P. Herdewijn, E. De Clercq, J. Desmyter, and M. Vandeputte, *Biochem. Biophys. Res. Commun.*, **142**, 128 (1987).
11. T.-S. Lin, R.F. Schinazi, and W.H. Prusoff, *Biochem. Pharmacol.*, **36**, 2713 (1987).
12. J. Balzarini, R. Pauwels, M. Baba, M.J. Robins, R. Zou, P. Herdewijn, and E. De Clercq, *Biochem. Biophys. Res. Commun.*, **145**, 269 (1987).
13. M. Baba, R. Pauwels, J. Balzarini, P. Herdewijn, and E. De Clercq, *Biochem. Biophys. Res. Commun.*, **145**, 1080 (1987).
14. H. Hartmann, G. Hunsmann, and F. Eckstein, *Lancet*, (I), 40 (1987).
15. J. Balzarini, M. Baba, R. Pauwels, P. Herdewijn, S.G. Wood, M.J. Robins, and E. De Clercq, *Mol. Pharmacol.*, **33**, 243 (1988).
16. J. Balzarini, M. Baba, R. Pauwels, P. Herdewijn, and E. De Clercq, *Biochem. Pharmacol.*, **37**, 2847 (1988).
17. J. Balzarini, P. Herdewijn, R. Pauwels, S. Broder, and E. De Clercq, *Biochem. Pharmacol.*, **37**, 2395 (1988).
18. K. Ono, H. Nakane, Z.-M. Meng, Y. Ose, Y. Sakai, and M. Mizuno, *Chem. Pharm. Bull.*, **37**, 1810 (1989).
19. K. Ono, H. Nakane, M. Fukushima, J.-C. Chermann, and F. Barré-Sinoussi, *Biomed. Pharmacother.*, **44**, 13 (1990).
20. K. Ono, H. Nakane, M. Fukushima, J.-C. Chermann, and F. Barré-Sinoussi, *Biochem. Biophys. Res. Commun.*, **160**, 982 (1989).
21. K. Nakajima, K. Ono, and Y. Ito, *Intervirology*, **3**, 332 (1974).
22. A. Matsukage, M. Sivarajan, and S.H. Wilson, *Biochemistry*, **15**, 5305 (1976).
23. K. Ono, A. Ohashi, K. Tanabe, A. Matsukage, M. Nishizawa, and T. Takahashi, *Nucleic Acids Res.*, **7**, 715 (1979).
24. M. Yamaguchi, A. Matsukage, and T. Takahashi, *J. Biol. Chem.*, **255**, 7002 (1980).
25. S. Okamura, F. Crane, H.A. Messner, and T.W. Mak, *J. Biol. Chem.*, **253**, 3765 (1978).
26. H. Nakane, J. Balzarini, E. De Clercq, and K. Ono, *Eur. J. Biochem.*, **177**, 91 (1988).
27. T.J. Lindell, F. Weinberg, P.W. Morris, R.G. Roeder, and M.J. Rutter, *Science (Washington, D.C., 1883–)*, **170**, 447 (1967).
28. K. Ono, H. Nakane, M. Fukushima, J.-C. Chermann, and F. Barré-Sinoussi, *Eur. J. Biochem.*, **190**, 469 (1990).

Received 5 February 1990