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Hypothesis

A kinetic model for comparing proteolytic processing activity and inhibitor resistance potential of mutant HIV-1 proteases

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Abstract A kinetic model is presented for the comparison of potential proteolytic processing activities of wild-type and mutant human immunodeficiency virus proteases in the presence and absence of protease inhibitors. The protease processing of *gag* substrate in the immature virions is assessed by the kinetic parameters, k_{cat}, K_m and K_i. The relationship of the estimated potential processing activities to the viability of the HIV mutants and their tendencies to resist inhibitors also are discussed. A fully developed model has the potential to simulate the results of inhibitor resistance either in vivo or in cell culture.

Key words: HIV protease; Processing activity; Mutation; Drug resistance

1. Introduction

Human immunodeficiency virus contains a protease which is responsible for the processing of the *gag* and *gag–pol* precursors to yield structural proteins and enzymes of the mature virus. The structure of the enzyme [1–3] and its specificity [4] have been well studied. This enzyme, which is essential for HIV maturation and infectivity [5,6], is an important target for AIDS therapy. Many potent HIV protease inhibitors have been tested [7–11]. In vitro, in vivo, and clinical experiments have shown that some of these inhibitors have anti-HIV properties [12–15]. Therapeutic use of an HIV protease inhibitor is adversely impacted by viral drug resistance resulting from mutations of protease gene and selection of resistant strains in the presence of inhibitors [16,17]. Resistance to protease inhibitors has also been demonstrated in HIV grown in cultured cells [18–24]. The resistance problem needs to be overcome before this line of therapy can be effective.

How structural mutation of HIV protease renders it inhibitor resistant is poorly understood. Resistant mutants can apparently retain sufficient enzyme activity but specifically reduce inhibition sensitivity. Consequently, potential resistant mutations can be analyzed by comparing the kinetic parameters for activity and inhibition of wild-type and mutant enzymes. Although there are several reports of kinetic data for HIV protease mutants, no method has been developed to assess in vivo processing activity and resistant potential for mutant enzymes.

2. A kinetic model for assessing the processing activity of viral mutants

In the presence of inhibitors, the overall processing activity in a virion is a balance of protease processing efficiency (k_{cat}/K_m) and inhibition efficiency (specified by inhibition constant, K_i, and inhibitor concentration, $[I]$). The processing activity, a_{MI}, is described by the following equation for a freely diffusible system where the effective substrate concentrations are significantly smaller than K_m:

$$a_{MI} = \sigma \frac{k_{cat}/K_m}{1 + \frac{[I]}{K_i}} \quad (1)$$

where σ is determined by initial substrate and protease concentrations.

Eqn. 1 can describe the intra-virion processing because the 'effective substrate concentration' inside of the immature virions is expected to be much lower than the total substrate concentration. In the immature virions, the p55 *gag* polyproteins form a '*gag* shell' with the p17 ends anchored in the outer lipid membrane. The p55 subunits are cylinder shaped and are densely packed in an icosahedral two-dimensional array [25]. The *gag* portion of the p190 *gag–pol* precursor co-assembles with p55 in the '*gag* shell' while the *pol* cylinder (including protease) is located in the space inside of the '*gag* shell' (Fig. 1). To initiate processing, the protease is likely freed by self-excision from the precursor [26,27] then by protease catalyzed release. The pores in the '*gag* shell' are too small [25] for the protease to penetrate. Thus, the processing must proceed through the accessible cleavage sites on the inner surface of the '*gag* shell'. This near sequential processing mechanism is supported by the observation that p39 (p17–p24) is the major processing intermediate [28,29] and is consistent with the final locations of individual *gag* subunits in the mature virion. The total concentration of a single p55 cleavage site inside the immature virion is estimated to be 5.6 mM [25]. The 'effective substrate concentration' is lower since p55 is membrane anchored. (a) The probability of enzyme–substrate collision is diminished by more than 50-fold. (b) The abolishment of gradient-driven substrate diffusion toward the enzyme after a transient low substrate concentration around the enzyme is created

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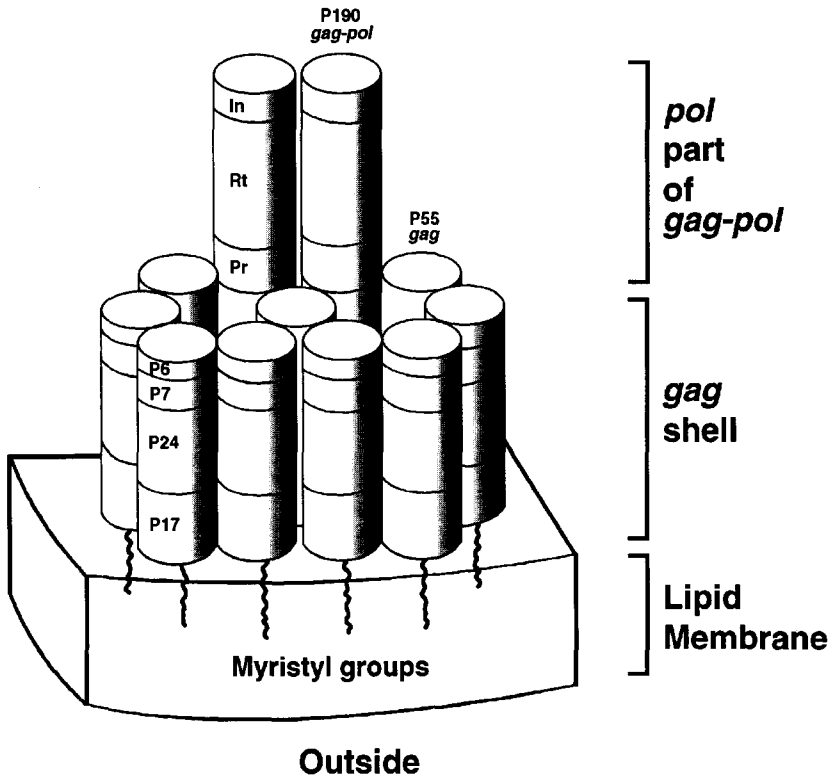

Fig. 1. Schematic presentation of the packing relationships of p55 *gag* and p190 *gag–pol* inside of a membrane section of immature HIV virion. Immediately inside of the lipid membrane is a layer '*gag* shell' which contains tightly packed, cylinder shaped p55 *gag* precursor units organized in icosahedral pattern. Each p55 unit is anchored in the membrane by an N-terminal myristoyl group. The two longer cylinders represent p190 *gag–pol* with the protease dimer located adjacent to the '*gag* shell'. The processed protease dimer (dimensions about 39 Å × 53 Å) is unable to enter the cavity (diameter about 35 Å) in the '*gag* shell', so the processing of the *gag* is assumed to be sequential from the p6 to p17 as marked on the p55 cylinder in Fig. 1. The packing dimensions of p55 in the '*gag* shell' is taken from the work of Nermut et al., [25]. In each immature virion there are about 1890 molecules of p55 of which 95 are in the p190 form.

by the conversion of substrate to products. Even without considering (b), the 'effective substrate concentration' is estimated at 80 μM[1]. Since the hydrolysis of bonds in *gag* polyprotein is sequential, the overall processing rate is limited by the slowest step, which is the site between p7 and p6 [30]. The K_m for the hydrolysis of this site is 530 μM, much higher than the 'effective substrate concentration' when all factors are considered. The kinetics for the hydrolysis of membrane-fixed *gag* should follow steady-state kinetics. In an analogous case, the hydrolysis of membrane-located phosphatidylcholine by phospholipase A_2 [31] conforms with Michaelis-Menton kinetics without possessivity by the enzyme toward the substrate surface. Therefore, the kinetics of the intra-virion processing should be appropriately described by kinetics characteristic of [S] $\ll K_m$.

Additionally, a teleologic argument supports dilute-substrate kinetics. Several HIV-1 protease mutants are 'K_m-mutants' (such as Val^{82} replaced by Glu, Ala, Asp or Gln) in that they have relatively unchanged k_{cat} values but large changes of K_m compared to the wild-type enzyme [32]. If substrate concentration were high inside the immature virions, processing velocities for 'K_m-mutants' would proceed at near V_{max}, and these mutants would have be observed as wild-types. This is not the case [33–35]. Taken together, these facts support use of Eqn. 1 to predict processing activity in the immature virions.

3. Potential processing activities of HIV-1 protease mutants and their relationships to viral viability and resistance to inhibitors

Eqn. 1 can be used to define the resistance potential of different mutants against an inhibitor or to compare the resistance potential of a mutant enzyme against different inhibitors. For the convenience of comparison, the a_{MI} values are calculated for a constant [*I*]. Then the potential processing activity can be experimentally defined with kinetic parameters, k_{cat}, K_m, and K_i. MMA, mutation modulated activity, is the ratio of a_{MI} for a particular mutant to the processing activity for uninhibited wild-type HIV protease expressed as a percentage.

We have calculated the potential processing activities of wild-

[1]Relative contact volumes between enzyme and substrate were used to estimate the predicted decrease of effective substrate concentration compared to total substrate concentration. The contact volume for the anchored substrate is a spherical shell adjacent to the p55 cylinder ends which has thickness equal to the HIV-PR radius. This volume is decreased by the fraction of the spherical surface which is filled by p55; i.e. the area between cylinder ends is omitted from the calculation. The diffusible substrate contact volume is the volume of a sphere of radius equal to the sum of the p55 and HIV-PR radii multiplied by the number of substrate molecules. For the surface model the volume of the spherical shell is 4.2×10^7 Å^3 and the fraction of the surface occupied by cylinder ends is 0.64. For the diffusible model, the contact volume is 1.9×10^9 Å^3. The effective substrate concentration is then:

$$5.6 \text{ mM} \left(\frac{4.2 \times 10^7}{1.9 \times 10^9}\right)(0.64) = 0.08 \text{ mM}.$$

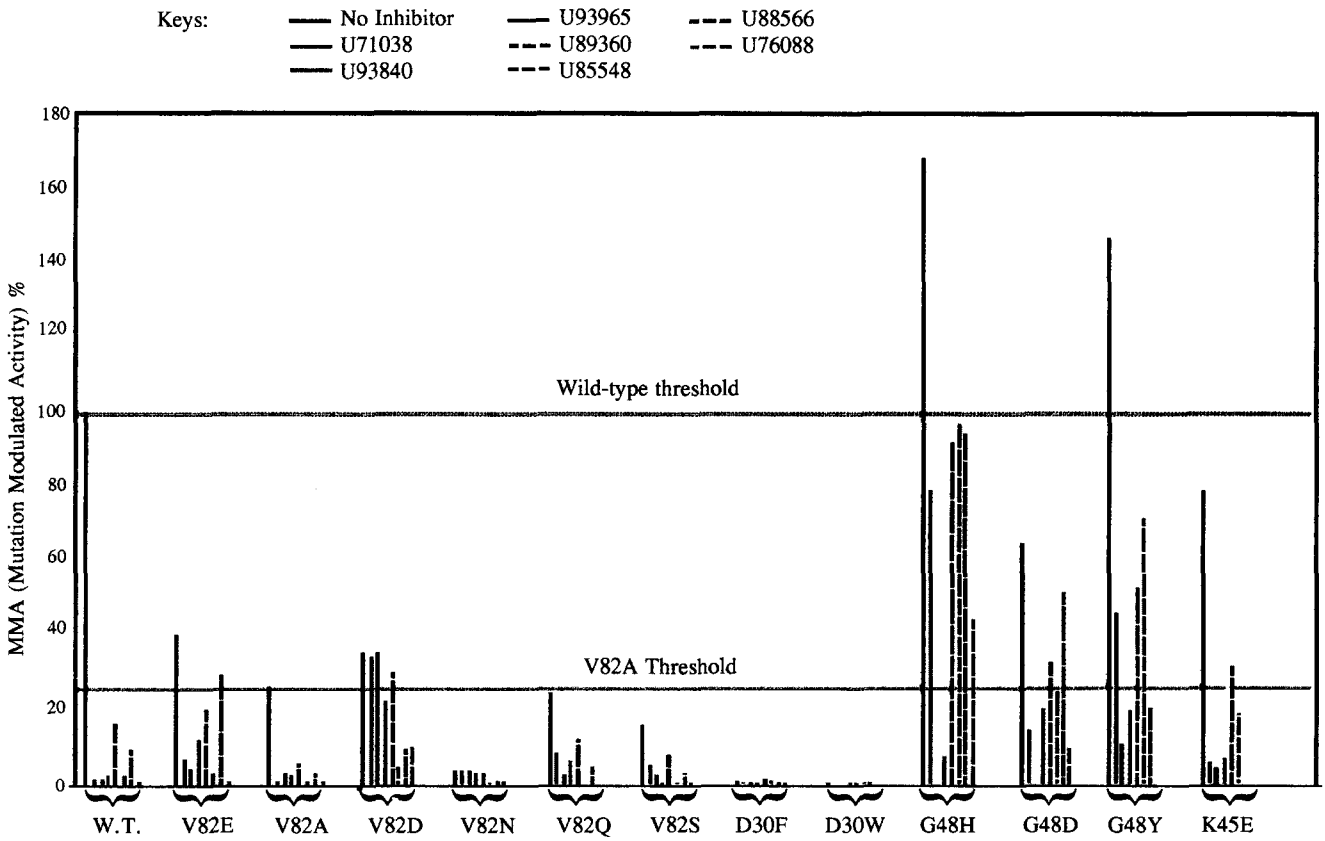

Fig. 2. 'Mutation modulated activity' (MMA) of HIV-1 protease and 12 mutants in presence and absence of inhibitors. The MMA value of the wild-type HIV-1 protease is taken as 100% (see footnote of Table 1). The MMA value of V82A in the absence of inhibitor (horizontal line at 26%) is taken as the minimal threshold that can support the HIV life cycle. For each enzyme, the first bar on the left is without inhibitor then the order of the inhibitors, from left to right, is U71038, U93840, U93965, U89360, U85548, U88566 and U76088.

type HIV-1 protease and 27 mutants against individual inhibitors based on reported kinetic data [32,36,37]. An inhibitor concentration of 10^{-7} M is used. Since the K_i of inhibitors of interest are in the low nmole range, the use of [*I*] 10–100-fold greater than K_i is required to achieve significant inhibition. In Fig. 2, all MMA values calculated from the work of [32] are plotted together. As can be seen, these values range from insignificant to over 170%.

It is useful to know the threshold MMA value of a viable mutant. We assume that the viral processing activity in patients is directly linked to viral maturation and infection of the new cells. Thus, a MMA value above the threshold level is required to attain a sufficient rate of new cell infection to counter the elimination of the infected cells by the immune system so that an in vivo steady state is attained [38]. When the in vivo threshold is adequately defined, the present model is a potentially useful tool for predicting viral viability and inhibitor resistance. Many laboratories have used in vitro selection of resistant strains of HIV in the presence of inhibitors. Unlike the in vivo selections where the mutant proteases must keep pace with cell destruction, the in vitro selections need only maintain the viral proliferation. So a protease mutant with a MMA value below the in vivo threshold may still be a successful resistant strain in vitro. However, the current model can be correlated to both in vivo and in vitro HIV viability and resistance because the difference is only the respective thresholds. There is not sufficient information to establish an in vivo threshold presently. An example of an in vitro model is the use of the MMA value of mutant V82A, 26%, as a threshold. Mutant V82A has been selected in cell culture in the presence of different inhibitors [21,39] so the activity of V82A must be able to support the proliferation of the virus in tissue culture in the absence of any inhibitor. The V82A threshold line in Fig. 2 serves to illustrate the importance of a threshold line to identify mutants which are viable in the absence of inhibitor and whether a mutant is resistant in the presence of inhibitors. For all 7 inhibitors at 10^{-7} M, the wild-type enzyme has MMA values below the V82A threshold. Several mutant enzymes, V82N, V82S, D30F, and D30W, have MMA values below the V82A threshold in the absence of inhibitor and are probably not viable mutations for HIV grown in tissue culture. Mutants V82D, V82A, V82D, G48H, G48D, G48Y, K45E and possibly V82Q are likely viable mutants. Resistance against some of the 7 inhibitors are seen for V82E, V82D, K45E and 3 G48 mutants. Interestingly, resistance in the case of V82E and V82D are achieved by loss of sensitivity to inhibitors compared to the wildtype enzyme. The resistance in G48H and G48Y mutants was achieved by the increase of uninhibited MMA value over the wild-type protease. Similar analysis can be made for kinetic data of other HIV mutants [36,37] to simulate in vitro resistance.

In vitro selection of HIV protease mutants in tissue culture in the presence of an inhibitor A-77003 with K_i values have been reported [21,22]. The relative k_{cat}/K_m values are available only for mutant enzymes V32I and V82I [36,37]. We have calculated

Table 1
Estimated[a] processing activity of resistant HIV-1 protease mutants identified from cell culture experiments[b]

Mutants	Inhibitor concentration			
	K_i (nM)	10^{-6} M	10^{-7} M	10^{-8} M
	Estimated MMA values			
From Kaplan et al. [22]				
Wild-type	0.5	0.05	0.50	4.8
R8Q	31	3.0	24	76
V32I	3.8	0.02	0.23	1.8
V32I/V82I	11	1.1	9.9	52
M46F	2.0	0.20	2.0	17
M46L	1.3	0.13	1.3	12
V82I	0.5	0.10	1.0	10
From Ho et al. [21]				
Wild-type	0.084	0.01	0.08	0.83
R8Q	2.7	0.27	2.6	21
M46I	0.12	0.01	0.12	1.2

[a]For mutants R8Q, V32I/V82I, M46F, M46L and M46I, the estimated MMA values were calculated assuming k_{cat}/K_m values for each mutant were the same as for the wild-type enzyme. For mutants V32I and V82I, the mutant to wild type ratio of k_{cat}/K_m was taken from the results of Sardana et al. [36].
[b]The cell culture experiments were those of Kaplan et al. [22] and Ho et al. [21]. The inhibitor used was A-77033 in both studies.

the MMA values for V32I and V82I and have estimated MMA for other mutant enzymes (R8Q, M46I, V32I/V82I, M46F and M46L) using the k_{cat}/K_m values of the wild-type enzyme. Since the k_{cat}/K_m values of most mutants are lower than that of the wild-type [32], the estimated MMA value is probably the maximal processing activity of a given mutant at the specified inhibitor concentration. Table 1 shows 7 mutants resistant to A-77003 in tissue culture [22]. These values varied greatly and only 1 (R8Q) has a MMA value approaching the V82A threshold at [*I*] equal 10^{-7} M. At 10^{-6} M inhibitor, the activities of all mutants are extremely low. The K_i value of R8Q determined by Ho et al. [21] is about 10 times lower than that reported by Kaplan et al. [22]. Only at 10^{-8} M inhibitor does the MMA (21%) approach the V82A threshold (26%) (Table 1). Apparently the final inhibitor concentration used to select resistant HIV strains, 10^{-5} M, is 100–1000-fold higher than that required to keep the activity of R8Q below the V82A threshold. It is possible that the inhibitor concentration at the processing site is limited by membrane penetration or the even the modification of the inhibitor by the host cells. If these are the only explanations, then all MMA values should be uniformly low, which is not the case. There are several possible explanations for this disparity. First, it is possible that HIV-cell culture with protease inhibitor selects, in addition to the resistant protease mutants, also phenotypes which can reduce the inhibitor concentration at the processing site. Another possibility is that HIV can mutate the processing site sequences in order to increase the MMA values. Although there are eight processing sequences, mutation in the sequence of the poorest substrate site could be sufficient to enable viral propagation. Finally, as discussed above, an in vitro selection system may allow low efficiency mutants to propagate slowly but still be selected.

4. Future experiments

The current kinetic analysis provides a quantitative model for predicting proliferation of HIV with mutant enzymes and the potential of the mutant enzymes to resist inhibitors in both in vivo and in vitro systems. There are a number of uncertainties in this model which need substantiation and improvement. First, the assumption that the effective concentration of the substrate is significantly smaller than K_m requires experimental verification. Second, the MMA values for inhibitor resistant mutants selected in vivo and in vitro need to be determined in order to establish better threshold values. This requires that kinetic parameters be collected for the wild-type and mutant HIV proteases using the same substrate, which is not widely available in the literature. Also, it is preferable to use substrates taken from the original processing sites. Third, resistance and the lack of it predicted by this kinetic model can be further tested in in vivo and in vitro experiments. These new experiments would help to further develop this model which may useful in quantitating and predicting the viability and the inhibitor resistance potentials of HIV protease mutants. This model may also aid the studies on structural changes in HIV protease mutations responsible for inhibitor resistance.

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References

[1] Wlodawer, A., Miller, M., Jaskolski, M., Sathyanarayana, B.K., Baldwin, E., Weber, I.T., Selk, L.M., Clawson, L., Schneider, J. and Kent, S.B.H. (1989) Science 245, 616–621.
[2] Navia, M.A., Fitzgerald, P.M.D., McKeever, B.M., Leu, C.-T., Heimbach, J.C., Herber, W.K., Sigal, I.S., Darke, P.L. and Springer, J.P. (1989) Nature 337, 615–620.
[3] Lapatto, R., Blundell, T., Hemmings, A., Overington, J., Wilderspin, A., Wood, S., Merson, J.R., Whittle, P.J., Danley, D.E., Geoghegan, K.F., Hawrylik, S.J., Lee, S.E., Scheld, K.G. and Hobart, P.M. (1989) Nature 342, 299–302.
[4] Poorman, R.A., Tomasselli, A.G., Heinrikson, R.L. and Kezdy, F.J. (1991) J. Biol. Chem. 266, 14554–14561.
[5] Kohl, N.E., Emini, E.A., Schleif, W.A., Davis, L.J., Heimbach, J.C., Dixon, R.A.F., Scolnick, E.M. and Sigal, I.S.(1988) Proc. Natl. Acad. Sci. USA 85, 4686–4690.
[6] Peng, C., Ho, B.K., Chang, T.W. and Chang, N.T. (1989) J. Virol. 63, 2550–2555.
[7] Debouck, C. and Metcalf, B.W. (1990) Drug Dev. Res. 21, 1–17.
[8] Petteway Jr., S.R., Dreyer, G.B., Meek, T.D., Metcalf, B.W. and Lambert, D.M. (1991) in: AIDS Research Review 1 (W.C. Koff, F. Wong-Staal and R.C. Kennedy eds.) pp. 267–288, Marcel Dekker, New York.
[9] Tomasselli, A.G., Howe, W.J., Sawyer, T.K., Wlodawer, A. and Heinrikson, R.L. (1991) Chimicaoggi-Chemistry Today 9, 6–27.
[10] Huff, J.R. (1991) J. Med. Chem. 34, 2305–2314.
[11] Wlodawer, A. and Erickson, J.W. (1993) Annu. Rev. Biochem. 62, 543–585.
[12] Ashorn, P., McQuade, T.J., Thaisrivongs, S., Tomasselli, A.G., Tarpley, W.G. and Moss, B. (1990) Proc. Natl. Acad. Sci. USA 87, 7472–7476.
[13] Roberts, N.A., Martin, J.A., Kinchington, D., Broadhurst, A.V., Craig, J.C., Duncan, I.B., Galpin, S.A., Handa, B.K., Kay, J., Krohn, A., Lambert, R.W., Merrett, J.H., Mills, J.S., Parkes, K.E.B., Redshaw, S., Ritchie, A.J., Taylor, D.L., Thomas, G.J. and Machin, P.J. (1990) Science 248, 358–361.
[14] Kempf, D.J., Norbeck, D.W., Codacovi, L., Wang, X.C., Kohlbrenner, W.E., Wideburg, N.E., Paul, D.A., Knigge, M.F., Vasavanonda, S., Craig-Kennard, A., Saldivar, A., Rosenbrook Jr., W., Clement, J.J., Plattner, J.J. and Erickson, J. (1990) J. Med. Chem. 33, 2687–2689.

[15] Vacca, J.P., Guare, J.P., deSolms, S.J., Sanders, W.M., Giuliani, E.A., Young, S.D., Darke, P.L., Zugay, J., Sigal, I.S., Schleif, W.A., Quintero, J.C., Emini, E.A., Anderson, P.S. and Huff, J.R. (1991) J. Med. Chem. 34, 1225–1228.
[16] Wei, X., Ghosh, S.K., Taylor, M.E., Johnson, V.A., Emini, B.H., Saag, M.S. and Shaw, G.M. (1995) Nature 373, 117–122.
[17] Ho, D.D., Neumann, A.U., Perelson, A.S., Chen, W., Leonard, J.M. and Markowitz, M. (1995) Nature 373, 123–126.
[18] Otto, M.J., Garber, S., Winslow, D., Cheng, Y.S.E., Reid, C.D., Aldrich, P. and Jadhav, P.K. (1992) Abstract to the VIII International Conference on AIDS, Amsterdam, July 19–24, 1992 Abstract ThA 1505.
[19] Dianzani, F., Antonelli, G., Turiziani, O., Riva, E., Dong, G. and Bellarosa, D. (1993) Antiviral Chem. Chemother. 4, 329–333.
[20] El-Farrash, M.A., Kuroda, M.J., Kitazaki, T., Masuda, T., Kato, K., Hatanaka, M. and Haarada, S. (1994) J. Biol. 68, 233–239.
[21] Ho, D.D., Toyoshima, T., Mo, H., Kempf, D.J., Norbeck, D., Chen, C.M., Wideburg, N.E., Burt, S.K., Erickson, J.W. and Singh, M.K. (1994) J. Virol. 68, 2016–2020.
[22] Kaplan, A.H., Michael, S.F., Wehbie, R.S., Knigge, M.F., Paul, D.A., Everitt, L., Kempf, D.J., Norbeck, D.W., Erickson, J.W. and Swanstrom, R. (1994) Proc. Natl. Acad. Sci. USA 91, 5597–5601.
[23] Jacobsen, H., Yasargil, K., Winslow, D.L., Craig, J.C., Krohn, A., Duncan, I.B. and Mous, J. (1995) Virology 206, 527–534.
[24] Markowitz, M., Mo, H., Kempf, D.J., Norbeck, D.W., Bhat, T.N., Frickson, J.W. and Ho, D.D. (1995) J. Virol. 69, 701–706.
[25] Nermut, M.V., Hockley, D.J., Jowett, J.B.M., Jones, I.M., Garreau, M. and Thomas, D. (1994) Virology 198, 288–296.
[26] Co, E., Koelsch, G., Lin, Y., Ido, E., Hartsuck, J.A. and Tang, J. (1994) Biochemistry 33, 1248–1254.
[27] Louis, J.M., Nashed, N.T., Parris, K.D., Kimmel, A.R. and Jerina, D.M. (1994) Proc. Natl. Acad. Sci. USA 91, 7970–7974.
[28] Gowda, S.D., Stein, B.S., Steimer, K.S. and Engleman, E.G. (1989) J. Virol. 63, 1451–1454.
[29] Gowda, S.D., Stein, B.S. and Engleman, E.G. (1989) J. Biol. Chem. 264, 8459–8462.
[30] Tozser, J., Blaha, I., Copeland, T.D., Wondrak, E.M. and Oroszlan, S. (1991) FEBS Lett. 281, 77–80.
[31] Kupferberg, J.P., Yokoyama, S. and Kezdy, F.J. (1981) J. Biol. Chem. 12, 6274–6281.
[32] Lin, Y., Lin, X., Hong, L., Foundling, S., Heinrikson, R.L., Thaisrivongs, S., Leelamanit, W., Raterman, D., Shah, M., Dunn, B. and Tang, J. (1995) Biochemistry 34, 1143–1152.
[33] Fontenot, G., Johnston, K., Cohen, J.C., Gallaher, W.R., Robinson, J. and Luftig, R.B. (1992) Virology 190, 1–10.
[34] Leelamanit, W., Westling, J., Farmerie, W.G., Goodnow, M.M. and Dunn, B.M. (1994) Protein Sci. 3 (Suppl.), 441S.
[35] Winslow, D.L., Stack, S., King, R., Scarnati, H., Bincsik, A. and Otto, M.J. (1995) AIDS Res. Human Retroviruses 11, 107–113.
[36] Sardana, V.V., Schlabach, A.J., Graham, P., Bush, B.L., Condra, J.H., Culberson, J.C., Gotlib, L., Graham, D.J., Kohl, N.E., LaFemina, L., Schneider, C.L., Wolanski, B.S., Wolfgang, J.A. and Emini, E.A. (1993) Biochemistry 33, 2004–2010.
[37] Vacca, J.P., Dorsey, B.D., Schleif, W.A., Levin, R.B., McDaniel, S.L., Darke, P.L., Zugay, J., Quintero, J.C., Blahy, O.M., Roth, E., Sardana, V.V., Schlabach, A.J., Graham, P.I., Condra, J.H., Gotlib, L., Holloway, M.K., Lin, J., Chen, I.-W., Vastag, K., Ostovic, D., Anderson, P.S., Emini, E.A. and Huff, J.R. (1994) Proc. Natl. Acad. Sci., U.S.A. 91, 4096–4100.
[38] Coffin, J.M. (1995) Science 267, 483–489.
[39] Otto, M.J., Garber, S., Winslow, D.L., Reid, C.D., Aldrich, P., Jadhav, P.K., Patterson, C.E., Hodge, C.N. and Cheng, Y.S. (1993) Proc. Natl. Acad. Sci. USA 90, 7543–7547.