DOI 10.1002/mnfr.200500216 Mol. Nutr. Food Res. 2006, *50*, 396 – 400

# Anti-human immunodeficiency virus activity of 3,4,5-tricaffeoylquinic acid in cultured cells of lettuce leaves

**Hirotoshi Tamura[1], Takashi Akioka[1], Koichi Ueno[1], Takeshi Chujyo[1], Katsu-ichiro Okazaki[2], Peter J. King[3] and W. Edward Robinson, Jr.[3]**

[1] Department of Biochemistry and Food Science, Kagawa University, Miki-cho, Kagawa, Japan
[2] Departmet of Life Science, Kagawa University, Miki-cho, Kagawa, Japan
[3] Department of Pathology, University of California, Irvine, CA, USA

3,4,5–Tricaffeoylquinic acid (TCQA) that is not found in intact plant of lettuce leaves was isolated from the cultured cells. The intact plant produced chicoric acid (dicaffeoyl tartaric acid: L-CCA) as well as chlorogenic acid (3-caffeoylquinic acid: 3-CQA) as the major metabolites. After subculturing of the cells for 40 days, the amount of 3,4,5-TCQA reached to 0.14 mg/g fresh weight. The inhibitory effect of 3,4,5-TCQA for human immunodeficiency virus (HIV) Type 1 integrase was assayed. Anti-HIV activity using HIV and MT-2 cells was 1.15 μM and $IC_{50}$ against HIV integrase was 0.063 μM whereas cell toxicity of this chemical was expressed as 5% death of all living cells to be 18.4 μM. The HIV inhibitory effect of 3,4,5-TCQA was the highest in values among L-CCA, and other dicaffeoylquinic acids. This data will provide a new possibility for creating a new drug design for HIV.

**Keywords:** Cultured cell / Integrase / *Lactuca sativa* / Lettuce / Tricaffeoylquinic acid

Received: October 21, 2005; revised: December 4, 2005; accepted: December 5, 2005

## 1 Introduction

Human immunodeficiency virus (HIV)/AIDS has been an epidemic of 40 million people at the end of 2003. HIV-1 integrase plays a crucial role in the HIV-1 virus replication cycle. An essential step in the viral life cycle is integration of the viral DNA into the host genome (Fig. 1). It catalyzes to snip the host DNA and also to incorporate virus genome to the snipped ends. Human cells do not have any needs to cut-and-paste DNA fragments into their genome. Therefore, the inhibition of integrase should be a good target for drug therapy, since it is not likely to interfere with the normal operation of human cells [1].

Robinson *et al.* [2] comparatively reported selective inhibitory effects of dicaffeoylquinic acids (DCQA) and chicoric acid (L-CCA) for HIV-1 integrase. Synthetic approach for seeking new HIV integrase inhibitors was also tried among caffeoyl derivatives [3, 4]. Cultured cells from certain plants may also give some possibility of providing the target drugs all seasons.

So, we would like to report herein that 3,4,5-tricaffeoylquinic acid (TCQA) from cultured cells of the lettuce (*Lactuca sativa* var. *crispa* L.) would be a potent inhibitor against HIV-1 integrase among mono-, di-, and tricaffeoyl derivatives.

**Correspondence:** Professor Hirotoshi Tamura, Department of Biochemistry and Food Science, Kagawa University, Miki-cho, Kagawa, 761-0795 Japan
**E-mail:** tamura@ag.kagawa-u.ac.jp
**Fax:**+81-87891-3021

**Abbreviations: 3-CQA,** 3-caffeoylquinic acid; **DCQA,** dicaffeoylquinic acids; **HIV,** human immunodeficiency virus; **3,4,5-TCQA,** 3,4,5-tricaffeoylquinic acid

## 2 Materials and methods

### 2.1 HPLC analysis of 3-caffeoylquinic acid (3-CQA) and the related compounds

3-CQA and the related compounds were analyzed by an HPLC instrument (980-PU, JASCO) under the gradient elution with a mixed solvent of AcOH : $CH_3CN$ : $H_2O$ : $H_3PO_4$ = 20 : 25 : 53.5 : 1.5 (this solvent system is expressed as 100%-P) and 1.5% $H_3PO_4$ aqueous solution at 1.0 mL/min. Metabolites were detected at 325 nm (UV-975, JASCO). The chromatography was effectively conducted by using a 5 μm Develosil Ph-HG-5 column, 250 mm × 4.6 mm id. During the initial 5 min, 40%-P solvent was eluted and then the solvent was gradually changed to 100%-P for 20 min. Finally, the 100% P solvent was kept flowing for 10 min.

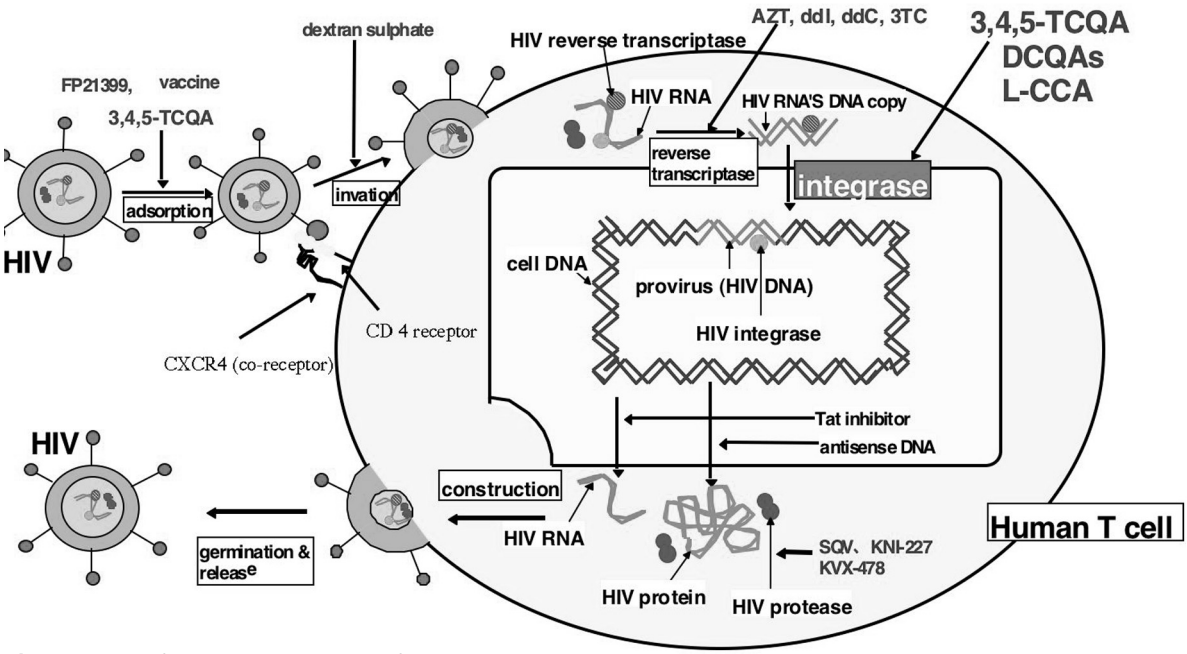


**Figure 1.** HIV life cycle and target site of anti-HIV agents.

### 2.2 Tissue culture of the lettuce leaves

The induction of callus cells from intact leaves of lettuce was conducted on a Gamborg B5 medium. Small pieces of the young leaves were inoculated on the Gamborg B5 medium containing 0.9% agar, 20% coconut milk, indoleacetic acid (IAA, 10 mg/L), and kinetin (10 mg/L) under 2000 lux at 25°C, and then the callus tissue was subcultured every 25 days under the same cultural conditions. The cultured cells were collected at 40 days after subculture.

### 2.3 Isolation of 3,4,5-TCQA

#### 2.3.1 General remarks

Cultured cells (1.5 kg) were homogenized and immersed in 3 L of a 1% TFA-50% $CH_3CN$ aqueous solution for 24 h and then filtrated. The residues were immersed again in the same volume of the solution. The combine mixture was concentrated into 0.7 L of the volume by a rotary evaporator. The concentrated solution was applied onto a column of Amberlite XAD-7 (360 mm × 78 mm id). The crude fraction (1.2 g) containing 3,4,5-TCQA was recovered by eluting at the rate of 20 mL/min with a 1% TFA – 40–50% $CH_3CN$ containing aqueous solution. Further purification of the metabolites was done by using ODS-5 μm and Ph-5 μm stainless steel columns (250 mm × 20 mm id), affording 35 mg of compound 3 (3,5-DCQA, 90% purity), 25 mg of compound 4 (4,5-DCQA, 92% purity), 44 mg of compound 5 (3,4-DCQA, 92% purity), and 24 mg of compound 6 (3,4,5-TCQA, more than 95% purity) as the white amorphous, respectively.

#### 2.3.2 Physical properties of isolated compounds

Compound 3 (3,5-DCQA): $^1$H-NMR (400 MHz, [D6]DMSO, TMS): $\delta_H$ 7.49 (1H, d, $J$ = 15.1 Hz), 7.45 (1H, d, $J$ = 14.6 Hz), 7.08 (1H, bd, $J$ = 2.5 Hz), 7.07 (1H, bd, $J$ = 2.5 Hz), 7.01(1H, m), 6.99 (1H, m), 6.82 (1H, d, $J$ = 7.5 Hz), 6.81 (1H, d, $J$ = 7.5 Hz), 6.26 (1H, d, $J$ = 16.1 Hz), 6.17 (1H, d, $J$ = 16.1 Hz), 5.23 (1H, bs), 5.15 (1H, bs), 3.86 (1H, bs), 2.1 (4H, m); $^{13}$C-NMR (100 MHz, [D6]DMSO): $\delta_C$ 175.4, 166.1, 165.6, 148.3, 148.2, 145.5 (2H), 145.2, 144.8, 125.6, 125.5, 121.5, 121.2, 115.8, 115.7, 114.7 (2H), 114.1, 95.3, 72.4, 70.9, 70.5, 34.6, 22.4; FAB-MS *m/z* 515 ($M^+$ – 1, neg).

Compound 4 (4,5-DCQA): $^1$H-NMR (400 MHz, [D6]DMSO, TMS): $\delta_H$ 7.46 (2H, d, $J$ = 15.6 Hz), 7.45 (2H, bd, $J$ = 16.1 Hz), 7.03 (2H, bd, $J$ = 3.4 Hz), 6.96 (2H, dd, $J$ = 3.4 Hz, 7.8 Hz), 6.75 (d, $J$ = 7.8 Hz), 6.74 (d, $J$ = 7.8 Hz), 6.24 (1H, d, $J$ = 16.1 Hz), 6.18 (1H, d, $J$ = 16.1 Hz), 5.42 (1H, bs), 4.93 (1H, bd, $J$ = 5 Hz), 4.08 (1H, bd, $J$ = 6 Hz), 2.1 (4H, m); $^{13}$C-NMR (100 MHz, [D6]DMSO): $\delta_C$ 175.6, 165.8, 165.7, 148.2, 148.1, 145.3 (2H), 145.2, 145.0, 125.4, 125.3, 121.3, 121.2, 115.6 (2H), 114.6, 114.5, 114.1, 113.8, 95.3, 72.5, 68.2, 64.1, 35.5, 22.3; FAB-MS *m/z* 515 ($M^+$ – 1, neg).

Compound 5 (3,4-DCQA): $^1$H-NMR (400 MHz, [D6]DMSO, TMS): $\delta_H$ 7.49 (1H, d, $J$ = 16.1 Hz), 7.43 (1H,

 

d, $J$ = 15.6 Hz), 7.04 (bs), 7.03 (bs), 6.98 (bd, $J$ = 9 Hz), 6.96 (bd, $J$ = 9 Hz), 6.75 (d, $J$ = 7.8 Hz), 6.74 (d, $J$ = 8.3 Hz), 6.24 (d, $J$ = 16.1 Hz), 6.14 (d, $J$ = 16.1 Hz), 5.34 (bs), 4.96 (dd, $J$ = 2.6, 7.6 Hz), 4.17 (bs), 2.1 (4H, m); $^{13}$C-NMR (100 MHz, [D6]DMSO): $\delta_C$ 174.7, 165.8, 165.4, 148.2 (2H), 145.3 (4H), 125.3 (2H), 121.3, 121.2, 115.6, 115.5, 114.7 (2H), 113.7, 113.5, 95.3, 73.2, 67.5, 66.0, 37.3, 22.3; FAB-MS *m/z* 515 ($M^+ - 1$, neg).

Compound 6 (3,4,5-TCQA$^1$H-NMR (400 MHz, [D6]DMSO, TMS): $\delta_H$ 7.49 (1H, d, $J$ = 15.9 Hz), 7.05 (2H, bs), 7.03 (1H, bs), 6.99 (2H, m), 6.95 (1H, bd, $J$ = 8.4 Hz), 6.77 (1H, d, $J$ = 8.4 Hz), 6.77 (1H, d, $J$ = 8.4 Hz), 6.72 (1H, d, $J$ = 8.4 Hz), 6.23 (1H, d, $J$ = 15.9 Hz), 6.22 (1H, d, $J$ = 15.9 Hz), 6.18 (1H, d, $J$ = 15.9 Hz), 5.46 (1H, bs), 5.45 (1H, bs), 5.25 (1H, bs, $J$ = 2.6 Hz, 7.6 Hz), 2.1 (4H, m); $^{13}$C-NMR (100 MHz, [D6]DMSO): $\delta_C$ 174.9, 165.8, 165.5, 165.3, 148.3 (2H), 148.2, 145.9, 145.7, 145.4 (4H), 125.5, 125.4, 125.3, 121.6, 121.4 (2H), 115.7, 115.6 (2H), 114.8 (2H), 114.7, 114.0, 113.4, 113.2, 72.3, 70.1, 68.1, 67.6, 36.6, 35.1; FAB-MS *m/z* 701 ($M^+$ + Na, pos; matrix glycerol).

### 2.4 Cells and virus

MT-2 cells are a CD4$^+$ T-lymphoblastoid cell line that is completely lysed by T-cell tropic isolates of HIV-1 [5]. $HIV_{LAI}$ is the LAI isolate of HIV-1. $HIV_{LAI}$ was grown in H9 cells, a CD4$^+$ T-lymphoblastoid cell line that supports chronic production of HIV. Cells were grown in RPMI-1640 containing 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) and supplemented with 11.5% fetal bovine serum and 2 mM L-glutamine. HIV was obtained by filtration through 0.45 μm NC filters.

### 2.5 Cell toxicity assays

Cell toxicity assay was performed as reported previously [2, 5]. Briefly, isolated compounds from lettuce leaf calli were dissolved in either $H_2O$ or 95% ethanol, diluted 1 : 5 in growth medium, filter sterilized, and further two-fold serially diluted from 1 : 10 to 1 : 1280 in triplicate wells of microtiter plate. To each 50 μL of diluted drug, 50 μL of growth medium was added followed by 100 μL of MT-2 cell suspension (2 × 105 cells). Cells were incubated with drug for 48 or 72 h at 37°C, and then harvested for cell viability in a neutral red dye assay as described previously [5]. The lethal dose was defined as 5% inhibition of MT-2 cell growth in 48 h ($LD_5$). Several of the compounds were not available in sufficient quantity or demonstrated a solubility profile that precluded determination of a true $LD_5$. For the compounds, cell toxicity is defined as greater than the maximum concentration of compound tested. The $LD_5$ is a better measure of toxicity than $LD_{50}$ as 5% inhibition of cell growth is within one SD of the cell controls. Thus, this is a truly nontoxic concentration of compound.

### 2.6 Anti-HIV assay

Anti-HIV assays were performed as described [2, 5]. Based upon cell toxicity data, DCQAs and 3,4,5-TCQA were diluted in growth medium such that a final 1:4 dilution of the sample would result in a concentration of sample equal to the $LD_5$. The compounds were then two-fold serially diluted in triplicate. To each 50 μL of diluted compound, 50 μL of $HIV_{LAI}$ was added and the virus-drug mixture was incubated for 1 h at 37°C. Next, 100 μL of MT-2 cell suspension ($2 \times 10^5$ cells) was added to each well and cells were incubated for 72 h at 37°C. Final multiplicity of infection (MOI) was 1 – 5. Cells were harvested to quantitate cytopathic effect using a neutral red dye assay as described [5]. The antiviral concentration reported is the concentration of sample necessary to protect MT-2 cells from 50% viral-induced cell death; this is referred to as the 50% effective dose ($ED_{50}$). Inhibition of virus-induced cell death has correlated well with virus replication as measured by synthesis of antigens, reverse transcriptase release, and production of infectious progeny virus [6].

### 2.7 Disintegration activity

The disintegration activities of integrase in the presence and absence of inhibitors was assayed *in vitro* as modified from Chow *et al.* [7]. The following oligonucleotides (GenoSys) were used as DNA substrates:

T1 (16mer):
5′-CAGCAACGCAAGCTTG-3′

T3 (30mer):
5′-GTCGACCTGCAGCCCAAGCTTGCGTTGCTG-3′

V2 (21mer):
5′-ACTGCTAGAGATTTTCCACAT-3′

V1/T2(33mer):
5′-ATGTGGAAAATCTCTAGCAGGCTGCAGGTCGAC-3′

The oligonucleotides were gel purified by the manufacturer. Oligonucleotide T1 was labeled at the 5′-end using T4 polynucleotide kinase and [γ-32P] ATP (6000 Ci/mmol, Amersham). The substrate for assaying disintegration activity, the Y-oligomer, was prepared by annealing the labeled T1 strand with oligonucleotides T3, V2, and V1/T2 [7]. In a 20 μL volume, the DNA (0.1 pmol) was incubated with 1.5 pM recombinant integrase for 60 min at 37°C in a buffer containing a final concentration of 20 mM HEPES pH 7.5,

 

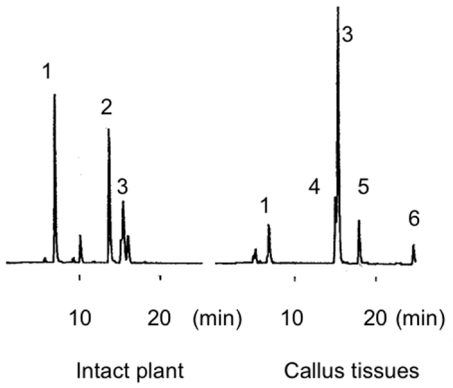


**Figure 2.** HPLC analysis of secondary metabolites in lettuce plant and the callus tissues. (1) Chlorogenic acid (3-CQA), (2) chicoric acid (L-CCA), (3) 3,5-DCQA, (4) 4,5-DCQA, (5) 3,4-DCQA, (6) 3,4,5-TCQA.

10 mM DTT, 0.05% NP-40, and 10 mM $MnCl_2$. To each 19 mL of reaction mixture, 1 L of inhibitor at various concentrations in solvent or solvent alone was added. The reaction was stopped by the addition of EDTA to a final 18 mM concentration. Reaction products were heated at 90°C for 3 min before analysis by electrophoresis on a 15% polyacrylamide gel with 7 M urea in Tris-borate-EDTA buffer. All reactions were performed at enzyme excess, and reactions were stopped within the linear range of the reaction [7]. All compounds were first tested at 25 μM. For active compounds, $IC_{50}$ analysis was performed using linear regression analysis on 0.5 log10 dilutions of inhibitor and the $IC_{50}$ was determined from triplicate experiments.

## 3 Results

### 3.1 Secondary metabolites of lettuce callus tissues

Structure elucidation of secondary metabolites of lettuce callus was mainly conducted by FAB-MS and 400 MHz NMR measurements. FAB-MS data showed the molecular ion at *m/z* 515 for compounds 3, 4, and 5, and at *m/z* 701 for compound 6, suggesting the presence of two caffeoyl moieties and one quinic acid moiety on the molecules of compounds 3, 4, and 5, and also the presence of three caffeoyl moieties and one quinic acid moiety on the molecule of compound 6. Typical proton signals in a lower field clearly indicated the presence of caffeoy moiety (<6 ppm). $^1$H-NMR of the quinic acid moieties of those compounds showed the characteristic signals around 2 and 4–5 ppm. The signals correspond to two sets of methylene group and three hydroxy groups. $^1$H-$^1$H COSY spectra of the quinic acid moieties of those compounds, 3, 4, 5, and 6 showed clear correlations between two sets of methylene protons, and 3- and 5-hydroxy groups. So, $C_4$ position of quinic acid moieties of the four chemicals with lacking correlation in those range of chemical shifts can be easily assigned by $^1$H-$^1$H COSY spectra. Furthermore as $^1$H-$^1$H correlation between proton signals on $C_3$-hydroxy and $C_4$-hydroxy groups of the quinic acid moieties is caused by larger dihedral angle of H-$C_3$-$C_4$-H (180°) of quinic acid, it enabled to distinguish both proton signals on $C_3$-hydroxy group and $C_5$-hydroxy group easily. So, all proton signals of compound 3, 4, 5, and 6 were completely assigned by $^1$H-$^1$H COSY spectra. Thus, chemical structures of 3,5-DCQA, 4,5-DCQA, 3,4-DCQA, and 3,4,5–TCQA were confirmed.

3,4,5-TCQA, which is not found in intact lettuce leaves, was newly produced in the cultured cells on the Gamborg B5 medium. The intact plant contained L-CCA and 3-CQA as the major metabolites whereas the major secondary metabolites from cultured cells is 3,5-DCQA and then the quantity of individual metabolites was monitored by HPLC (Fig. 2). Consequently, the optimal amounts of 3,5-DCQA and 3,4,5-TCQA productions were 0.83 mg/g fresh weight (for 3,5-DCQA) at 20 days and 0.14 mg/g fresh weight (for 3,4,5-TCQA) at 40 days, respectively (Fig. 3). The amount of 3,5-DCQA from callus tissues increased to 3.8-folds larger than that of the intact plant.

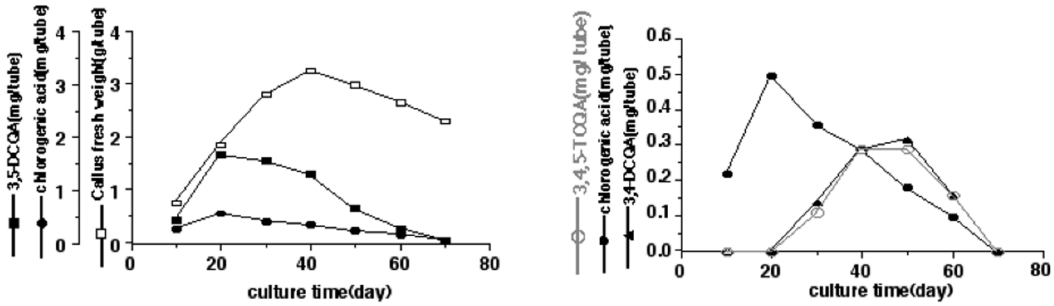


**Figure 3.** Time course change of callus weight and the secondary metabolites during the cultivation.

 

### 3.2 Inhibitory effect of 3,4,5-TCQA for HIV-1

The concentration for 5% death of MT-2 cells was 18.4 μM and expressed as the cell toxicity ($LD_5$). In Table 1, 50% of inhibitory effect of 3,4,5-TCQA against HIV Type 1 activity was to be 1.15 μM and expressed as $ED_{50}$. The difference between $LD_5$ and $ED_{50}$ was 17.2 μM (16-fold difference) and the difference indicated the safe and effective zone of the dose for anti-HIV tests. $IC_{50}$ against HIV integrase was assayed by disintegration method and found to be 0.063 μM. The inhibitory effect of 3,4,5-TCQA was the lowest in values among L-CCA, DCQA, and other similar analogs (Table 1).

**Table 1.** Anti-HIV activities of 3,4,5-TCQA, DCQAs and their analogs

| Compound | Cell toxicity $LD_5$ (μM) | Anti-HIV $ED_{50}$ (μM) | Anti-HIV integrase activity (in vitro) $IC_{50}$ (μM) |
|---|---|---|---|
| Quinic acid[a)] | 911 | >1822 | NT |
| Caffeic acid[a)] | 1389 | >1389 | >278 |
| Chlorogenic acid[a)] | 250 | >499 | >142 |
| 1-MO-3,5-DCQA[a)] | 372 | 7 | 0.47 |
| 1,5-DCQA[a)] | 145 | 4 | 0.84 |
| 4,5-DCQA[a)] | 145 | 4 | 0.30 |
| 3,5-DCQA[a)] | 290 | 2 | 0.66 |
| 3,4-DCQA(synthetic)[a)] | 116 | 12 | 0.71 |
| L-CCA[a)] | 264 | 4 | 0.15 |
| Curcumin[b)] | | 13.6 | 136 |
| AZT[c)] | | 1 | |
| 3,4,5-TCQA | 18.4 | 1.15 | 0.063 |

NT: not tested
a) [12]
b) [13]
c) [14]

## 4 Discussion

Disintegration activity of aurintricarboxylic acid [8], cosalene analogs [9], flavones, caffeic acid phenethyl ester [10], and other compounds [11] was reported to be effective. However, some of them interfered with retro-virus transcriptase and showed low selectivity in the active sites or could not detect any increase of vital cells against HIV infection. Only curcumin shows the reliable anti-HIV activity at the higher concentration (13.6 μM) [2]. On the other hand, 3,4,5-TCQA contributed to higher cell toxicity ($LD_5$ = 18.4 μM) but the difference in concentration between $LD_5$ and $ED_{50}$ still provides a safety zone for dose of it whereas the anti-HIV activity was found to be the lowest in the minimum requirement for $ED_{50}$ and the amount was close to that of AZT with a different active site. Furthermore, higher positive correlation between $ED_{50}$ and $IC_{50}$ was observed. Especially 3,4,5-TCQA inhibited anti-HIV integrase activity specifically. $IC_{50}$ of 3,4,5-TCQA is the lowest value at present. A tight substrate-enzyme interaction can be formed. So, it will be concluded that anti-HIV activity of 3,4,5-TCQA attributes to the specific anti-HIV integrase activity.

From the viewpoint of chemical therapy, chemicals inhibiting HIV integrase such as 3,4,5-TCQA and DCQAs will be potent drugs for combination therapy of HIV infection. Drug design for stable DCQA derivatives is realized by syntheses of the analogs [3, 4] but nobody tried to make drug analogs for 3,4,5-TCQA and its derivatives, yet this data will provide a new possibility for creating a new drug design.

*The authors would like to thank Ms. B. R. McDougall for excellent technical support. This work was supported in part by the fund from Taisho technos, Shizuoka in Japan.*

## 5 References

[1] King, P. J., Robinson, W. E., Jr., *J. Virol*. 1998, *72*, 8420 – 8424.
[2] Robinson, W. E., Jr., Cordeiro, M., Abdel-Malek, S., Jia, Q., *et al*., *Mol. Pharmacol*. 1996, *50*, 846 – 855.
[3] Xu, Y.-W., Zhao, G.-S., Shin, C.-G., Zang, H.-C., *et al.*, *Bioorg. Med. Chem.* 2003, *11*, 3589 – 3593.
[4] Lee, J. Y., Yoon, K. J., Lee, Y. S., *Bioorg. Med. Chem. Lett.* 2003, *13*, 4331 – 4334.
[5] Montefiori, D. C., Robinson, W. E., Jr., Schuffman, S. S., Mitchell, W. M., *J. Clin. Microbiol.* 1988, *26*, 231 – 235.
[6] Robinson, W. E., Jr., Montefiori, D. C., Gillespie, D. H., Mitchell, W. M., *J. Acquir. Immune Defic. Syndr.* 1989, *2*, 33 – 42.
[7] Chow, S. A., Vincent, K. A., Ellison, V., Brown, P. O., *Science* 1992, *255*, 723 – 726.
[8] Cushman, M., Sherman, P., *Biochem. Biophys. Res. Commun*. 1992, *185*, 85 – 90.
[9] Cushman, M., Golebiewski, W. M., Pommier, Y., Mazumder, A., *et al.*, *J. Med. Chem*. 1995, *38*, 443 – 452.
[10] Fesen, M. R., Kohn, K. W., Leteurtre, F., Pommier, Y., *Proc. Natl. Acad. Sci. USA* 1993, *90*, 2399 – 2403.
[11] Fesen, M. R., Pommier, Y., Leteurtre, F., Hiroguchi, S., *et al*., *Biochem. Pharmacol*. 1994, *48*, 595 – 608.
[12] McDougall, B., King, P. J., Wu, B.W., Hostomsky, Z., *et al.*, *Antimicrob. Agents Chemother.* 1998, *42*, 140 – 146.
[13] Mazumder, A., Raghavan, K., Weinstein, J., Kohn, K. W., *et al*., *Biochem. Pharmacol*. 1995, *49*, 1165 – 1170.
[14] Robinson, W. E., *Antiviral Res.*, 1998, *39*, 101 – 111.

 