Indonesian Journal of Biotechnology, December, 2010 Vol. 15, No. 2, pp. 64-71

Comparison of Cytotoxic and Antiproliferative Effects of Benzylidenecyclopentanone Analogues of Curcumin on RBL-2H3 Cells

Agung Endro Nugroho[1,3*], Sardjiman[2], and Kazutaka Maeyama[3]

[1]Department of Pharmacology and Clinical Pharmacy, Faculty of Pharmacy, Gadjah Mada University, Yogyakarta, Indonesia
[2]Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Gadjah Mada University, Yogyakarta, Indonesia
[3]Department of Pharmacology, Informational Biomedicine, School of Medicine, Ehime University Japan

Abstract

Curcumin is a natural yellow pigment isolated from the rhizomes of *Curcuma longa* L. (turmeric), and has several pharmacological effects and no toxicity in both in animal and human clinical study. However, the problem of curcumin is its stability because of its active methylene moiety. Modification of this moiety to cyclopentanone is expected to increase the stability. Previous study reported that benzylidenecyclopentanone analogues of curcumin showed inhibitory effect on histamine release from RBL-2H3 (rat basophilic leukemia) cells, a tumor analog of mast cells. One of them, the hydroxy-methoxy analog (PGV-0), showed more potent effect than that of curcumin. In the present study, some benzylidenecyclopentanone analogues of curcumin were evaluated for their effects on the viability and proliferation of RBL-2H3 cells. Viable cells were counted under a light microscope with a cells-counting chamber or using the cell viability reagent WST-1. The results showed that mast cell viability and histamine content were not affected by curcumin and benzylidene cyclopentanone for 30 min incubation, however, impaired for overnight incubation. The hydroxy-dimethyl benzylidene analog (PGV-1) strongly decreased the mast cells viability for overnight incubation, and its effect was highest among the other analogues. In the proliferation study, this compound also strongly inhibited the proliferation of mast cells, whereas curcumin and hydroxy-methoxy benzylidene analog inhibited the proliferation slightly. There were no inhibitory effects on mast cells proliferation treated by dibenzylidene; dihydroxybenzylidene; and hydroxy-diethylbenzylidene cyclopentanone.

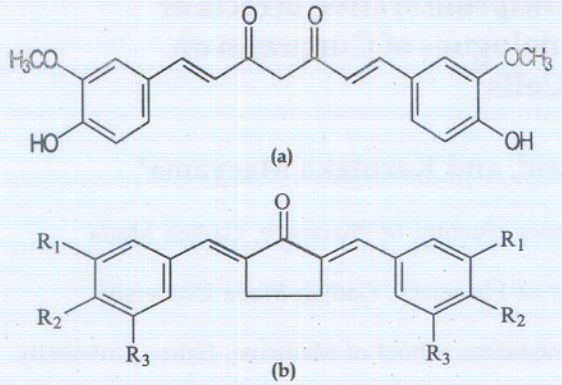
Keywords : viability, proliferation, curcumin, benzylidene cyclopentanone, RBL-2H3 cells

Introduction

Curcumin, chemically also named as 1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione] is a natural yellow pigment isolated from the rhizomes of the plant *Curcuma longa L.* (turmeric) widely found in south and southeast Asia (Figure 1a).

*** Corresponding author** :
Agung Endro Nugroho,
Department of Pharmacology and Clinical Pharmacy, Faculty of Pharmacy, Gadjah Mada University, Yogyakarta, Indonesia
E-mail : agungendronugroho@yahoo.com

Reportly, curcumin is a potent anticancer in various systems. The mechanisms underlying this effect are inhibition on transformation, tumor initiation, tumor promotion, invasion, angiogenesis, and metastasis processes. Curcumin has been shown to suppress tumorigenic activity of a wide variety of carcinogens in cancers of the colon, duodenum, esophagus, forestomach, stomach, liver, breast, leukaemia, oral cavity, and prostate.



Compound.	R1	R2	R3	Chemical name	MW
1.	H	H	H	2,5-Dibenzylidenecyclopentanone	260.12
2.	H	OH	H	2,5-Bis(4-hydroxybenzylidene)cyclopentanone	292.11
3.	OCH_3	OH	H	2,5-Bis(4-hydroxy-3-methoxybenzylidene)cyclopentanone	352.13
4.	CH_3	OH	CH_3	2,5-Bis(4-hydroxy-3,5-dimethylbenzylidene)cyclopentanone	348.17
5.	C_2H_5	OH	C_2H_5	2,5-Bis(4-hydroxy-3,5-diethylbenzylidene)cyclopentanone	404.24

Figure. 1. Structure, chemical name and molecular weight of curcumin (a) and its benzylidenecyclopentanone analogues (b)

Besides, curcumin also inhibits the proliferation of a wide variety of cancer cell types *in vitro*, including cells from cancers of the bladder, breast, lung, pancreas, prostate, cervix, head and neck, ovary, kidney, and brain; and osteosarcoma, leukaemia and melanoma (Shishodia *et al.*, 2005; Goel *et al.*, 2008).

Although curcumin has potent anticancer effects, its use is limited by the compounds instability. Stability of curcumin is strongly affected by some aspects such pH and light (Van der Goot *et al.*, 1995). The kinetics of the-pH-dependent degradation of curcumin in aqueous medium with various pH was studied by Tonnesen and Karlsen (Tonnesen *et al.*, 1985). Curcumin in aqueous solution with pH less than 7 is quite stable, but at pH more than 7 curcumin will be decomposed with increasing pH. Modification of the middle site of curcumin becomes1,4-pentadiene-3-ones; cyclopentanone or cyclohexanone still maintains the hydroxy moiety responsible for antioxidant activities. The compounds resulted from these modification are named 1,5-diphenyl-1,4-pentadiene-3-one analogs, benzylidenecyclopentanone, and benzylidene hexanone, respectively (Sardjiman *et al.*, 1997).

Two benzylidenecyclopentanone curcumin analogues, 2,5-Bis(4-methoxybenzylidene) cyclopentanone and 2,5-Bis(4-hydroxy-3,5-dimethylbenzylidene) cyclopentanone, also named as pentagamavunon-0 (PGV-0) and pentagamavunon-1 (PGV-1) respectively (Reksohadiprodjo *et al.*, 2004), were reported to possess cytotoxic and antiproliferative properties againts T47D human breast cancer cell line. These compounds induced apoptosis on T47D cell line by increasing p53 and Bax expression, and decreasing Bcl-2 expression at the protein level. Besides, they have been shown that possess antiangiogenesis properties by decreasing of VEGF and COX-2 expressions (Melannisa, 2004; Nurulita and Meiyanto, 2006). Moreover, PGV-0 also inhibited cell cycle progression through G1 arrest (Meiyanto and Dai, 2006). Whereas, PGV-1 inhibited cell cycle progression through G2/M arrest, and induced dephosphorilation of CDK-1 on T47d cell line (Dai *et al.*, 2006).

Investigation of anticarcinogenic properties of benzylidenecyclopentanone analogues have been studying against other kinds of cancer cells. In present study, some benzylidenecyclopentanone analogues of curcumin including PGV-0 and PGV-1 were evaluated for their effects on the viability and proliferation of RBL-2H3 (rat basophilic leukemia) cells, a tumor analog of mast cells.

Materials and Methods

Materials

Curcumin was purchased from Sigma-Aldrich (St. Louis USA), and benzylidenecyclopentanone analogues of curcumin were kindly supplied by Dr. Sardjiman (Faculty of Pharmacy, Gadjah Mada University). The chemical structures

of the compounds are shown in Figure 1b. Eagle's minimum essential medium (MEM) and antibiotics (combination of penicillin G sodium and streptomycin sulfate) were purchased from Gibco (Grand Island, NY, USA). Fetal calf serum was obtained from JRH Biosciences (Kansas, USA). Piperazine-1,4-bis(2-ethanesulfonic acid) (PIPES) was purchased from Dojindo (Kumamoto, Japan), and o-phthalaldehyde was from Wako Pure Chemical Industries (Osaka, Japan). WST reagent used for cytotoxic and antiproliferative assays was purchased from Roche Diagnostics (Mannheim, Germany).

Assay of cell viability

RBL-2H3 cells (Department of Pharmacology, School of Medicine, Ehime University Japan) were cultured in MEM containing 15% fetal calf serum and antibiotics (penicillin and streptomycin) in a flask in a humidified atmosphere (5% CO2) at 37°C as described by Barsumian *et al.* (1981).The flask of 25 cm^2 containing approximately 1 x 10^5 cells/ml were used in the experiments. All addition into the medium was sterilized by filtration in a 0.22 mm millipore filter. For the assay, cells were seeded into well culture plates at a density of 1.25 x 10^5 cells/200 ml per each well either without (as a negative control) or with the drug. The drug concentrations were 1, 10 and 100 mM. Then, the cells were incubated for 30 min or 24 h at 37°C. The adherent cells were scraped and collected, the cells number were counted under a light microscope with a cells-counting chamber (Improved Neubauer Deep). Besides, cell viability after overnight incubation was also assayed directly using the cell viability reagent WST-1.

Assay of cell proliferation

RBL-2H3 Cells were cultured in 24-well plates at the density of 2 x 10^4 cells/400 ml per well in presence or absence of the drugs. The cells were incubated in a humidified atmosphere of 5% of CO_2 at 37°C for certain period time. For the study, the cell number was determined after 0, 24, 48 and 72 h incubation, and using drug concentrations at low-toxic concentrations according to previous assay, 1 and 3 mM. The number of cells was determined under a light microscope. After incubation, the RBL-2H3 cells were washed twice with 500 l of PIPES buffer, finally added by 500 ml of this buffer. The adherent cells were scraped and collected, the cells number were counted under a light microscope with a cells-counting chamber (Improved Neubauer Deep).

WST assay

In the WST assay, after incubation the WST dye solution was added directly into the 96-well plate. After 4 h incubation at 37°C, the formazan was quantified by absorption values detected at 450 nm by microplate autoreader. The optical density was measured on a microplate reader with a wavelength of 450 nm. In this assay, the viable cells cleave the tetrazolium salts to formazan by cellular enzyme, mitochondrial dehydrogenases. The augmentation in enzyme activity leads to an increase in the amount of formazan dye formed, which directly correlates to the number of metabolically active cells in the sample.

Assay of histamine release

Histamine contents were measured by HPLC-fluorometry as described previously study (Yamatodani *et al.*, 1985). After certain incubation, the adherent cells were scraped and collected. The plate was centrifuged at 3,000 rpm for 5 min and 50 ml of the supernatant was mixed with 250 ml of 3% perchloric acid containing 5mM Na_2-EDTA. After addition of 30 ml of 2 M KOH/1 M KH_2PO_4 and centrifugation at 10,000 x g for 15 min at 4°C, 50 ml of the supernatant was injected directly onto a column packed with TSKgel SP-2SW cation exchanger (Tosoh, Tokyo). Histamine was

eluted with 0.25 M potassium phosphate at a flow rate of 0.6 ml/min, and post-labeled with *o*-phthalaldehyde under alkaline conditions and detected using a F1080 Fluorometer (Hitachi, Tokyo) at excitation and emission wavelengths of 360 and 450 nm, respectively.

Statistical analysis

All data were expressed as mean ± SEM. One-way analysis of variance (ANOVA) followed by the least significant difference (LSD) test were used for statistical analyses. *P*-values less than 0.05 were considered significant.

Results and Discussion

Effects on the viability of RBL-2H3 cells

Figure 2-4 show the effects of a series concentration of benzylidenecyclopentanone analogues of curcumin on the viability of RBL-2H3 (rat basophilic leukemia) cells, a tumor analog of mast cells for 30 min and 24 h incubations.

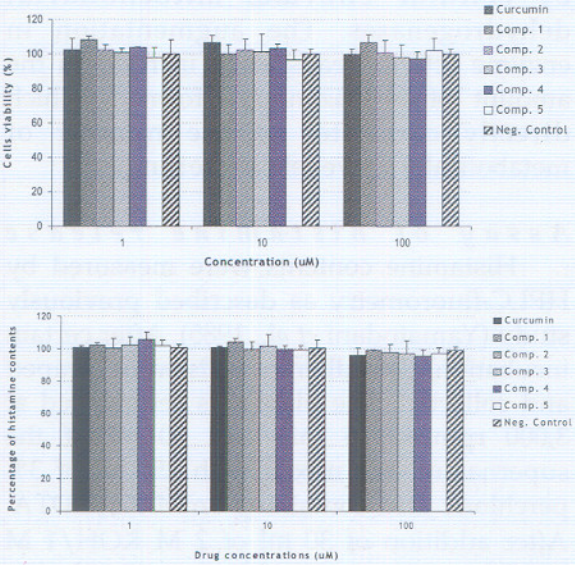


Figure 2. Effects of benzylidenecyclopentanone analogues of curcumin on the density of RBL-2H3 cells (a) and the histamine contents (b) during 30 min incubation. Results are the meanSEM of three experiments. The x-axis is logarithmic scale.

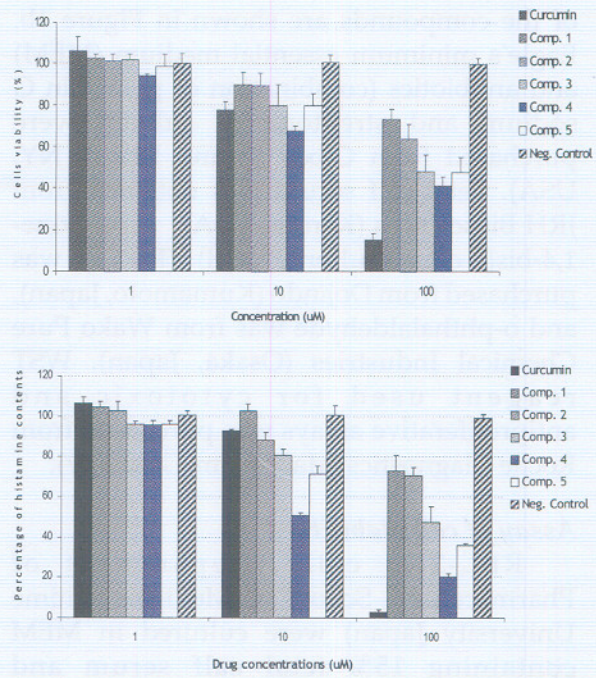


Figure 3. Effects of benzylidenecyclopentanone analogues of curcumin on the density of RBL-2H3 cells (a) and the histamine contents (b) during 24 h incubation. Results are the mean±SEM of three experiments. The x-axis is logarithmic scale.

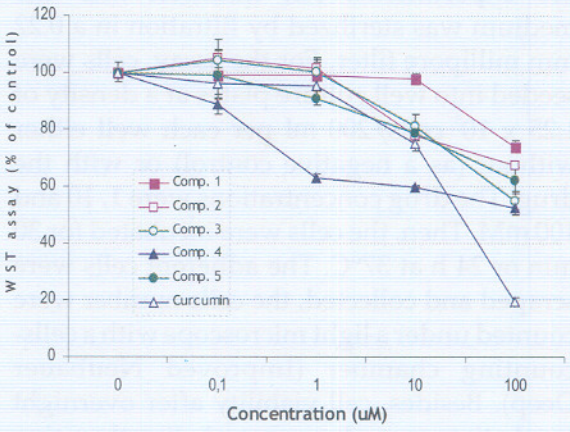


Figure 4. Effects of benzylidenecyclopentanone analogues of curcumin on RBL-2H3 cell. Cells were incubated for 24 h with various concentrations of benzylidenecyclopentanone analogues of curcumin. Cell proliferations were examined by WST assay. Results are the mean?SEM of three experiments. The x-axis is logarithmic scale.

Treatment with various concentrations of benzylidenecyclopentanone analogues of curcumin (1, 10, or 100 μM) for 30 min did not influence the viability of RBL-2H3 cells. However, they markedly decreased the viability of RBL-2H3 cells after 24 h incubation in dose-dependent manner. It indicates that benzylidenecyclopentanone analogues of curcumin caused cytotoxic effects against RBL-2H3 cells, especially at concentration of 10 and 100 μM. The cytotoxic effects of most analogues are still less potent than this of curcumin. However, compound 4 showed more potent cytotoxic effect at low concentration (10 μM) in compare to this of curcumin.

Effects on the histamine contents in RBL-2H3 cells

Treatment with a series concentration of curcumin (1, 10, or 30 μM) for 30 min did not influence histamine contents in RBL-2H3 cells. However, they markedly decreased the viability of RBL-2H3 cells after 24 h incubation. The histamine content in mast cells was inversely correlated with the concentration, indicating its dose-dependent inhibitory effect. The decrease in histamine contents due to 24 h incubation of the compounds are paralleled to the decrease in viable mast cells. The effects of most analogues on the histamine content were less potent than this of curcumin. However, the effect of compound 4 was more potent than this of curcumin at concentration (10 μM).

Effects on the proliferation of RBL-2H3 cells

In the study, the cell number was determined after 0, 24, 48 and 72 h incubation. The drug concentrations used in the study were low-toxic concentration according to previous assay, 1 and 3 μM. At the concentration of 1 μM, only compound 4 attenuated the proliferation of RBL-2H3 cells. It obviously inhibited the proliferation after 48 h incubation. In the other hand, curcumin and all its analogues did not influence the proliferation of the cells. However, at the concentration of 3 μM curcumin and compound B1 slightly attenuated the proliferation of RBL-2H3 cells after 72 h incubation. At the same concentration, compound 4 showed cytotoxic effect on the cells after overnight incubation. The result is shown in table 1.

Table 1. The effects of the treatment of benzylidenecyclopentanone analogues of curcumin at doses of 1 μM (a) and 3 μM (b) on RBL-2H3 cells growth during 24, 48, and 72 h

(a). Dosage 1 μM			
Treatment	Number of cell (10^4 per mL)		
	24 h	48 h	72 h
Neg. Control	7.02 ± 0.70	23.79 ± 0.72	33.25 ± 0.73
Comp .1	7.02 ± 0.41	24.47 ± 0.72	33.80 ± 0.64
Comp. 2	6.79 ± 0.38	24.69 ± 1.56	31.81 ± 1.42
Comp. 3	6.87 ± 0.61	22.82 ± 0.53	32.84 ± 1.21
Comp. 4	6.56 ± 0.33	8.63 ± 0.85*	5.83 ± 0.97*
Comp. 5	7.02 ± 0.76	23.19 ± 0.65	33.16 ± 0.68
Curcumin	6.95 ± 0.76	23.11 ± 2.31	32.84 ± 0.40
(b). Dosage 3 μM			
Treatment	Number of cell (10^4 per mL)		
	24 h	48 h	72 h
Neg. Control	7.02 ± 0.70	23.79 ± 0.72	33.25 ± 0.73
Comp .1	7.17 ± 0.59	23.42 ± 0.29	33.79 ± 0.15
Comp. 2	6.92 ± 0.54	22.33 ± 1.70	32.29 ± 0.49
Comp. 3	7.08 ± 0.66	23.25 ± 0.52	28.58 ± 2.33*
Comp. 4	3.04 ± 0.40*	4.29 ± 0.36*	4.25 ± 0.36*
Comp. 5	7.04 ± 0.37	22.79 ± 0.41	31.62 ± 1.61
Curcumin	6.75 ± 0.19	23.12 ± 1.12	28.00 ± 1.45*

In all plates, 5.0 × 10^4 cells were seeded per ml. The results are presented as mean ± SEM of 3 experiments.

* Significant difference ($P<0.05$) compared to the negative control value

In present study, benzylidenecyclopentanone curcumin analogues (Figure 1b) were investigated for their effects on viability and proliferation of RBL-2H3 (rat basophilic leukemia) cells, a tumor analog of mast cells. They did not influence the viability of the cells after 30 min incubation, however, suppressed the viability after 24 h incubation. Parallelly, they showed same pattern effects on the histamine con-

tents in the cells. The effects of most analogues were less potent than these of curcumin. However, compound 4 showed more potent effects than these of curcumin at concentration of 10 μM. Previously, this compound also showed more potent cytotoxic effect than this of curcumin in T47D human breast cancer cell line. Moreover, this compound also markedly attenuated the proliferation of RBL-2H3 cells at low concentration (1 μM), whereas, curcumin and its other benzylidenecyclopentanone analogues did not influence the proliferation at this dose. It indicates that compound 4 is prospective to be developed as an anti carcinogenic agent.

β-diketon and double bond moieties of curcumin have a main role on its anticancer and antimutagenic (Majeed *et al.*, 1995). For the purpose of curcumin instability, attemp to modify the middle site of curcumin becomes cyclopentanone resulted in benzylidenecyclopentanone analogues (Sardjiman *et al.*, 1997). This attemp still maintans hydroxy moiety at aromatic rings responsible for several biological activities, and double bond moiety. Present study, compound 3 which has most similar structure to curcumin shows possessing lower cytotoxicity in RBL-2H3 than this of curcumin. Lack of β-diketon is suggested to contribute attenuate its cytotoxicity effect. Compound 4 is more potent than this of compound 3, probably because has four methyl moieties, whic are more hydrophobic compared to the two methoxy moieties in aromatic rings of compound 3. In compound 5, four ethyl moieties in the aromatic rings adjacent to the hydroxyl moieties cause hydrophobicity than the four methyl moieties in compound 4. However, in the fact the potency of compound 5 is less potent than this of compound 4. It suggests that the steric factor of ethyl is more prominent that its hydrophobicity.

Curcumin is reported to possess potent anticarcinogenic activities. Curcumin suppressed tumorigenic activity of a wide variety of carcinogens in cancers *in vitro* and *in vivo* (Shishodia *et al.*, 2005; Goel *et al.*, 2008).

The mechanisms of cytotoxic and antiproliferative properties of benzylidenecyclopentanone curcumin analogues might be similar to these of curcumin. Curcumin down-regulated NF-κB and NF-κB-regulated gene products (IκBα, Bcl-2, Bcl-XL, cyclin D1, and interleukin-6) in human multiple myeloma leading supression of proliferation and arrest of cells at the G_1/S phase of the cell cycle. The compound induced apoptosis of the cells by activating caspase-7 and -9, and inducing polyadenosine-5'-diphosphate-ribose polymerase cleavage (Bharti *et al.*, 2003). The compound also induced apoptosis human renal carcinoma Caki cells by down-regulating antiapoptotic Bcl-2, Bcl-X_L and IAP proteins, stimulating release of cytochrome c and activation of caspase 3, generating reactive oxygen species, and dephosphorilating Akt (Woo *et al.*, 2003). Curcumin potently suppressed cyclooxigenase-2 enzyme that stimulate antiapoptotic Bcl-2 protein (Gafner *et al.*, 2004).

In previous studies, two benzylidenecyclopentanone curcumin analogues i.e. compound 3 or 2,5-Bis(4-methoxybenzylidene)cyclopentanone and compound 4 or 2,5-Bis(4-hydroxy-3,5-dimethylbenzylidene) cyclopentanone have been shown to possess cytotoxic and antiproliferative properties againts T47D human breast cancer cell line. These compound are also named as pentagamavunon-0 (PGV-0) and pentagamavunon-1 (PGV-1) respectively. These compounds induced apoptosis on T47D cell line by increasing p53 and Bax expression, and decreasing Bcl-2 expression at the protein level. They have been shown to possess antiangiogenesis properties by decreasing of VEGF and COX-2 expressions (Melannisa, 2004; Nurulita and Meiyanto, 2006). Moreover, PGV-0 also

inhibited cell cycle progression through G1 arrest (Meiyanto and Dai, 2006). Whereas, PGV-1 inhibited cell cycle progression through G2/M arrest, and induced dephosphorilation of CDK-1 on T47d cell line (Dai *et al.*, 2006).

We conclude that the curcumin analog i.e. compound 4 or 2,5-Bis(4-hydroxy-3,5-dimethylbenzylidene) cyclopentanone, also named as pentagamavunon-1 (PGV-1) has been shown to possess more potent cytotoxicity and antiproliferative properties than these of other benzylidenecyclopentanone analogues of curcumin on RBL-2H3 cell.

References

Barsumian, E.L., Isersky, C., Petrino, M.G., Siraganian, R.P. 1981. IgE-induced histamine release from rat basophilic leukemia cell lines. *Eur. J. Immunol.* **11**(4), 317-323.

Bharti, A.C., Donato, N., Singh, S., and Aggarawal, B.B. 2003. Curcumin (diferlomethane) down-regulates the constitutive action of nuclear factor-kB and IkBa kinase in human multiple myeloma cells, leading to suppression of proliferation and induction of apoptosis. *Blood.* **101**(3),1053-1062.

Dai M, Kawaichi M, and Meiyanto E. 2006. PGV-1 Induces G2/M Arrest through P21 Expression with P53-Independent on T47D Cells, *Proceedings of The International Symposium on The Recent Progress in Curcumin Research,* 11-12 September 2006, Garuda Inn Hotel, Yogyakarta, Indonesia.

Goel, A., Kunnumakkara, A.B., Aggarwal, B.B., 2008. Curcumin as "Curecumin": from kitchen to clinic. *Biochem Pharmacol.*, **75**(4), 787-809.

Gafner, S., Lee, S.K., Cuendet, M., Barthelemy, S., Vergnes, L., Labidalle, S., Mehta, R.G., Boone, C.W., Pezzuto, J.M. 2004. Biologic evaluation of curcumin and structural derivatives in cancer chemoprevention model systems. *Phytochemistry.* **65**, 2849–2859.

Majeed, M., Badmaev, V., Shivakumar, U., dan Rajendran, R. 1995. *Curcuminoids: Antioxydant Phytonutrients,* Piscataway, New Jersey : Nutri Science Publisher Inc.

Melannisa R. 2004. Pengaruh PGV-1 pada Sel Kanker Payudara T47D yang diinduksi 17α-Estradiol: Kajian antiproliferasi, pemacuan apoptosis, dan antiangiogenesis, *Thesis,* Fakultas Farmasi UGM, Yogyakarta.

Meiyanto E and Dai M. 2006. Curcumin and PGV-0 Induce G1 Arrest and Having Synergistic Effect with Doxorubucin on T47D, *Proceedings of The International Symposium on The Recent Progress in Curcumin Research,* 11-12 September 2006, Garuda Inn Hotel, Yogyakarta, Indonesia.

Nurulita NA and Meiyanto E. 2006. Anticancer Effect of Pentagamavunon-0 (PGV-0) on T47D cells Induced By 17-B-Estradiol Through Apoptosis Induction and Angiogenesis Suppression, *Proceedings of The International Symposium on The Recent Progress in Curcumin Research,* 11-12 September 2006, Garuda Inn Hotel, Yogyakarta, Indonesia.

Reksohadiprodjo MH, Timmerman H, Sardjiman, Supardjan AM., Sudibyo M., Sugiyanto, Hakim L., Nurlaila, Hakim AR, Puspitasari I, Purwantiningsih, Nurrochmad A, Oetari, Yuwono T. 2004. Derivatives of Benzylidene Cyclohexanone, Benzylidene Cyclopentanone, and Benzylidene Acetone, and Therapeutics Use Thereof, *US Patent,* US 6,777,447 B2.

Sardjiman, Reksohadiprodjo, M.S., Hakim, L., Van der Goot, H., Timmerman, H. 1997. 1,5-Diphenyl-1,4-pentadiene-3-

ones and cyclic analogues as antioxidatine agent. Synthesis and structure-activity relationship. *Eur J Med Chem*. **32**, 625-630.

Shishodia, S., Sethi, G., and Aggarwal, B.B. 2005. Curcumin: Getting Back to the Roots. *Ann. N.Y. Acad. Sci.* **1056**, 206–217.

Tonnesen, H.H., Karlsen, J. 1985. Studies on curcumin and curcuminoid. VI. Kinetics of curcumin degradation in aqueous solution. *Z Lebensm Unters Forsch*. **180**(5), 402-404.

Van der Goot H. 1995.The chemistry and qualitative structure-activity relationship of curcumin. In Recent development in Curcumin Pharmacochemistry. *Proceeding of The International Symposium on Curcumin Pharmacochemistry (ISCP).* , August 29-31 1995, Yogyakarta Indonesia. p23-33.

Woo, J.H., Kim, Y.H., Choi, Y.J., Kim, D.G., Lee, K.S., Bae, J.H., Min, D.K., Chang, J.S., Jeong, Y.J., Lee, Y.H., Park, J.W., Kwon, T.K. 2003. Molecular mechanisms of curcumin-induced cytotoxicity: induction of apoptosis through generation of reactive oxygen species, down-regulation of Bcl-XL and IAP, the release of cytochrome c and inhibition of Akt. *Carcinogenesis*. **24**(7), 1199-1208.

Yamatodani, A., Fukuda, H., Wada, H., Iwaeda, T., Watanabe, T. 1985. High-performance liquid chromatographic determination of plasma and brain histamine without previous purification of biological samples : cation-exchange chromatography coupled with post-column derivatization fluorometry. *J Chromatogr*. **344**, 115-123.