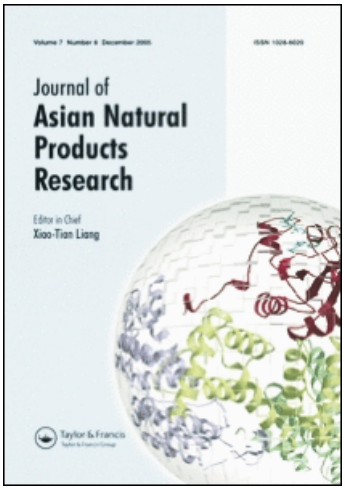

Journal of Asian Natural Products Research
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713454007

Activation of the SIRT1 pathway and modulation of the cell cycle were involved in silymarin's protection against UV-induced A375-S2 cell apoptosis

L.-H. Li[a]; L.-J. Wu[b]; S.-I. Tashiro[c]; S. Onodera[c]; F. Uchiumi[d]; T. Ikejima[a]
[a] China-Japan Research Institute of Medical and Pharmaceutical Sciences, Shenyang Pharmaceutical University, Shenyang, China [b] Department of Phytochemistry, Shenyang Pharmaceutical University, Shenyang, China [c] Department of Clinical and Biomedical Sciences, Showa Pharmaceutical University, Tokyo, Japan [d] Department of Gene Regulation, Faculty of Pharmaceutical Sciences, Tokyo University of Science, Noda, Chiba, Japan

To cite this Article Li, L.-H. , Wu, L.-J. , Tashiro, S.-I. , Onodera, S. , Uchiumi, F. and Ikejima, T.(2007) 'Activation of the SIRT1 pathway and modulation of the cell cycle were involved in silymarin's protection against UV-induced A375-S2 cell apoptosis', Journal of Asian Natural Products Research, 9: 3, 245 — 252
To link to this Article: DOI: 10.1080/10286020600604260
URL: http://dx.doi.org/10.1080/10286020600604260

Journal of Asian Natural Products Research, Vol. 9, No. 3, April–May 2007, 245–252

Activation of the SIRT1 pathway and modulation of the cell cycle were involved in silymarin's protection against UV-induced A375-S2 cell apoptosis

L.-H. LI†, L.-J. WU‡, S.-I. TASHIRO¶, S. ONODERA¶, F. UCHIUMI§ and T. IKEJIMA†*

†China–Japan Research Institute of Medical and Pharmaceutical Sciences, Shenyang Pharmaceutical University, Shenyang 110016, China
‡Department of Phytochemistry, Shenyang Pharmaceutical University, Shenyang 110016, China
¶Department of Clinical and Biomedical Sciences, Showa Pharmaceutical University, Tokyo 194 8543, Japan
§Department of Gene Regulation, Faculty of Pharmaceutical Sciences, Tokyo University of Science, 2641 Yamazaki, Noda, Chiba 278-8510, Japan

(Received 7 September 2005; revised 29 November 2005; in final form 7 December 2005)

Silymarin, derived from the milk thistle plant, *Silybum marianum*, has been traditionally used in the treatment of liver disease. Our previous study demonstrated that silymarin has an anti-apoptotic effect against UV irradiation. In this study, SIRT1, a human deacetylase that was reported to promote cell survival, was activated by silymarin (5×10^{-4} mol/L) in UV-irradiated human malignant melanoma, A375-S2 cells, followed by down-regulated expression of Bax and decreased release of cytochrome *c*. Cleavage of procaspase-3 and digestion of its substrates, the inhibitor of caspase-activated DNase (ICAD) and poly(ADP-ribose) polymerase (PARP), were also reduced. Consistent with its protective effect on UV-induced apoptosis, silymarin (5×10^{-4} mol/L) also increased G_2/M phase arrest, possibly providing a prolonged time for efficient DNA repair. Consequently, that silymarin protected A375-S2 cell against UV-induced apoptosis was partially through SIRT1 pathway and modulation of the cell cycle distribution.

Keywords: Silymarin; A375-S2 cell; UV irradiation; Anti-apoptosis; SIRT1; Cell cycle arrest

1. Introduction

Extensive research within the last few years has shown that silymarin (figure 1), a polyphenolic flavanoid, exhibits potent antioxidant activity [7], in addition to hepatoprotective [1,2] and anti-inflammatory [8] effects. Besides these, silymarin was found to have a cytoprotective effect, especially against UV irradiation-induced apoptosis [3]. Numerous studies show that ageing is associated with increased rates of stress-induced apoptosis [9] and the cumulative effects of cell loss have been implicated in various diseases including neurodegeneration, retinal degeneration, cardiovascular disease, and frailty

*Corresponding author. Email: ikejimat@vip.sina.com

Journal of Asian Natural Products Research
ISSN 1028-6020 print/ISSN 1477-2213 online © 2007 Taylor & Francis
http://www.tandf.co.uk/journals
DOI: 10.1080/10286020600604260

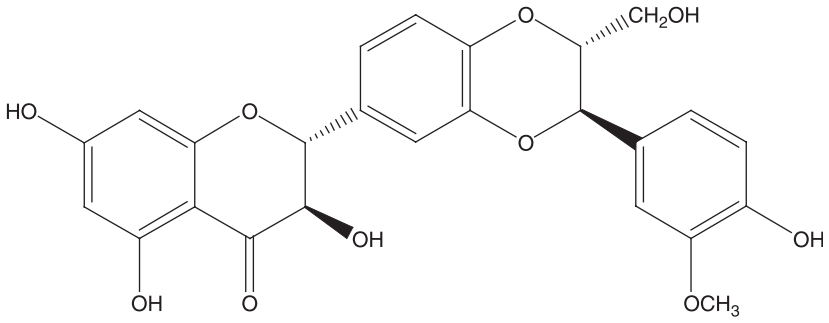

Figure 1. Structure of silymarin.

[9–11]. Therefore, searching for the active compounds from Chinese herbal medicine that inhibit disease-associated apoptosis or protect cells from various causes of death is the aim of our study.

Here, we found that silymarin's inhibitory mechanism on UV-induced A375-S2 cell apoptosis has a relationship with SIRT1, a member of the conserved sirtuin family of nicotinamide adenine dinucleotide (NAD^+)-dependent deacetylases, which is a key regulator of cell defences and survival in response to a variety of stresses [4–6] as well as molecules on its pathway (Bax, cytochrome *c*, procaspase-3, caspase-activated DNase [ICAD] and poly(ADP-ribose) polymerase [PARP]). The effect of silymarin on UV-induced cell cycle modulation was also examined in this study.

2. Results and discussion

2.1 Silymarin protected A375-S2 cells against UV-induced cell death

Table 1 shows the viability ratio of A375-S2 cells, which were treated with various concentrations of silymarin for 1 h and then further incubated for 12 h after UV irradiation. It was found that silymarin protected UV-irradiated A375-S2 cells from death in a dose-dependent manner (cell viability ratio was increased to 92.5% at the concentration of 5×10^{-4} mol/L) and that it had no cytotoxic effect on the cells.

Table 1. Protective effect of silymarin on cell viability in UV-irradiated A375-S2 cells (%).

	Cell viability (%)	
Silymarin (mol/L)	*UV irradiation*	*Without UV irradiation*
0	18.2 ± 3.4	100.0 ± 2.8
1×10^{-5}	17.8 ± 2.6	100.3 ± 2.4
5×10^{-5}	18.9 ± 4.3	98.5 ± 3.7
1×10^{-4}	28.3 ± 2.5	103.1 ± 1.6
2×10^{-4}	34.7 ± 4.2	105.7 ± 4.9
3×10^{-4}	45.6 ± 1.6	105.4 ± 4.1
4×10^{-4}	69.7 ± 3.8	107.3 ± 6.2
5×10^{-4}	92.5 ± 5.6**	103.5 ± 3.1

$\bar{x} \pm s$, $n = 3$. $^{**}P < 0.01$ *vs* 0 mol/L.

2.2 Silymarin reversed UV irradiation-induced morphologic changes in A375-S2 cells

In response to cellular insults, cells attempt to repair and defend themselves, but if unsuccessful, they often undergo programmed cell death, or apoptosis. Therefore, in order to determine whether silymarin protected A375-S2 cells against UV-induced cell death through anti-apoptotic pathway, the morphologic changes were observed. When A375-S2 cells were cultured for 12 h after UV irradiation, marked morphologic changes were observed as compared with the untreated control (figure 2a,c). The majority of cells became round, and some of these cells showed membrane blebbing (figure 2c), which were hallmarks of apoptosis, while silymarin pre-treatment (5×10^{-4} mol/L) reversed these morphologic changes (figure 2d).

2.3 The expression of SIRT1 was up-regulated in UV-irradiated A375-S2 cells after silymarin pre-treatment

SIRT1 play an important role in cell defences and survival in response to stress [4–6]. To investigate whether SIRT1 might be responsible for the ability of silymarin to protect A375-S2 cells from UV-induced apoptosis, the expression of SIRT1 was examined by Western blot analysis, which was found to be markedly up-regulated by silymarin (5×10^{-4} mol/L) in UV-irradiated A375-S2 cells as compared to that of silymarin-untreated cells (figure 3), suggesting that silymarin's protection against UV irradiation might be through SIRT1 pathway.

2.4 The protein expressions involved in SIRT1 pathway

Since up-regulated SIRT1 activity [6] could deacetylate the DNA repair factor Ku70, causing it to sequester the proapoptotic factor Bax away from the outer mitochondrial membrane to

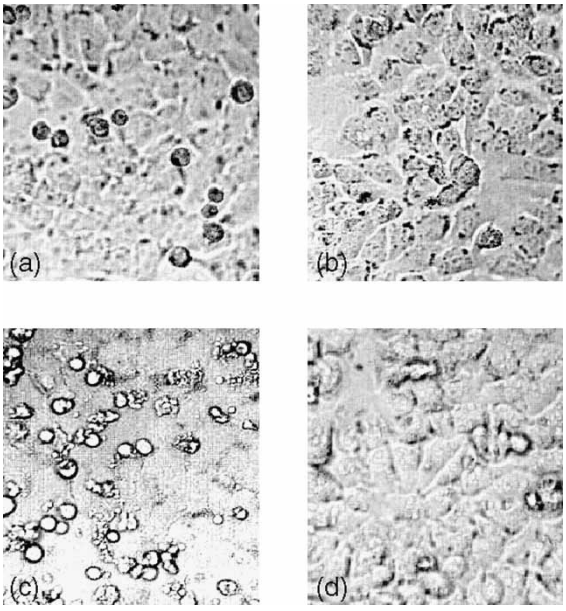

Figure 2. UV irradiation-induced morphologic changes of A375-S2 cells were reversed by silymarin. (a) Medium control; (b) the cells were incubated with silymarin (5×10^{-4} mol/L) for 12 h; (c) the cells were incubated for 12 h after UV irradiation (52.1 J/m^2); (d) the cells were irradiated by UV with silymarin pre-treatment (5×10^{-4} mol/L), then incubated for 12 h. Morphologic changes were observed by phase-contrast microscopy (original magnification × 200).

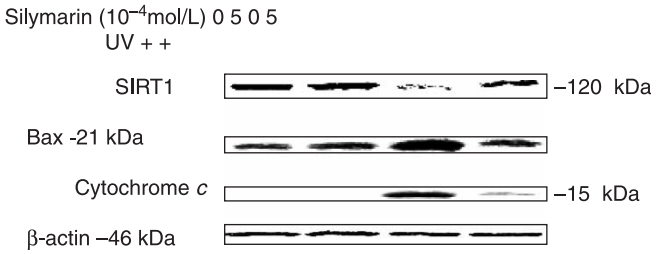

Figure 3. The expressions of SIRT1 activity, Bax and cytochrome *c* in UV-irradiated A375-S2 cells after silymarin pre-treatment. The cells were treated with or without silymarin (5×10^{-4} mol/L) for 1 h, then incubated for 12 h after UV-irradiation (52.1 J/m^2). Cell lysates were separated by 12% SDS-PAGE, and SIRT1, Bax and cytochrome *c* protein bands were detected by Western blot analysis. β-Actin was used as an equal loading control.

the cytoplasm, forming a complex with Ku70, the subsequent release of cytochrome *c* was inhibited as the result of Bax protein relocalisation. Downstream events including caspase activation and cleavage of ICAD and PARP were attenuated, thereby inhibiting stress-induced apoptotic cell death. In our study, it was found that the expression of Bax and release of cytochrome *c* from mitochondria were attenuated in UV-irradiated A375-S2 cells after silymarin pre-treatment (figure 3). Cleavage of procaspase-3 to caspase-3 (figure 4) and digestion of its substrates, ICAD and PARP, were also inhibited subsequently (figure 5).

Therefore, it can be concluded that silymarin may act as an agonist of SIRT1, suppressing UV-induced A375-S2 cell apoptosis through SIRT1 cooperating with mitochondria, and caspase pathway: activated SIRT1 by silymarin pre-treatment decreased the expression of Bax through deacetylation of Ku70, followed by inhibition of Bax-mediated apoptosis (release of cytochrome *c* and activation of caspase-3) in UV-irradiated cells.

2.5 The effect of silymarin on UV-induced cell cycle modulation

Cell cycle progression is important for maintaining homeostasis, especially when there is an insult to DNA [12,13]. Physiological stress or an insult to DNA could cause arrest in different stages of the cell cycle. Since UV irradiation is known to damage DNA directly, the effect of UV irradiation and silymarin pre-treatment on cell cycle progression was assessed. It was found that UV exposure caused a S arrest (29.62 versus 16.00% in control) at the expense of

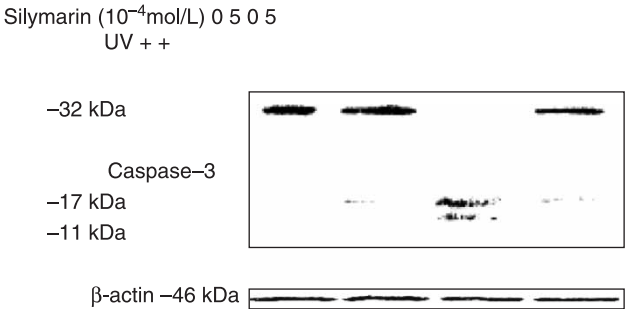

Figure 4. Silymarin inhibited the activation of caspase-3 in UV-irradiated A375-S2 cells. The cells were treated with or without silymarin (5×10^{-4} mol/L) for 1 h, then incubated for 12 h after UV-irradiation (52.1 J/m^2). Cell lysates were separated by 12% SDS-PAGE, and procaspase-3 and caspase-3 bands were detected by Western blot analysis. β-Actin was used as an equal loading control.

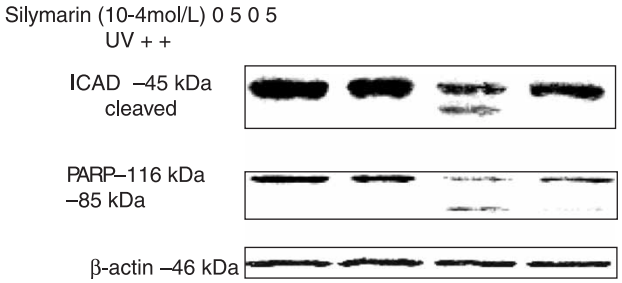

Figure 5. Cleavage of ICAD and PARP by caspase-3 in UV-irradiated A375-S2 cells were attenuated by silymarin treatment. The cells were treated with or without silymarin (5×10^{-4} mol/L) for 1 h, then incubated for 12 h after UV-irradiation (52.1 J/m^2). Cell lysates were separated by 12% SDS-PAGE, and the expressions of ICAD and PARP were detected by Western blot analysis. β-Actin was used as an equal loading control.

a decrease in G_2/M phase cells (0 versus 7.86% in control) (figure 6a,c, table 2). Pre-treatment with silymarin (5×10^{-4} mol/L), however, reversed the UV-induced S arrest, resulting in an increase in G_2/M phase cells (7.45% in silymarin + UV versus 0 in UV alone) (figure 6c,d, table 2). In general, an arrest in G_2/M phase of the cell cycle allows cells more time to repair damaged DNA before mitosis (M phase), until the damage of the genome is

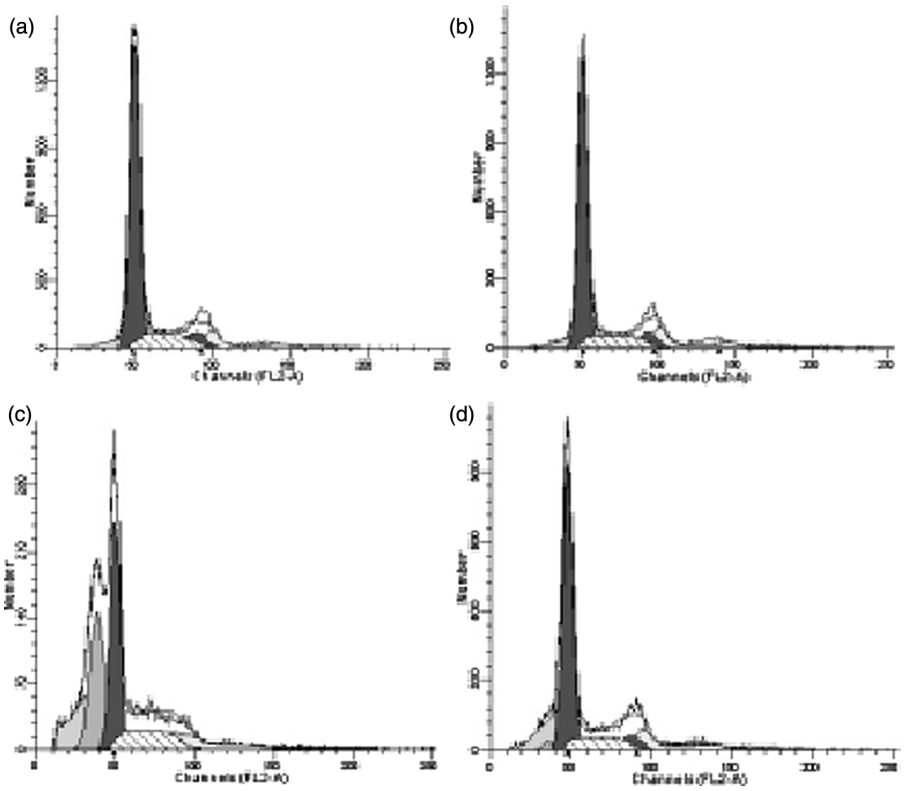

Figure 6. Flow cytometric analysis of silymarin-treated A375-S2 cells. (a) Medium control; (b) the cells were incubated with silymarin 5×10^{-4} mol/L for 12 h; (c) the cells were incubated for 12 h after UV irradiation (52.1 J/m^2); (d) the cells were irradiated by UV with silymarin pre-treatment (5×10^{-4} mol/L), then incubated for 12 h. Table 2 shows the cell cycle distribution in A375-S2 cells. Dark grey areas show cells at sub-G_0/G_1 phage, while striated areas show cells at S phage and black areas on the left or right of striated areas show cells at G_0/G_1 phage, or G_2/M phage, respectively.

Table 2. Effects of silymarin on cell cycle distribution (%).

	Cell phage (%)		
Group	G_0/G_1	*S*	G_2/M
Medium control	76.14	16.00	7.86
Silymarin (5×10^{-4} mol/L)	75.60	16.45	7.95
UV irradiation (52.1 J/m^2)	70.38	29.62	0
Silymarin (5×10^{-4} mol/L) + UV irradiation (52.1 J/m^2)	76.39	16.16	7.45

repaired. Since silymarin treatment resulted in an accumulation of UV-irradiated cells at G_2/M phase, part of the protective effect of silymarin against UV-induced apoptosis might be due to its effect on cell cycle distribution. However, detailed studies remain to be conducted to delineate the molecular mechanism involved in this action of silymarin. In addition, apoptotic sub-G_0/G_1 phase peak, caused by UV irradiation, was reduced obviously by silymarin pre-treatment (figure 6c,d).

In conclusion, silymarin promoted UV-irradiated A375-S2 cell survival partly through SIRT1 pathway. It also modulated the distribution of the cell cycle to allow more time for the damaged cells to repair. Present results may broaden silymarin's potential therapy use for many diseases in the future.

3. Experimental

3.1 Chemicals

Silymarin (mol. wt. = 482.4) was obtained from the Beijing Institute of Biologic Products (Beijing, China), the purity of which is determined to be about 98% by HPLC. Propidium iodide (PI) and 3,3-diaminobenzidine tetrahydrochloride (DAB) were purchased from Sigma Chemical (St Louis, MO, USA). Rabbit polyclonal antibodies against SIRT1, Bax, cytochrome *c*, procaspase-3, ICAD, PARP and horseradish peroxidase-conjugated secondary antibodies (goat anti-rabbit) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

3.2 Cell culture

The cells were cultured in RPMI-1640 medium (Hyclone, Logan, UT, USA) supplemented with 10% foetal bovine serum (Dalian Biological Reagent Factory, Dalian, China), L-glutamine (2×10^{-3} mol/L, Gibco, Grand Island, NY, USA), penicillin (100 U/ml) and streptomycin (100 μg/ml) and maintained at 37°C with 5% CO_2 in a humidified atmosphere.

3.3 Cell growth assay

The cells were dispensed in 96-well flat-bottomed microtitre plates (Nunc, Roskilde, Denmark) at a density of 1×10^4 cells/well. After 12 h incubation, the cells were incubated with various concentrations of silymarin for 1 h. Before UV irradiation, the medium contained with silymarin was removed, and the cells were washed with PBS twice and then added by PBS, followed by UV irradiation (52.1 J/m^2). The cells were further incubated for 12 h with the medium containing silymarin, which had been added before UV irradiation.

Cell growth was measured using the MTT assay as described [14] with an ELISA reader (TECAN SPECTRA, Wetzlar, Germany).

The percentage of cell viability was calculated as follows:

$$\text{Cell viability}(\%) = \frac{(A_{490} - A_{490,\text{blank}})}{(A_{490,\text{control}} - A_{490,\text{blank}})} \times 100$$

3.4 Observation of morphologic changes

375-S2 cells in RPMI-1640 containing 10% FBS were seeded into 60 × 15 mm cell culture dishes (Corning, NY, USA) and incubated overnight. Silymarin (5×10^{-4} mol/L) was added to the cell culture for 1 h prior to UV irradiation (52.1 J/m^2), and further incubated for 12 h. The cellular morphology was observed using phase-contrast microscopy (Leica, Germany).

3.5 Western blot analysis

After incubation for 12 h, both adherent and floating A375-S2 cells were collected. Western blot analysis was carried out as previously described [15] with some modification. The cells were lysed on ice in lysis buffer [50 mM HEPES (pH 7.4), 1% Triton X-100, 2 mM sodium orthovanadate, 100 mM sodium fluoride, 1 mM EDTA, 1 mM EGTA, 1 mM phenylmethanesulfonyl fluoride (PMSF)], supplemented with the proteinase inhibitors, aprotinin 100 μg/ml, leupeptin 10 μg/ml and pepstatin 100 μg/ml for 1 h. The protein concentration was determined using the folin assay. The lysate was centrifuged at 16,000*g* at 4°C for 10 min, equal amounts of total proteins were mixed in 2 × loading buffer [50 mM Tris–HCl (pH 6.8), 2% SDS, 10% 2-mercaptoethanol, 10% glycerol, and 0.002% bromphenol blue], boiled for 5 min, and subjected to a 12% SDS–polyacrylamide gel electrophoresis. Proteins were electrotransferred onto nitrocellulose membranes and detected with antibodies against SIRT1, Bax, cytochrome *c*, procaspase-3, ICAD and PARP followed by the addition of horseradish peroxidase (HRP)-conjugated secondary antibody and DAB as the HRP substrate.

3.6 Flow cytometric analysis of cell cycle

The cells (1×10^6 cells) were harvested and washed once in cold phosphate-buffered saline (PBS). The cell pellets were fixed in 75% ethanol at 4°C overnight and washed in cold PBS. Then the pellets were suspended in 1 ml of PI solution containing PI 50 μg/ml, 0.1% (w/v) sodium citrate and 0.1% (v/v) Triton X. Cell samples were incubated at 4°C in the dark for at least 15 min, and analysed by a FACScan flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA).

References

[1] P. Ferenci, B. Dragosics, H. Dittrich, H. Frank, L. Benda, H. Lochs, S. Meryn, W. Base, B. Schneider. *J. Hepatol.*, **9**, 105 (1989).
[2] A. Valenzuela, A. Garrido. *Biol. Res.*, **27**, 105 (1994).
[3] L.H. Li, L.J. Wu, B. Zhou, Z. Wu, S. Tashiro, S. Onodera, F. Uchiumi, T. Ikejima. *Biol. Pharm. Bull.*, **27**, 1031 (2004).

[4] H. Vaziri, S.K. Dessain, E. Ng Eaton, S.I. Imai, R.A. Frye, T.K. Pandita, L. Guarente, R.A. Weinberg. *Cell*, **107**, 149 (2001).
[5] J. Luo, A.Y. Nikolaev, S. Imai, D. Chen, F. Su, A. Shiloh, L. Guarente, W. Gu. *Cell*, **107**, 137 (2001).
[6] H.Y. Cohen, C. Miller, K.J. Bitterman, N.R. Wall, B. Hekking, B. Kessler, K.T. Howitz, M. Gorospe, R.D. Cabo, D.A. Sinclair. *Science*, **305**, 390 (2004).
[7] A. Valenzuela, R. Guerra, L.A. Videla. *Planta Med.*, **6**, 438 (1986).
[8] J.S. Kang, Y.J. Jeon, S.K. Park, K.H. Yang, H.M. Kim. *Biochem. Pharmacol.*, **67**, 175 (2004).
[9] Y. Higmi, I. Shimokawa. *Cell Tissue Res.*, **301**, 125 (2000).
[10] Y.P. Zhang, B. Herman. *Mech. Aging Dev.*, **123**, 245 (2002).
[11] J. Campisi. *Exp. Gerontol.*, **38**, 5 (2003).
[12] C.J. Norbury, I.D. Hickson. *Annu. Rev. Pharmacol. Toxicol.*, **41**, 367 (2001).
[13] J. Qin, L. Li. *Radiat. Res.*, **159**, 139 (2003).
[14] W. Lasek, A. Wankowicz, K. Kuc, W. Feleszko, J. Golab, A. Giermasz, W. Wiktor-Jedrzejczak, M. Jakobisiak. *Cancer Immunol. Immunother.*, **40**, 315 (1995).
[15] K. Suzuki, M. Hino, H. Kutsuna, F. Hato, C. Sakamoto, T. Takahashi, N. Tatsumi, S. Kitagawa. *J. Immunol.*, **167**, 5940 (2001).