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62 *The Open Plant Science Journal* , 2017, *10*, 62-69

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The Open Plant Science Journal

Content list available at: www.benthamopen.com/TOPSJ/

DOI: 10.2174/1874294701710010062

RESEARCH ARTICLE

Synergistic Antitumor Effect of Genitinib (Iressa®) with Flavonoids from the *Scutellaria baicalensis* Root on the Non-Small Cell Lung Cells

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Received: October 23, 2016 Revised: February 09, 2017 Accepted: March 16, 2017

Abstract:

Background:

As was reported, gefitinib (Iressa®) showed a strong growth inhibitory effect on non-small-cell lung cancer PC-9 cells with mutant EGFR, but did not so much on A549 cells with wild-type EGFR.

Observation:

We here demonstrated by isobolograms and combination index analyses that the paired combinations of gefitinib with flavonoids from Wogon (*Scutellaria baicalensis* root): wogonin, oroxylin A and chrysin exerted synergistic anti-tumor effects against A549 cells.

Result:

The cell cycle analyses revealed that the combination of gefitinib and oroxylin A in A549 cells induced more apoptotic cells than other paired combinations as well as gefitinib alone. Thus, it is anticipated that oroxylin A could help to enhance the remission rate in the gefitinib therapy for the patients with non-small cell cancer cells with wild-type EGFR which provide a poor prognosis.

Conclusion:

Since the Wogon flavonoids, wogonin, oroxylin A and chrysin, accelerated the acetylation of Lysine residues of histone proteins, it is suggested that they put forth anti-tumor activities through inhibition of histone deacetylases which mediated the post-translational modification of histones.

Keywords: Wogon, *Scutellaria baicalensis* root, Wogonin, Non-small-cell lung cancer cell A549, PC9, Mutant EGFR, Synergistic antitumor effect.

1. INTRODUCTION

Wogon (*Scutellaria baicalensis* root) is used as a component of Chinese traditional medicines including Wogon-to and Sho-saiko-to. Since Wogon exerts anti-inflammatory and antipyresis activities, it has been widely applied for the

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treatments of diarrhea and stomach pains since ancient times. The flavonoids containing an unsubstituted B-ring from Wogon, such as wogonin, oroxylin A and chrysin are biosynthesized starting from phenylalanine by a shikimate-malomate pathway involving a pinocembrin chalcone as an intermediate [1]. Baicalin, one of the Wogon flavonoids, has been reported to suppress induction of IL-1 β and TNF-α *via* an NF-κβ pathway associated with inflammation [2]. Another Wogon flavonoid, wogonin, suppressed the growth of the non-small-cell lung cancer cell line A549 through a tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) pathway [3]. It was recently reported that Wogon-to (consisting of the natural drugs: *Scutellaria baicalensis* root, *Paeonia lactiflora*, *Ziziphus jujuba* and *Glycyrrhiza uralensis*) (US investigational new drug code PHY906) showed synergistic antitumor activities in combination with Irinotecan (CPT-11), capecitabine or 5-FU against renal cancer [4]. These paired combinations led to reduction in the side effects due to gastrointestinal disturbance and peripheral neuralgia [4]. These results prompted us to examine the combination effects of gefitinib (Iressa®) and Wogon flavonoids Fig. (**1**) against metastatic non-small-cell-lung cancer cell lines. Gefitinib was launched as an EGFR molecular target agent for the treatment of non-small cell lung cancers in 2002. Although this drug had marked effectiveness on lung cancers with mutant EGFR [5], the side effects, such as interstitial pneumonia, cardiac toxicity and neurotoxicity limit therapy in lung cancer patients. Thus, combination therapy has been longing that can reduce the dose of gefitinib.

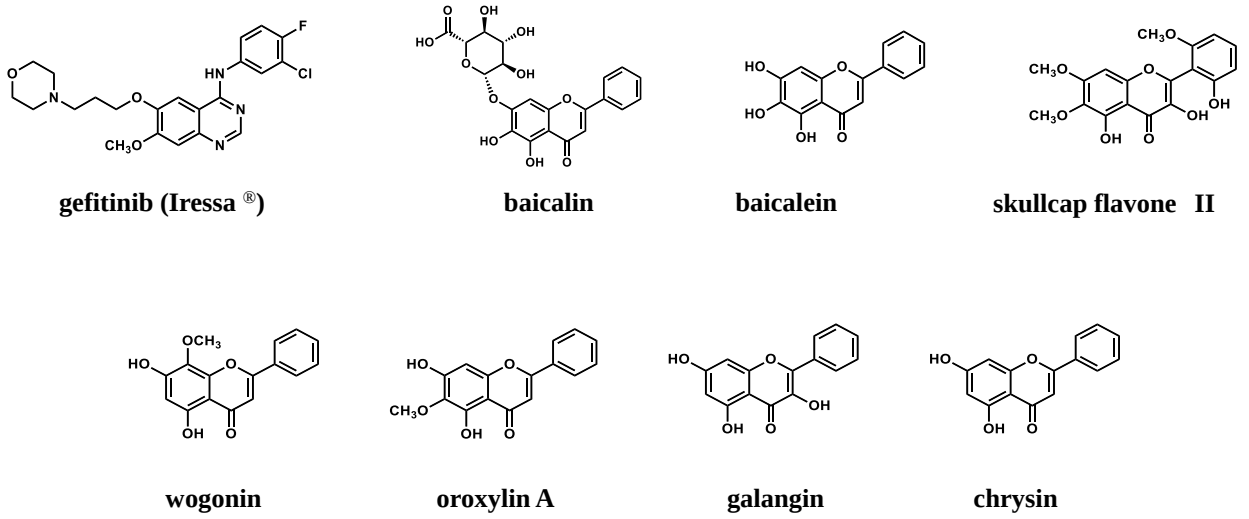

Fig. (1). Structures of gefitinib (Iressa®) and Wogon flavonoids.

During a course of pharmacological study on natural products, we have exploited application of the Wogon flavonoids for clinical therapy. Although most of the Wogon flavonoids were reported to show anticancer activities [5], these activities were too weak to be applied as an individual medicine for the clinical treatment of cancer. Since gefitinib reportedly showed a strong growth inhibition on PC-9 cells with mutant EGFR, but not on A549 cells with wild-type EGFR [6], we tried to examine the anti-tumor effects of paired combinations of gefitinib and the Wogon flavonoids on A549 cells by using isobolograms and combination index analyses [7 - 9]. We demonstrated herein that gefitinib exerted synergistic inhibitory effects against the growth of A549 cells in combination with wogonin, oroxylin A or chrysin. Of these couples and gefitinib alone, however, only the combination of gefitinib and oroxylin A increased significantly the sub-G/G_1 phase cells (corresponding to apoptotic cells) in the cell cycle analyses of A549 cells. Subsequently, we examined the influence of the Wogon flavonoids on acetylation of Lysine residues of histone proteins in A549 cells, since wogonin was reported to inhibit expression of histone deacetylase (HDAC) 1 and 2 in A549 cells [10]. It was found out that wogonin, oroxylin A and chrysin accumulated acetylated Lysine of histone proteins as did the HDAC Class I inhibitor MS-275 [11, 12]. Taken together, we speculate that some Wogon flavonoids exerted an anti-tumor effect by inhibiting HDACs which are involved in post-translational modification of histones. We anticipate that the present combinational treatments of gefitinib and Wogon flavonoids would pave the way to the treatment for non-small cell lung cancers with wild type EGFR.

2. MATERIALS AND METHODS

2.1. Chemicals

Gefitinib was purchased from Sigma-Aldrich, USA. MS-275 was synthesized according to the reported procedure [12]. The Wogon flavonoids were previously isolated in Prof. Taniguchi's laboratory [13, 14].

2.2. Cell Lines and Antibodies

A549 and PC9 cells were obtained from the American Type Culture Collection. Anti-Acetylated-Lysine polyclonal antibody was purchased from Merck-Millipore, USA. Anti-β actin antibody was purchased from Sigma-Aldrich, USA.

2.3. Inhibition of Gefitinib or Wogon Flavonoids Against the Growth of A549 and PC9 Cell Lines

A549 and PC9 cells were cultured in DMEM and RPMI1640 mediums (Thermo, USA) with 10% fetal bovine serum (FBS) (Biowest, France), respectively. Cells (1×10^4/well) were inoculated onto standard 96-well microtiter plates. After culturing for 1 day, serially diluted samples (gefitinib, 100, 10, 1 and 0.1 μM for A549; 100, 10, 1, 0.1 nM for PC9: Wogon flavonoid, 100, 10, 1 and 0.1 μM for A549 and PC9) were added to the wells, and the culture was continued for another 3 days. Cell growth was evaluated with the assay of WST-8 (Dojindo, Japan), and IC_{50} values were calculated.

2.4. Isobolograms and Combination Index Analyses for the Interaction between Gefitinib and Wogon Flavonoids in A549 Cells

The IC_{50} values of the respective substances were as follows: 10.6 μM (gefitinib); 48.2 μM (Wogonin); 4.9 μM (oroxylin A); 61.9 μM (galangin); 29.1 μM (chrysin); 51.4 μM (skullcap flavone II). Then, IC_{50} values of gefitinib (10 μM) were measured on the addition of the respective concentrations of Wogon flavonoids (20.0, 40.0, 75.0, and 100.0 μM for chrysin; 10.0, 25.0, 50.0, and 75.0 μM for other Wogon flavonoids). The IC_{50} value of gefitinib alone is expressed as 1.0 on the Y axis of the isobologram, while that of Wogon flavonoid alone is expressed as 1.0 on the X axis. The IC_{50} values thus obtained were plotted to make a line graph. A concave isobologram indicates synergy, a convex isobologram reflects antagonism and a straight line connecting the X-axis intercept to the Y-axis intercept represents additivity between two substances.

Combination indexes (CIs) were calculated utilizing the following equation [7 - 9].

$$\mathbf{CI} = \frac{\mathbf{C_{A,x}}}{\mathbf{IC_{x,A}}} + \frac{\mathbf{C_{B,x}}}{\mathbf{IC_{x,B}}}$$

CA,x and CB,x represent the concentrations of drug A and drug B used in combinations to achieve x% drug effect. ICx,A and ICx,B are the concentrations for single agents to achieve the same effect. CIs of less than, equal to, and more than 1 indicate synergy, additivity, and angatonism, respectively. The CI values calculated at each concentration point of gefitinib and Wogon flavonoids were as follows: 0.83, 0.67, 0.92 (wogonin); 0.98, 0.57, 0.76 (oroxylin A); 0.99, 0.75. 0.60 (chrysin); 1.03, 0.96, 0.72 (galangin); 0.93, 0.98, 0.96 (skullcap flavone II).

2.5. Western Blot Analysis

A549 cells were plated onto 60 mm diameter dishes (1.0×10^6/dish). After incubating for 24 h, the cells were treated with Wogon flavonoids (each 100 μM), gefitinib (10 μM), MS-275 (10 μM) or control (0.1% DMSO) and incubated for 24 h. The medium was discarded, and floating and adhered cells were washed with 1×PBS (2 × 1 mL), scraped, and resuspended in 200 μL of modified Covance Research lysis buffer (pH 8.0 and 1% Nonidet P-40 were adopted in place of pH 7.4 and 1% Triton-X 100, respectively) at 0 °C for 1 h. The lysates were centrifuged at 14000 rpm for 15 min at 4 °C. The protein concentration was determined with a BCA protein assay kit (Thermo Fisher Scientific, USA). An equal amount (10 μg) of protein was then resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a PVDF membrane (Merck-Millipire, USA). The blots (1 × Tris-HCl buffer saline, 5% nonfat milk and 0.05% Tween 20) were probed with an antibody recognizing acetylated-Lysine and were detected by using LAS3000 detection system (GE health care, USA).

2.6. Cell Cycle Analysis

A549 cells were plated onto 60-mm diameter dishes (1.0×10^6/dish). After incubating for 24 h, the cells were washed with serum-free medium (2 × 1 mL) and immersed in serum-free medium (5 mL) for 24 h. After removal of the medium, the cells were washed with serum-free medium (2 × 1 mL) and incubated with gefitinib (5 μM) alone or with the paired combinations of gefitinib (5 μM) and the Wogon flavonoids (each 50 μM) in the serum-free medium (5 mL) for another 24 h. For the control, 0.1% DMSO was used. The medium (about 5 mL) was transferred to Centrifuge Tubes (Iwaki, Japan). The adhered cells, after being washed twice with cold PBS (each 1 mL), were treated with 0.25% trypsin (Invitrogen) (200 μL) for 3 min and transferred to the above tubes. The cells in the tubes were treated with a Cycle Test™ Plus DNA reagent Kit (Cat. No. #340242) according to the procedure recommended by Becton Dickinson and Company. DNA content was measured with a FACS™Cant II.

3. RESULTS

3.1. Inhibition of the Wogon Flavonoids Against the Growth of A549 and PC9 Cells

The IC_{50} value (0.01 μM) of gefitinib against the growth of PC9 cells with mutant EGFR was much smaller than the value (10.6 μM) against the growth of A549 cells with wild-type EGFR as shown Table **1**. Furthermore, the Wogon flavonoids, wogonin, oroxylin A, galangin, chrysin, and skullucap flavone II had inhibitory activities against A549 and PC9 cells, whereas both baicalin and baicalein had no or weak activity.

Table 1. Inhibitory activites of Wogon flavonoids and Gefitinib against A549 and PC9 cells.

IC_{50} (μM)		
	A549	PC9
Baicalin	> 100	> 100
Baicalein	70.3	54.2
Wogonin	48.2	10.9
Oroxylin A	4.9	11.4
Galangin	61.7	46.8
Chrysin	29.1	14.4
Skullcap flavone II	51.4	30.5
Gefitinib	10.6	0.01

3.2. Isobologram and Combination Index Analyses of the Interaction Between the Wogon Flavonoids and Gefiitinib in A549 Cells

In order to assess the combination effects of the gefitinib with Wogon flavonoids against the growth of A549 cells, isobolograms [7 - 9] were prepared according to the procedure indicated in Materials and Methods. As shown in Fig. (**2**), gefitinib were combined with the various concentrations of Wogon flavonoids for the determination of antiproliferative activity against A549 cells. The Y-axis expresses the ratio of the IC_{50} of gefitinib in the presence of a fixed concentration of Wogon flavonoid to the IC_{50} of gefitinib in the absence of Wogon flavonoid. The X-axis represents the ratio of the fixed concentration of Wogon flavonoid to the IC_{50} of Wogon flavonoid in the absence of geftinib.

The paired combinations of gefitinib with wogonin, oroxylin A and chrisin showed a plotted curve below the straight line, whereas those of gefitinib with galangin and skullcap flavone II exhibited nearly straight line connecting the X-axis intercept to the Y-axis intercept.

Additionally, combination indexes (CIs) were calculated from the isobolograms, utilizing the equation [7 - 9] in Materials and Methods. The CI values in the paired combinations of gefitinib with wogonin, oroxylin A and chrysin were less than 1 and thus represent synergy. By contrast, those of gefitinib with galangin and skullcap flavone II were nearly 1, indicating additivity.

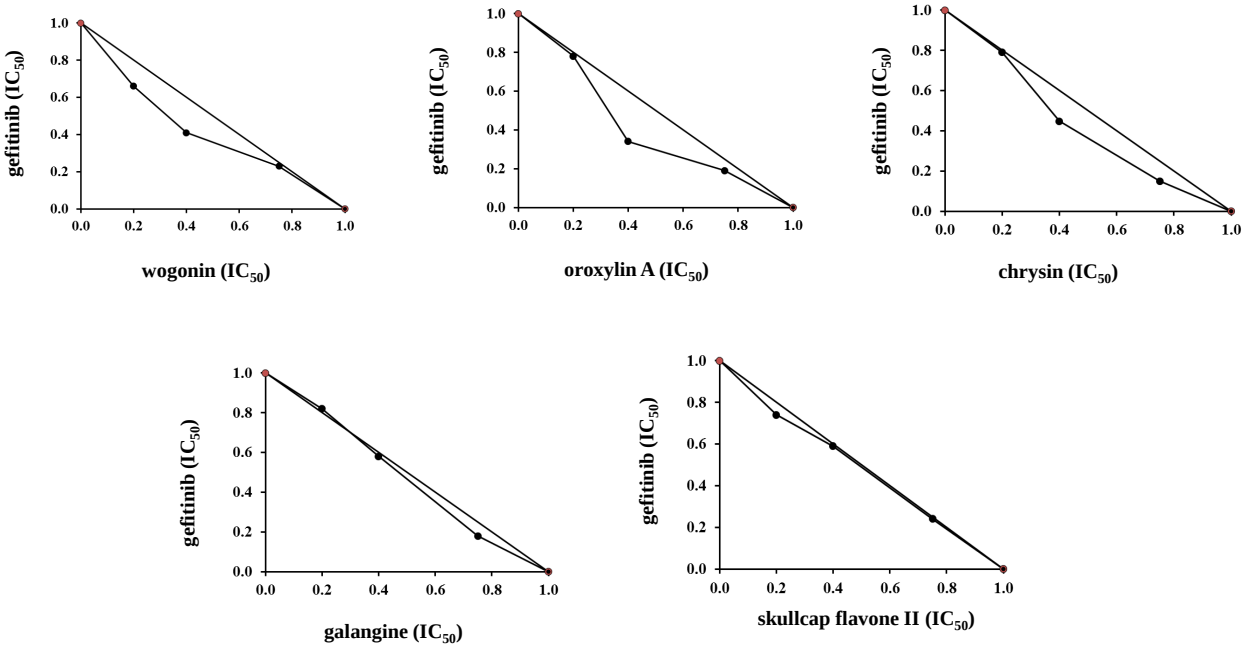

Fig. (2). IC_{50} isobolograms of gefitinib and Wogon flavonoid against A549 cells. The IC_{50} value of gefitinib alone is expressed as 1.0 on the Y-axis of the isobologram, while that of Wogon flavonoid (wogonin, oroxylin A, galangin, chrysin or sculcap flavone II) alone is expressed as 1.0 on the X-axis.

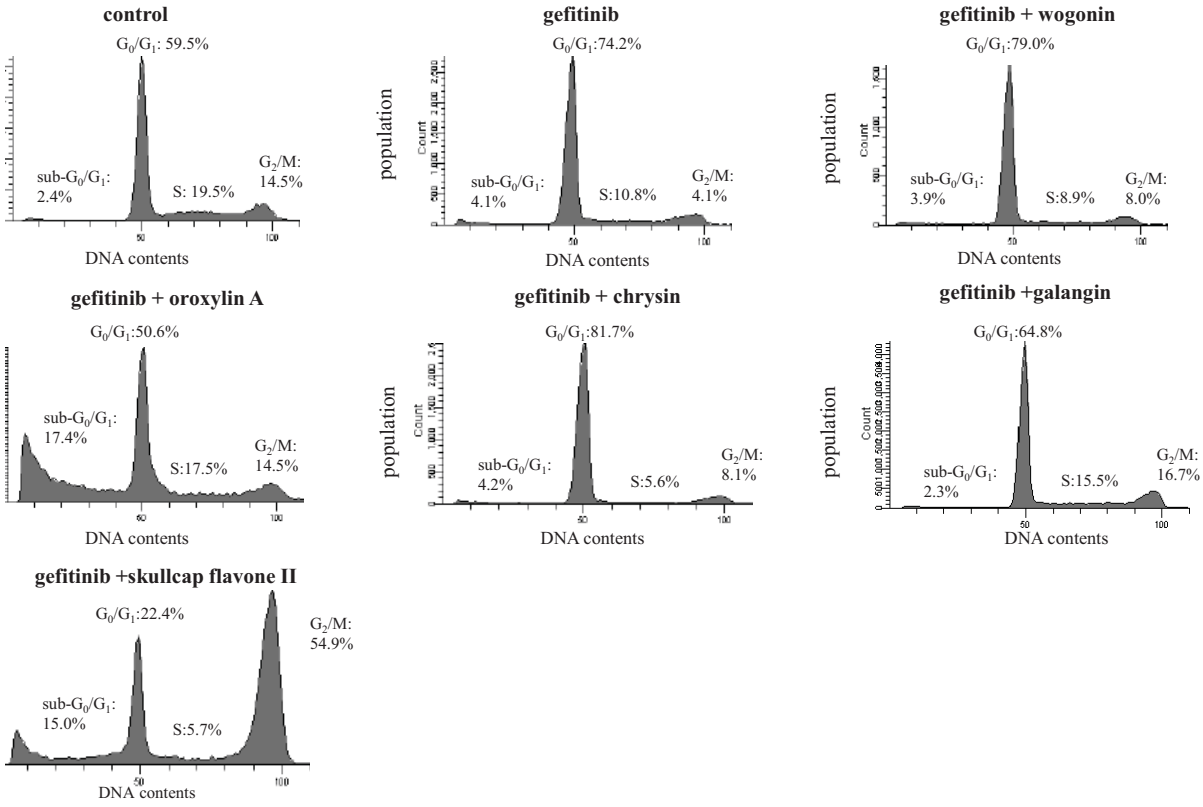

Fig. (3). Cell cycle analyses of A549 cells. Cells were treated with 0.1% DMSO (control), gefitinib (5 μM) or paired combinations of gefitinib (5 μM) and Wogon flavonoids (each 50 μM) for 24 h. The percentages in the graphs represent the rates of cells of each phase to the total cells.

3.3. Cell Cycle Analysis Using Flow Cytometer

We next tried to analyze the influence of the Wogon flavonoids on the cell cycle of A549 cells using flow cytometry. As shown in Fig. (**3**), the paired combinations of gefitinib (5 μM) with wogonin and chrysin (each 50 μM) increased the populations of $G_{0/}G_1$ phase cells by 4.8 and 7.5%, but practically did not vary those of sub-G/G_1 phase cells, relative to gefitinib (5 μM) alone, respectively. It is noteworthy that the couple of gefitinib (5 μM) and oroxylin A (50 μM) reduced G/G_1 phase cells by 23.6% and, instead, increased sub-G/G_1 cells by 13.3% relative to gefitinib (5 μM). The couple of gefitinib (5 μM) and skullcap flavone II (50 μM) reduced G/G_1 cells by 51.8% and increased G2/M cells by 50.8% relative to gefitinib (5 μM) alone.

3.4. Detection of Acetylated Lysine of Histone Protein by Western Blotting

Fig. (**4**) showed the level of acetylated Lysine of histone proteins in A549 cells which were incubated with gefitinib or Wogon flavonoids. Among the flavonoids, wogonin, oroxylin A and chrysin which exhibited synergistic antitumor activities with gefitinib increased acetylated Lysine level. It was therefore suggested that these flavonoids have an inhibitory effect against HDACs.

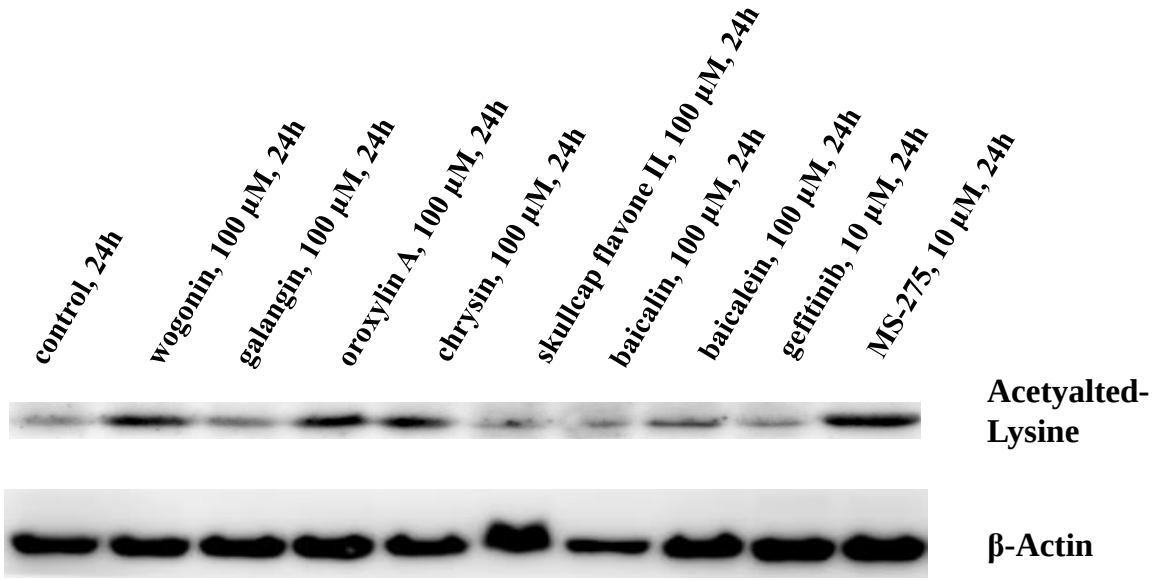

Fig. (4). Western blot analysis of A549 cells. Cells were treated with 0.1% DMSO (control), gefitinib, MS-275 and Wogon flavonoids for 24h, and acetylated-Lysine was detected.

CONCLUSION

Although the molecular target drug gefitinib (Iressa ®) enhanced the remission late in non-small cell lung cancers patients [15], the side effects such as interstitial pneumonia, cardiac toxicity and neurotoxicity limited therapy in lung patients [16]. Reportedly, gefitinib showed a strong growth inhibitory effect on non-small-cell lung cancer PC9 cells with mutant EGFR, but did not on A549 cells with wild-type EGFR [16]. Our study revealed that the paired combinations of gefitinib with the Wogon flavonoids: wogonin, oroxylin A and chrysin showed synergistic anti-tumor effects against A549 cells. Especially, the combined incubation of gefitinib and oroxylin A with A549 cells significantly augmented apoptotic cells as compared with the sole treatment of gefitinib (Fig. **3**). It is noted that the A549 cells given gefitinib and skullcap flavone II exhibited only an additive effect, in spite of the augmented accumulation of G_2/M phase cells. Since skullcap flavone II had no HDAC inhibitory activity Fig. (**4**), it remains to be resolved how this paired combination increased the G_2/M phase cells.

Our results provide a new way of the application of the Wogon flavonoids which do not have enough effectiveness on cancer cells as an individual dose. According to the report [7], wogonin lowered the expression levels of mRNAs and proteins of HDAC1 and 2 *via* interference with the *c*-Myc/ S-phase kinase-associated protein 2 (Skp2) pathway. This event, we feel, could be attributed, at least in part, to the following molecular interaction: wogonin disrupted the activities of HDAC1/2 enzymes by chelating with a Zinc ion at the bottom of HDAC enzymes and by binding to an

inner cavity of the enzymes as shown in Fig. (**5**) in the same manner as Merck compound, typical 2-aminobenzamide-type HDAC1/2-selective inhibitor [17 - 20]. In our study, wogonin, oroxylin A and chrysin accumulated the acetylated Lysine of histone proteins in A549 cells. It was thus suggested that these Wogon flavonoids inhibited the A549 cells by the mechanism involving HDAC inhibition. The findings obtained so far lead us to anticipate that the combinational treatments of gefitinib and the Wogon flavonoids would provide a clue to the treatment for non-small cell lung cancers with wild-type EGFR which give a poor prognosis.

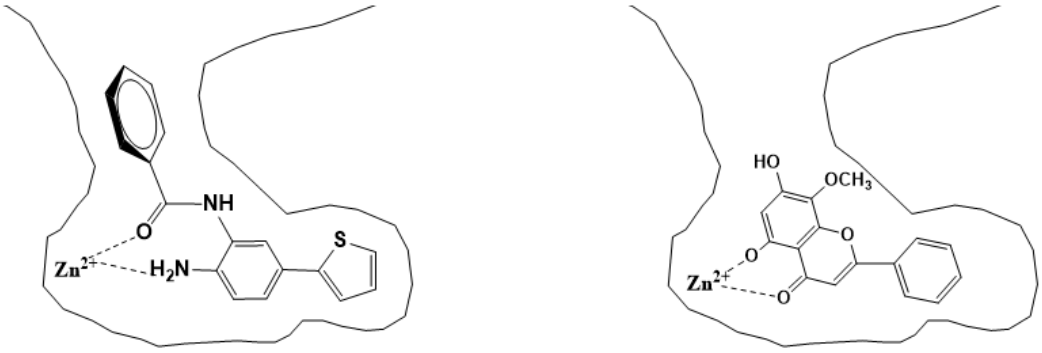

Fig. (5). Proposed models of Merck compound (A) and wogonin (B) bound to HDAC1 (or 2).

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

HUMAN AND ANIMAL RIGHTS

No Animals/Humans were used for studies that are base of this research.

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

ACKNOWLEDGEMENTS

Part of this work was supported by the following grants: Grand-in-Aid for Scientific Research (B) (24390222) from 'Ministry of Education, Culture, Sports, Science, and Technology' – 'Japan' and Takeda Science Foundation (T.S.).

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