Combined effects of low-dose gambogic acid and NaI131 in drug-resistant non-small-cell lung cancer cells

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Abstract

Background

Radioactive seed is a method for treating drug-resistant, late-stage non-small cell lung cancer (NSCLC), but has undesirable side effects. Gambogic acid (GA), an ingredient of traditional Chinese medicine, exerts broad-spectrum antitumour activities via several pathways. This study aimed to elucidate the mechanism involved in the combined effect of low-dose GA and NaI¹³¹ to sensitize the antitumour activity of NaI¹³¹ in drug-resistant NSCLC cells.

Methods

Human NSCLC cell line A549 and drug-resistant cell lines A549/DDP and A549/Taxol were treated with NaI¹³¹, low-dose GA or a combination of both; control group of each cell line was treated with phosphate-buffered saline. Following treatment, cell proliferation, apoptosis, cell cycle, and levels of expression of apoptosis-related proteins namely CDK1, Cyclin B, mtp53, HSP90, and Bax, Bcl-2 respectively, and P-glycoprotein 1 (P-gp) known to confer resistance to chemotherapy, were detected using western blotting and immunofluorescence. mRNA levels of mtp53 and HSP90 were measured using qRT-PCR.

Results

Compared to the control group, A549, A549/DDP, and A549/Taxol cells treated with NaI¹³¹, GA or combination of drugs exhibited G2/M arrest, increased percentage of total apoptotic cells, significantly reduced protein levels of CDK1, Cyclin B, mtp53, HSP90, Bcl-2 and P-gp, increased protein levels of Bax and decreased mRNA levels of mtp53 and HSP90. The changes in the combination group were significantly different from the other groups.

Conclusion

In NSCLC cell lines, low-dose GA could enhance the effect of NaI¹³¹ on G2/M arrest, promote cell apoptosis, reduce drug-resistance and hence could be explored as a potential radionuclide sensitizer.

Background

Lung cancer (LC), the most common type of cancer in the world is a global burden and a public health issue due to its high fatality rates. In USA, it constitutes about 13% and 14% of newly detected cancers in men and women respectively. [1] In China, the age-standardized incidence rate of LC was 36.71 per 100,000 with men having higher incidence than women. [2] Non-small cell lung cancer (NSCLC) accounts for 85% of all LC with an overall 5-year survival rate of 16%; the treatment mainly includes surgical

resection, targeted therapy, chemotherapy, radiotherapy and immunotherapy, although use of personalized medicine has been speculated. [3] It is estimated that 30–55% of patients with NSCLC experience relapse in spite of curative resection leading to subsequent mortality. [4]

Cancer chemotherapy resistance, be it innate or acquired, can be a hindrance during the treatment phase. It occurs due to a variety of reasons such as amplified drug target molecules, decreased drug accumulation and enhanced drug export, alterations in drug metabolism by modifying signalling transduction molecules, DNA damage, and evasion of apoptosis. [5] Avoiding apoptosis by upregulating anti-apoptotic proteins, such as Bcl-2, and inactivation of pro-apoptotic proteins such as Bax is commonly seen in cancer cells leading to a lack of response to chemotherapy. [6] Overexpression of P-glycoprotein 1 (P-gp) leading to the transport of anticancer drugs out of the cancer cells has been known to confer resistance to chemotherapy in NSCLC. [7] While conventional radiotherapy and stereotactic body radiation therapy are used for inoperable cases, iodine-125 seed brachytherapy has been proved to be a safe and effective technique with improved overall survival and quality of life. [8–10] It has been shown that the combination of Iodine-125 brachytherapy and chemotherapy is better than chemotherapy alone with improved therapeutic efficacy for NSCLC. [11, 12]

Although ionizing radiation is commonly used for lung cancer treatment, reports suggest that it might have secondary undesirable effects such as promoting cancer malignancy, incidence of cardiac events etc. [13, 14] Therefore, it is desirable to find a safe radionuclide sensitizer to reduce the side-effects of radiotherapy without influencing the antitumour efficacy in clinical practice.

Gambogic acid (GA) extracted from the gamboge resin of *Garcinia hanburyi* has been used in traditional Chinese medicine to treat infections and tumours. It exerts antitumour, antiangiogenic, and antimetastatic activities in several kinds of cancers including lung cancer [15–17]; in addition, conventional doses do not influence the functions of other cells in the body. [18, 19] GA exerts its anticancer activities via numerous targets and signalling pathways, promoting apoptosis, inducing cell cycle arrest, inhibiting angiogenesis, and activating lymphocytes. [20] GA in low doses causes autophagy in mutant p53 (mtp53) degradation in cancer cells [21] and protein levels of mtp53 reduce following GA exposure. [22]

Our previous results showed that a combination of low-dose GA and NaI¹³¹ was able to significantly inhibit cell proliferation as well as induce cell apoptosis in A549/DDP cells. [23] Therefore, we aimed to further study the plausible cellular mechanisms related to the effect of the combination therapy on two drug-resistant lung cancer cell lines. Additionally, we also explored if the combination therapy conferred inhibition to therapy resistance in NSCLC, so as to achieve better therapeutic results. In the current *in vitro* study, we aimed to elucidate whether low-dose GA could sensitize NaI¹³¹ to enhance its influence on cell cycle regulation, apoptosis and drug resistance in two NSCLC cell lines namely A549/Taxol and A549/DDP cells, that are resistant to taxol and cisplatin respectively.

Methods

Cell culture

Human NSCLC cell line A549 was a gift from the Laboratory of Pathology at the School of Medicine of Southeast University. The cisplatin-resistant A549/DDP cell line and the Taxol-resistant cell line A549/Taxol were procured from KeyGen Biotech (Jiangsu, China). Cells were cultured in RPMI1640 medium supplemented with 10% FBS and incubated in a humidified CO₂ (5%) incubator at 37 °C. When the cells reached a confluency of 80–90%, the original culture medium was discarded, the cells were treated with trypsin (0.25%) for 1–2 min, and the digestion was terminated by adding an equal volume of serum-containing culture medium. Cells were dispersed by gentle pipetting, and the cell suspension was transferred to a 15-ml centrifuge tube, after which the suspension was centrifuged at 1000 rpm for 5 min. The supernatant was discarded, and 1–2 ml of culture medium was added to resuspend the cells. The cells were then transferred to a culture flask for continuous culture.

Experimental groups and drug intervention

A549, A549/DDP and A549/Taxol cells were harvested in the logarithmic phase of growth. 5×10^4 cells per well were seeded in 6-well plates and cultured at 37°C, 5% CO₂ for 24 h and divided into the NaI¹³¹ group, GA group, combination group and control group. The NaI¹³¹ group was treated with 10.3 MBq NaI¹³¹, the GA group with 2.9 µg/ml GA, and the combination group with 10.3 MBq NaI¹³¹ and 0.3 µg/ml GA as described previously [23]. The control group was treated with PBS (2 ml per well). The cells were harvested after 48 h of drug treatment.

Cell cycle assay

Cells were fixed in 70% alcohol for 2 h. 100 µl of RNase A was added to the cells for 30 min followed by addition of 400 µl of propidium iodide stain (KeyGen Biotech; Jiangsu, China) and incubated at 4 °C in dark for 30 min. Cellular DNA content was measured using a flow cytometer with fluorescence-activated cell sorting (FACS) (Becton-Dickinson; Calibur, USA). The cell cycle profiles were analysed using Canto diva (BD Biosciences, San Jose, CA, USA).

Cell apoptosis assay

Annexin V-APC/7-AAD cell apoptosis detection kit (KGA1024, KeyGen Biotech, Jiangsu, China) was used for detection of apoptosis and the protocol mandated by the manufacturer was followed. After drug treatment, cells were mixed with 500 µl of binding buffer, 5 µl of Annexin V-APC and 5 µl of 7-AAD (7-Aminoactinomycin D), and incubated at room temperature in dark for 5–15 min. Cell apoptosis was measured using flow cytometry (Becton-Dickinson; Calibur, USA). Data was analysed using Canto diva (BD Biosciences, San Jose, CA, USA).

Immunofluorescence (IF)

Cells were air-dried on a glass slide and fixed in 4% paraformaldehyde for 30 min. Slides were rinsed thrice in PBS, followed by blocking with 50–100 µl of goat serum and incubated at room temperature for 20 min. Then, 50–100 µl of primary antibodies namely, anti-mtp53 (1:200, Abcam, ab32509) or anti-

HSP90 (1:200, Santa Cruz Biotechnology, sc-69703) was added and incubated at 37 °C for 2 h under humidified conditions. Slides were rinsed thrice in PBS, followed by addition of 50–100 µl of horseradish peroxidase (HRP)-conjugated anti-mouse or anti-rabbit secondary antibody (1:200 KeyGen Biotech) and incubated at 37 °C for 1 h at room temperature. After rinsing the slides thrice in PBS, 50–100 µl of DAPI staining solution (KeyGen Biotech; Jiangsu, China) was added and incubated at room temperature for 5 min in dark. Sections were mounted using anti-fade mounting medium (Sangon Biotech Shanghai, China). Protein expression in cells was observed under a fluorescence microscope (OLYMPUS BX61, Japan). Five areas that exhibited strong signals were photographed, and the images were analysed using ImageJ software.

Quantitative real-time RT-PCR

Total RNA was extracted from cells using TRIzol™ reagent (15596026, Invitrogen, Carlsbad, CA, USA). cDNA synthesis was performed using RevertAid First Strand cDNA Synthesis Kit (K1622, ThermoFisher Scientific). Specific mRNA quantification was performed by real-time PCR using the Thunderbird SYBR® qPCR Mix (QPS-201, TOYOBO Bio-Technology, CO., LTD) in a real-time PCR detection system (ABI Step one plus Real time-PCR system, USA) using the following reaction conditions: holding stage at 95 °C for 5 min; cycling stage at 95 °C for 15 s, 6 °C for 20 s, and 72 °C for 40 s for a total of 40 cycles; and melting curve stage at 95 °C for 15 s, 60 °C for 1 min, and 95 °C for 15 s. The $2^{-\Delta\Delta Ct}$ method was performed to calculate the relative mRNA expression of the target genes.

1) *mtP53*: Forward Primer: 5'-CCGCAGTCAGATCCTAGCG-3'; Reverse Primer: 5'-AATCATCCATTGCTTGGGACG-3'

2) *HSP90*: Forward Primer: 5'-CATAACGATGATGAGCAGTACGC-3'; Reverse Primer: 5'-GACCCATAGGTTACCTGTGT-3'

3) *GAPDH* primers: Forward primer was 5'-CATCTTCTTTTGGCTCGCCA-3'; reverse primer was 5'-TTAAAAGCAGCCCTGGTGACC-3'.

The experiment was repeated 3 times.

Western blot

Total cellular protein was extracted using RIPA lysis buffer, and the protein concentration was determined using the bicinchoninic acid (BCA) method. Proteins separated on a 12% sodium dodecyl sulphate–polyacrylamide gel were transferred onto a nitrocellulose membrane, blocked with 5% skim milk for 2 h, followed by addition of primary antibody namely, anti-CDK1 (1:300, Abcam, ab131450), anti-Cyclin B (1:300, Abcam, ab172317), anti-Bcl-2 (1:300, Abcam, ab692), anti-Bax (1:300, Abcam, ab77566) or anti-P-gp (1:300, BOSTER Biological Technology) and incubated overnight at 4 °C. The following day, the membrane was washed thrice with 5% TBST for 10 min each. Horseradish peroxidase-labelled goat anti-mouse IgG secondary antibody (1:5000, KeyGen Biotech, Jiangsu, China) was added, and the membrane was incubated at room temperature for 30 min. After the membrane was washed 3 times with 5% TBST

for 10 min, protein bands were visualized using the ECL chemiluminescence Kit (KGP116, Jiangsu, China). Gel-Pro32 software was used to analyse the band intensities.

Statistical analysis

Statistical analyses of the data were performed using SPSS software (Version 22.0. Armonk, NY: IBM Corp.) software. All data were expressed as the mean $\pm$ standard deviation (mean $\pm$ SD). Comparisons of the mean values between two groups were performed using the paired t-test. Comparisons of data among multiple groups were performed using analysis of variance (ANOVA). $P < 0.05$ indicated a statistical difference.

Results

Analysis of stages in the cell cycle

We first tested whether the combination treatment may have effect on cell cycle, since our previous results showed that treatment of A549/DDP cells with low-dose GA together with NaI^{131} was able to significantly inhibit cell proliferation.[23] NaI^{131} , GA, and a combination of both were used to treat A549, A549/DDP, and A549/Taxol cells and control group was treated with PBS. The cell cycle conditions in all groups were observed after 48 h (Fig. 1A, B). Data showed that in A549, A549/DDP, and A549/Taxol cells, the percentage of cells in the G2/M phase were considerably increased in NaI^{131} group, GA group, and combination group compared to that in the control group. The increase in the combination group was significantly higher than that in the NaI^{131} and GA group, individually ($P < 0.001$). Moreover, the highest percentage of cells in the G2/M phase was found in A549/Taxol cells. Additionally, the percentage of cells among these groups in G0/G1 and S phase were not significantly different. These results suggested that G2/M arrest occurred in cells in all the groups but was most pronounced in the combination group.

Analysis of cell apoptosis

Annexin V-APC/7-AAD assay was used to revalidate the effect of low-dose GA combined with NaI^{131} on cell apoptosis. NaI^{131} and GA could both induce apoptosis in A549, A549/DDP, and A549/Taxol cells. The results after 48 h of incubation are shown in Fig. 2A, B. Compared to the control group, the percentage of total apoptotic cells in the NaI^{131} group, GA group, and combination group increased appreciably; while the percentage of apoptotic cells was almost similar in the NaI^{131} and GA group with about 40–50% cell death. Cell apoptosis was most evident in the combination group ($P < 0.001$); late-stage apoptotic activity was highest among all the groups.

Expression levels of P-gp and, proteins related to cell cycle control, apoptosis

To better understand the combination treatment on drug-resistant NSCLC cells, proteins levels related to cell cycle, apoptosis and drug resistance, namely CDK1, cyclin B, Bcl-2, and P-gp were measured using Western blot (Fig. 3A). The expression levels of CDK1, cyclin B, Bcl-2, and P-gp decreased in the NaI¹³¹ group, GA group, and combination group compared to the control group. The expression levels were the lowest in the combination group in comparison to the other groups ($P < 0.001$). In contrast, the expression levels of Bax were elevated in all the groups compared to control, but the increase was most evident in the combination group ($P < 0.001$) (Fig. 3B-F).

Expression of mRNA and protein levels of mtp53 and HSP90

We wanted to check whether the changes in cell cycle and apoptosis brought about by the combination treatment on drug-resistant NSCLC cells was by influenced by mtp53. Results of RT-qPCR and immunofluorescence demonstrated that the expression levels of *mtp53* decreased in all the treatment groups as compared to control (Fig. 4A-C). The lowest signal was detected in the combination group ($P < 0.001$). While the expression level of *mtp53* was similar in A549 and A549/DDP cells treated with NaI¹³¹ or GA, it decreased slightly in A549/Taxol cells treated with GA as compared to control and NaI¹³¹ treatment ($P < 0.01$; Fig. 4C).

Additionally, we tested the expression of *HSP90* in drug-resistant NSCLC treated with the combination of drugs. mRNA expression of *HSP90* (Fig. 4D) was significantly reduced in the combination group as compared to individual treatment with either NaI¹³¹ or GA ($P < 0.001$). Immunofluorescence results was in-line with the RT-qPCR results, in that, the protein expression levels of HSP90 (Fig. 4E, F) were reduced in all the groups as compared to the control. The relative expression levels of HSP90 remained almost similar in A549 and A549/Taxol cells treated with NaI¹³¹ and GA, whereas the expression level increased in A549/DDP cells treated with GA as compared to control and NaI¹³¹ treatment ($P < 0.001$; Fig. 4F). These results indicated that the effect of NaI¹³¹ combined with GA on drug-resistant NSCLC cells may be associated with mtp53 and HSP90.

Protein expression of (B): mtp53 and (E): HSP90, in all groups were detected using immunofluorescence (IF) after treatment of A549, A549/DDP, and A549/Taxol cells with NaI¹³¹, GA, or combination of both. Graphical representation of the results of IF for (C): mtp53 and (F) HSP90. * refers to compared with the control group; # refers to compared with the NaI¹³¹ group, and Δ refers to compared with the GA group; *, #, Δ indicate $P < 0.05$; **, ##, $\Delta\Delta$ indicate $p < 0.01$; ***, ###, $\Delta\Delta\Delta$ indicate $p < 0.001$.

Discussion

The present study investigated the efficacy of using GA as a radionuclide sensitizer to NaI¹³¹ in an *in vitro* system. As an extension to our previous study which showed that GA below the conventional dose can enhance the apoptosis-promoting function of NaI¹³¹ in A549/DDP cells, the current study revealed that

compared to control, in the combination group of all the three NSCLC lines (A549, A549/DDP, A549/Taxol) there was significant increase in the percentage of cells in the G2/M phase, increase in the number of apoptotic cells, decrease in the levels of protein of CDK1, cyclin B, Bcl-2, P-gp, mtp53 and HSP90 and a significant increase in the protein levels of Bax.

A crucial function of the *p53* gene is induction of cell cycle arrest and promotion of cell apoptosis and DNA repair. [24] The cell cycle-related regulatory proteins CDK1 and cyclin B are the major regulatory proteins expressed in cells in S phase and during entry into G2/M phase. [25] *p53* can cause G2/M phase arrest through downregulation of cyclin B/CDK1 expression. [26] Mtp53 can drive mitosis in tumour cells and promote tumour cell passage through M phase via various pathways to cause tumour cell proliferation. [27] Since γ radiation can be persistently released after radioactive seed implantation in the tumour body, these cells in the active proliferation at G2 and M phases are more sensitive to this radiation causing cyclin B1-mediated G2/M arrest and elevated Bax/Bcl2 ratio. [28] GA acting on tumour cells can arrest cancer cells at G1/S phase [29] or G2/M phase. [30] It has been shown that *p53* has a crucial role in the induction of cell cycle arrest and promotion of cell apoptosis and DNA repair. [24] GA in low doses was reported to cause autophagy in mtp53 degradation in cancer cells. [21] Our experimental results showed that, in A549, A549/DDP, and A549/Taxol cells, GA could decrease levels of cyclin B, CDK1, and mtp53 in all the groups and increase the number of cells in G2/M phase, indicating that GA could cause G2/M arrest in these cells. In the NaI¹³¹ combined with GA group, the reduction of CDK1 and cyclin B levels was the highest, suggesting that low-dose GA might contribute to an increase in the number of cells in the radiation sensitive stage by influencing cell cycle proteins and mtp53 to increase the lethal effect of NaI¹³¹ on tumour cells.

Mtp53 protein is upregulated in many drug-resistant tumour cell lines and is thus involved in tumour drug resistance. [31, 32] Mtp53 protein can selectively upregulate the expression of multiple drug resistance gene 1 (*mdr1*). [33] P-gp encoded by *mdr1* can promote cellular excretion of chemotherapeutic drugs to produce drug resistance. [6] Our study results showed that, in the combination group, levels of mtp53 and P-gp in all the cell types decreased, indicating that the two might have common drug-resistance pathways. Moreover, GA is shown to down-regulate mtp53 at the post-transcription level. [22] Our study concurs with the previous study and demonstrates that the combinational effect of GA and NaI¹³¹ might possibly aid in overcoming drug-resistance leading to a better response to chemotherapy. In normal cells, wild type *p53* protein is subject to ubiquitination and degradation mediated by mouse double minute 2 (MDM2). [26] However, mtp53 protein cannot directly activate the MDM2 ubiquitination degradation pathway but can bind to heat shock protein 90 (HSP90). HSP90 and its associated chaperones can stabilize mtp53 protein to prevent its degradation, whereas inhibition of the function of HSP90 reduces mtp53 protein levels. [34, 35] GA is shown to prevent Hsp90/mtp53 complex formation. [22] Our study showed that, after intervention by NaI¹³¹ and GA, the expression of *HSP90* and *mtp53* at the levels of both mRNA as well as proteins, decreased in all the cell types, suggesting that these two kinds of intervention could reduce cellular expression of HSP90 and mtp53. In addition, the reduction was more evident in the combination group, indicating that low-dose GA combined with NaI¹³¹ could significantly

reduce HSP90 and mtp53 levels in drug-resistant cells likely due to the ability of GA to destabilize and degrade mp53. [22]

In the Bcl-2 family, Bcl-2 and Bax are the most representative apoptosis-inhibitory and apoptosis-promoting proteins, respectively. GA can decrease the level of Bcl-2 and increase the level of Bax in a cancer cell lines. [36, 37] Our study results conform to the previous studies in that, cell apoptosis results showed that NaI¹³¹ and GA could synergistically increase apoptosis in A549, A549/DDP, and A549/Taxol cells, with amplified effects observed in the combination of the two drugs. Moreover, the combination drugs promote late-stage apoptosis as evidenced by our cell apoptosis assay. Inhibition of apoptosis facilitates the survival and proliferation of cancer cells resulting in resistance to conventional chemotherapy agents. [6] As our study results reveal, a combination of NaI¹³¹ and GA can assist in increasing late-stage apoptosis by altering the expression levels of apoptosis-related proteins such as Bax and Bcl-2, and thus positively influencing the response to chemotherapy.

Our study is limited to *in vitro* cell line system and hence further validation in *in vivo* rodent models as well as in patient samples is warranted. Moreover, a comprehensive study related to more genes and proteins related to apoptosis and cell-cycle needs to be done to clarify the various signal-transduction mechanisms and related pathways.

Conclusions

In conclusion, our study illustrates that GA can be investigated as a favourable radionuclide sensitizer and a combination of GA along with NaI¹³¹ can have advantages in enhancing the effects of NAI¹³¹ on tumour cells by way of governing cell cycle stages, promoting apoptosis and reducing drug-resistance, all of which are necessary cellular factors for cancer treatment in clinical settings.

Abbreviations

NSCLC

non-small cell lung cancer

GA

Gambogic acid

LC

Lung cancer

P-gp

P-glycoprotein 1

mtp53

mutant p53

mdr1

multiple drug resistance gene 1

MDM2

Declarations

Ethics approval and consent to participate:

Not applicable

Consent for publication:

Not applicable

Availability of data and materials:

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests:

The authors declare that they have no competing interests.

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Authors' contributions:

JH and XZ designed the experiment and drafted the work, MD and YX carried out the experiment and obtained the data, SY for statistics, YW for analysis and interpretation, JH and SC made substantial revisions to the manuscript. All authors read and approved the final manuscript.

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Figures

Figure 1

(A): (a) Control group, (b) NaI131 group, (c) GA group, (d) combination group. A549, A549/DDP, and A549/Taxol cells were treated with PBS (control), NaI131, GA, or the combination of both, for 48 h and the stages of cell cycle in each group was detected: the first peak represents G0/G1 phase, the second peak represents G2/M phase, and the plateau represents S phase. (B) Graphical representation of the results. *

refers to compared with the control group; # refers to compared with the Nal131 group, and Δ refers to compared with the GA group; *, #, Δ indicate $P < 0.05$; **, ##, $\Delta\Delta$ indicate $p < 0.01$; ***, ###, $\Delta\Delta\Delta$ indicate $p < 0.001$.

Figure 2

(A) (a) Control group, (b) Nal131 group, (c) GA group, (d) combination group. A549, A549/DDP, and A549/Taxol cells were incubated with PBS (control), Nal131, GA, or the combination of both, for 48 h and the percentages of cells at all stages of apoptosis were detected by flow cytometry. The upper right quadrant suggests the number of apoptotic cells in the late stage, while the lower right quadrant suggests the number of apoptotic cells in the early stage. (B) Graphical representation of the results. * refers to compared with the control group; # refers to compared with the Nal131 group, and Δ refers to compared with the GA group; *, #, Δ indicate $P < 0.05$; **, ##, $\Delta\Delta$ indicate $p < 0.01$; ***, ###, $\Delta\Delta\Delta$ indicate $p < 0.001$.

Figure 3

After treatment of A549, A549/DDP, and A549/Taxol cells with NaI¹³¹, GA, or the combination of both, for 48 h, the expression of intracellular CDK1, Cyclin B, Bcl-2, Bax and P-gp protein in all groups was detected. (A): Lanes 1-4: correspond to the following groups: A549 cells treated with PBS, NaI¹³¹, GA, and combination respectively; Lanes 5-8: A549/DDP cells treated with PBS, NaI¹³¹, GA, and combination respectively; Lanes 9-12: A549/Taxol cells treated with PBS, NaI¹³¹, GA, and combination respectively. Graphical representation of the results is presented in (B): CDK1, (C) Cyclin B, (D) Bcl-2, (E) Bax, (F) P-gp. * refers to compared with the control group; # refers to compared with the NaI¹³¹ group, and Δ refers to compared with the GA group; *, #, Δ indicate $P < 0.05$; **, ##, $\Delta\Delta$ indicate $p < 0.01$; ***, ###, $\Delta\Delta\Delta$ indicate $p < 0.001$.

Figure 4

(a) Control group, (b) Nal131 group, (c) GA group, (d) combination group. Relative mRNA levels of (A): mtp53 and (D): HSP90, in all the groups were detected by qRT-PCR after treatment of A549, A549/DDP, and A549/Taxol cells with Nal131, GA, or combination of both. Protein expression of (B): mtp53 and (E): HSP90, in all groups were detected using immunofluorescence (IF) after treatment of A549, A549/DDP, and A549/Taxol cells with Nal131, GA, or combination of both. Graphical representation of the results of IF for (C): mtp53 and (F) HSP90. * refers to compared with the control group; # refers to compared with the Nal131 group, and Δ refers to compared with the GA group; *, #, Δ indicate $P < 0.05$; **, ##, $\Delta\Delta$ indicate $p < 0.01$; ***, ###, $\Delta\Delta\Delta$ indicate $p < 0.001$.

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