Inhibition of endogenous nitric oxide promotes p53-dependent apoptosis induced by cisplatin in human colon cancer cells

Ji Hee Byun [1], Min Young Kim [1,2,*]

[1.] Laboratory of Pharmacology and Toxicology, Faculty of Biotechnology, College of Applied Life Science and [2.] Subtropical Horticulture Research Institute, Jeju National University, Jeju 690-756, Republic of Korea
jeffmkim@jejunu.ac.kr

Abstract: Endogenous nitric oxide (NO•), a reaction product of NO• synthase (NOS) isoenzymes, appears to suppress apoptosis and promote growth of cancer cells, suggesting that its inhibition is a rational therapeutic approach. We report here the chemoadjuvant potential of NO• modulators on cisplatin-mediated cell killing/apoptosis in two isogenic HCT116 human colon carcinomas with distinct p53 status. Cells were treated with either cisplatin alone or in combination with three NOS isoform-selective inhibitors, namely an inducible NOS inhibitor 1400W, a neuronal NOS inhibitor Vinyl-L-NIO and an endothelial NOS inhibitor gallotanin, and an NO• scavenger carboxy-PTIO. We found that inhibition of endogenous NO• enhanced cisplatin-induced apoptosis in cells harvested 48 hours after treatments through induction of cell cycle change and down-expression of X-linked inhibitor of apoptosis (X-IAP). Apoptosis was delayed in p53-null HCT116 cells, underscoring the importance of p53 modulation of the response. The data suggests that inhibition of endogenous NO• may improve the efficacy of chemotherapy for colon cancer by inducing p53-mediated apoptosis.
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1. Introduction

Colon cancer is one of the most common malignancy of the gastrointestinal system which has traditionally been a leading cause of cancer mortality in western countries. However, during the past few decades, there have been remarkable changes in the incidence of colorectal cancer in Asian counties (Sung et al., 2005). For instance, colon cancer was the third most commonly diagnosed cancer in Korea (13% of all cancer diagnoses, Korea National Cancer Information Center, Cancer incidence 2009). Current evidence indicates that dysregulated production of nitric oxide (NO•) is an important factor in the etiology of this disease (Ekmekcioglu et al., 2005; Hussain and Harris, 2007)

NO• is produced by three isoforms of the enzyme nitric oxide synthase (NOS), neuronal NOS (nNOS, NOS1), inducible NOS (iNOS, NOS2) and endothelial NOS (eNOS, NOS3), which can regulate a number of important biological functions, as well as playing critical roles in the anti-pathogen response of the immune system (Xu et al., 2002; Wink et al., 1998; Nathan and Xie, 1994). However, the role of NO• in cancer is multi-dimensional, depending on timing, location and concentration (Wink et al., 1998). Some reports have indicated that low levels of NO• produced endogenously by tumor cells may reduce their metastatic potential (Kubes et al., 1991; Kubes et al., 1993). By contrast, NO• has been proposed to be an important mediator of tumor growth (Mordan et al., 1993; Gottke and Chadee, 1996). These observations suggest that manipulation of NO• may offer exciting opportunities to improve the effectiveness of cancer treatment.

Cisplatin is a potent antineoplastic agent used for the treatment of a wide range of cancers (Saad et al., 2004; Wang et al., 2004). Recent reports indicate that constitutive production of endogenous NO• has an antiapoptotic function promoting survival of human melanoma cells and the depletion of endogenous NO• leads to increased sensitivity to cisplatin (Salvucci et al., 2001; Tang and Grimm, 2004). In the present study, we investigated the susceptibility of human colon cancer cells to cisplatin induced apoptosis by modulating endogenous NO• using three NOS isoform-selective inhibitors, namely an iNOS inhibitor 1400W, a nNOS inhibitor Vinyl-L-NIO and an eNOS inhibitor gallotanin, and an NO• scavenger carboxy-PTIO (c-PTIO). In addition, wild-type p53 (p53+/+) and p53-null (p53-/-) HCT116 cells were used experimentally to evaluate the influence of p53 status.

2. Material and Methods

2.1. Cell cultures and chemicals

Two isogenic HCT116 colon carcinoma cells (p53+/+ and p53-/-), kindly provided by Dr. G.N. Wogan (Massachusetts Institute of Technology, USA), were cultured in McCoy's 5A medium supplemented with 10% heat-inactivated fetal bovine

serum, 100 units/mL penicillin, 100 µg/mL streptomycin and 2 mM L-glutamine at 37 ºC with 5% CO_2 in a humidified atmosphere. Sources of reagents were as follows: cell culture reagents, Lonza (Walkersville, MD, US); 1400W dihydrochloride, 2-(4-Carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (c-PTIO), gallotannin, sodium nitroprusside (SNP), tri reagent and GenElute™ mammalian genomic DNA miniprep kit, Sigma Chemical (St. Louis, MO, USA); Vinyl-L-NIO hydrochloride, Enzo Life Sciences (Ann Arbor, MI, US); N-methyl-L-arginine monoacetate (NMA) and cisplatin, CalBiochem (Salt Lake City, UT, USA); cell proliferation kit I (MTT) from Roche (Indianapolis, IN, USA); annexin V-FITC apoptotic assay kit, Clontech Laboratories (Palo Alto, CA, US); TOP script™ one-step RT PCR kit (Enzynomics, Daejeon, Korea).

2.2. Cell viability assay

Cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay. Cells were plated one day before treatment in 96-well plates at a density of 5×10^4 cells/well and were treated with indicated concentrations of NO• modulators alone or in combination with cisplatin and/or SNP for the designated time points. After the cultivation, 10 µL of MTT (5 mg/ml) was added and incubated at 37 ºC in the dark for 4 h, and the formazan product was dissolved by mixing it with 100 µL of 10% SDS in 0.01M HCl. Absorbance was determined spectrophotometrically at 550 nm with a Packard EL340 microplate reader (Bio-Tek Instruments, Winooski, VT, US). The relative percentage of cell survival was calculated by dividing the absorbance of treated cells by that of the control in each experiment. Results from the MTT assay has been used to derive the 50% effective concentrations (EC_{50}) of each drug to induce growth inhibition.

2.3. Measurement of nitrite production

NO• production was assessed by measuring nitrite in media fractions by the Griess reaction as previously described (Jorens et al., 1991). Nitrite concentrations were determined from a standard curve using sodium nitrite at concentrations ranging from 1.25 to 20 µM. Optical density was measured using a microplate reader at 540 nm, with fresh culture media serving as the blank. Results are expressed in pmoles per 10^7 viable (trypan blue-excluding) cells.

2.4. Apoptosis detection

Two million cells were incubated in a 60-mm tissue culture dish containing cisplatin alone or in combination with NO• modulators. Cells were harvested by trypsinization and centrifugation, then analyzed in a FACScan flow cytometer (BD Bioscience, San Jose, CA, USA; excitation at 488 nm) equipped with CellQuest software after staining with annexin V-FITC and propidium iodide by annexin V-FITC apoptotic assay kit following the manufacturer's protocols (Clontech). Apoptotic cells stained with annexin V (early apoptosis) or with both annexin V and propidium iodide (late apoptosis), necrotic cells stained with propidium iodide, and living cells did not contain either stain.

2.5. Cell cycle analysis

Cells were seeded in 100-mm tissue culture dishes at a density of 2×10^6 cells/mL and treated with cisplatin alone or in combination with NO• modulators at the indicated times. The cells were harvested, washed twice with PBS and fixed in 70% ethanol on ice for 30 min. The cells were further stained with 500 µg/mL of PI on ice, incubated with 10 µg/mL of ribonuclease A digestion at 37 ºC for 30 min and subjected to flow cytometric analysis. Phase distributions were calculated from the resultant DNA histogram using CellQuest software, and expressed as a percentage of cells in the respective phases.

2.6. Semiquantitative RT–PCR analysis

Total RNA was extracted by using the Tri reagent (Sigma) and semiquantitative RT-PCR analysis was performed using the TOP script™ one-step RT PCR kit (Enzynomics, Daejeon, Korea) following the manufacturer's protocol. Primer sequences were as follows: for X-IAP, sense 5′-ACACCATATACCCGAGGAAC-3′, antisense, 5′-CTTGCATACTGTCTTTCTGAGC-3′; for actin, sense 5′-GGTCATCTTCTCGCGGTTGGCCTTGGGGT-3′, antisense 5′-CCCCAGGCACCAGGGCGTGAT-3′. A total of 2 µg of total RNA was used in each reaction and was amplified within the exponential phase of the PCR (X-IAP, 32 cycles; actin, 30 cycles). Reactions were normalized by evaluating the level of amplification of the actin transcript. Amplified DNA was electrophoresed on 1.5% agarose gels and visualized by ethidium bromide staining.

2.7. Statistical analysis.

Data are presented as mean ± SD. Comparisons between treatment groups were made with the one-way analyses of variance followed by Mann-Whitney U test (SPSS 12.0). Values of $p < 0.05$ were considered statistically significant.

3. Results and Discussion

Cells treated with gallotannin (0.01-0.06 mM), c-PTIO (0.1-0.4 mM), 1400W (1-4 mM), Vinyl-L-NIO (1-4 mM) and NMA (1-4 mM) for 48 h responded with respect to viability, in that all treatments decreased the percentage of viable cells dose-dependently (Figure 1A). The median effective concentration EC_{50} of with gallotannin, c-PTIO, 1400W, Vinyl-L-NIO and NMA to cause cell growth inhibition was found to be 0.04, 0.19, 2.21, 2.34 and 4.03 mM, respectively, which indicates that endogenous NO• was implicated in increased colon cancer cell growth.

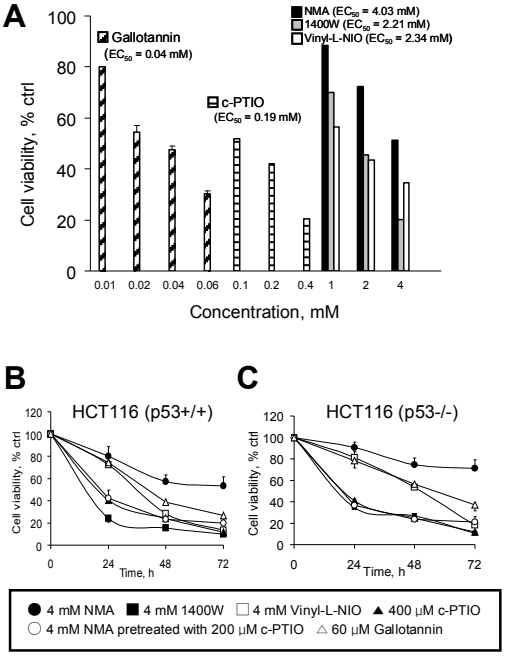


Fig. 1. Cell viability of two isogenic HCT116 cell lines after NOS inhibition by NMA, Vinyl-L-NIO, 1400W and Gallotannin, or NO• scavenging by c-PTIO. (A) The p53+/+ HCT116 cells were treated with the various concentrations of NO• modulators for 48 h. The p53+/+ (B) and p53-/- (C) HCT116 cells were incubated at indicated concentrations (*box*) of NO• modulators for 24, 48 and 72 h. Results are presented as a percentage of control cells (mean ± SD, n=3).

We then assessed the time-course of responses to doses of which showing below EC_{50}, with gallotannin, c-PTIO, 1400W, Vinyl-L-NIO and NMA, in HCT116 (p53+/+ and p53-/-) cells treated for 24, 48 and 72 h (Figures 1B and C). As expected, viability decreased in a time-dependent manner, and HCT116p53$^{-/-}$ cells were resistant to NO• modulators-induced cell death in comparison with HCT116 with wild-type p53, suggesting a role for the p53 status in cancer cell death induced by NO• modulators (Figures 1B and C). The tumor suppressor p53 is a sensor of diverse cellular stresses, which is the most commonly mutated gene in a broad spectrum of cancers, and its inactivation is frequently associated with tumor progression, resistance to therapy, and poor prognosis (Bargotti and Manfredi, 2002; Tang and Grimm, 2004).

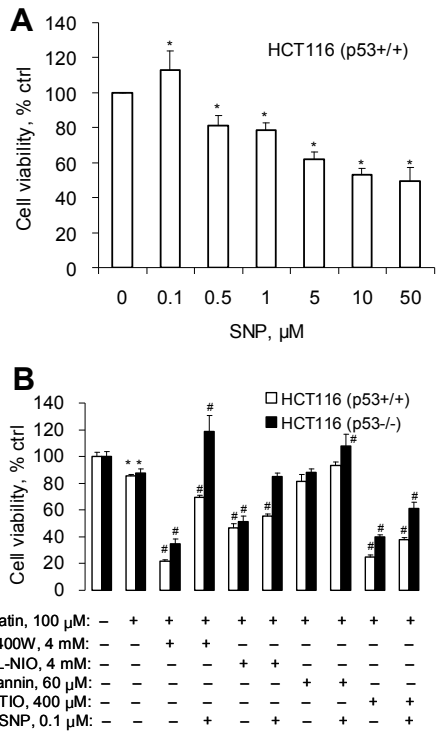


Fig. 2. Regulation of HCT116 cell growth by endogenous NO•. (A) HCT116 cells were treated with 0-50 µM of SNP (NO• donor) for 24 h, and cell viability was determined by MTT assay. (B) HCT116 cells (p53+/+ and p53-/-) were treated with 100 µM of cisplatin alone or combined with NO• modulators and/or 0.1 µM of SNP. Results are presented as a percentage of control cells (mean ± SD, n=3). $^{*}p < 0.05$ versus control; $^{\#}p < 0.05$ versus cisplatin.

Next, we evaluated whether endogenously produced NO• enhanced the proliferation of colon cancer cells. The NO• donor SNP was used to modulate NO• level in the cells for 24 h, after which cell viability was measured. The addition of 0.1 µM SNP led to an increase in cell growth ($p < 0.05$) (Figure 2A). Treatment of cells with more than 0.1 µM SNP caused a dose-dependent decrease in cell viability, suggesting that low levels of NO• enhances cell growth, whereas higher NO• concentrations lead to inhibition of growth ($p < 0.05$) (Figure 2A).

To assess whether endogenous NO• modulation regulates growth inhibition after cisplatin treatment, we incubated cells with cisplatin alone or in combination with 1400W, Vinyl-L-NIO, gallotannin and c-PTIO (Figure 2B). Cell viability significantly decreased by 85 and 87% after cisplatin treatment in HCT116 (p53+/+ and p53-/-) cells,

respectively ($p < 0.05$). When cells were co-incubated with both cisplatin and NO• modulators, viability significantly decreased by 4- and 2.5-folds over that which was induced by cisplatin alone in HCT116 (p53+/+ and p53-/-) cells, respectively ($p < 0.05$), suggesting an additive or synergistic effect between cisplatin treatment and endogenous NO• depletion. The concomitant addition of SNP, a NO• donor, with NO• modulators caused cell re-growth, which indicates that the antiproliferative effect was specific to endogenous NO• depletion (Figure 2B).

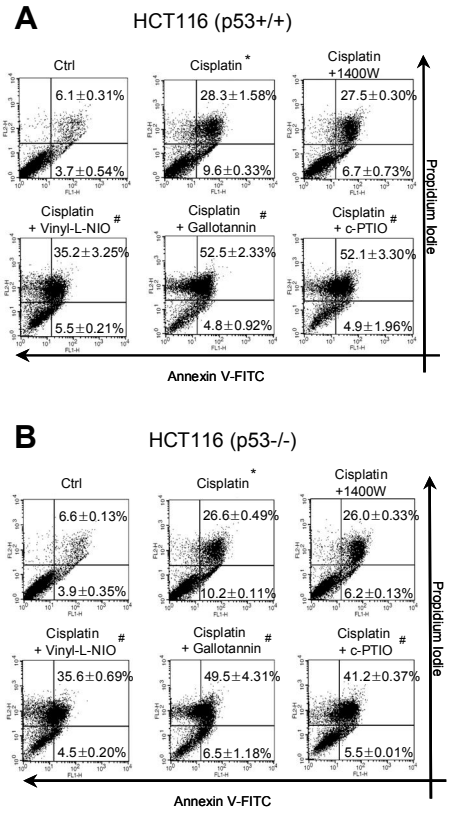


Fig. 3. Effects of NO• modulators on cellular nitrite level by cisplatin treated for 48 h in HCT116 cells (p53+/+ and p53-/-). Results are presented as a percentage of control cells (mean ± SD, n=3). $^{*}p < 0.05$ versus control; $^{\#}p < 0.05$ versus cisplatin.

To confirm that antiproliferative effects of NO• modulators on cisplatin, we measured nitrite levels in HCT116 (p53+/+ and p53-/-) cells (Figure 3). Cisplatin (100 µM, 48 h) significantly decreased nitrite generation by around 85%, as compared with controls, and the increases were further enhanced by combination treatment with NO• modulators in HCT116 cells ($p < 0.05$) (Figure 3). HCT116p53-/- cells produced nitrite at higher concentrations than HCT116p53+/+ cells (Figure 3); for example, the concentration in HCT116p53-/- cells (5 pmoles/10^7 cells) was 1.6 times that of HCT116p53+/+ cells (3 pmoles/10^7 cells) after 48 h of co-treatment with cisplatin and 1400W (Figure 3).

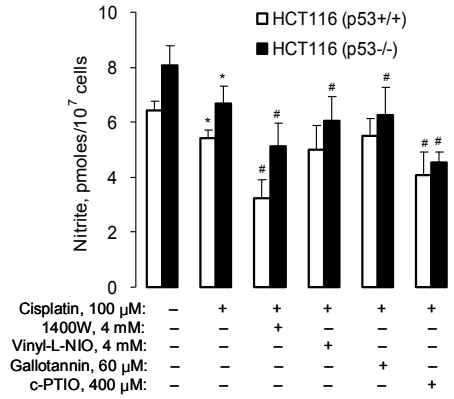
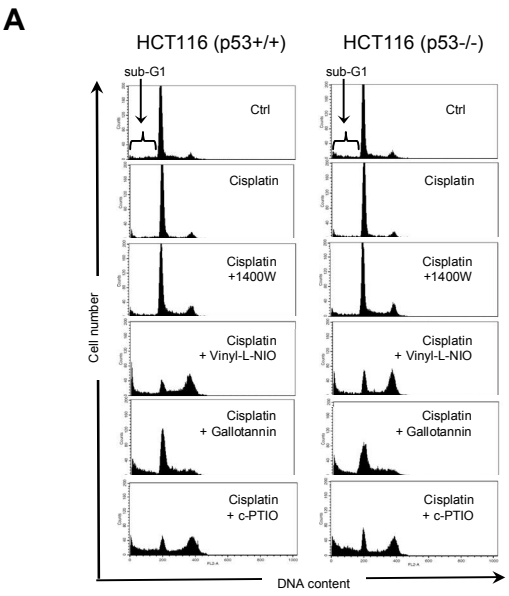


Fig. 4. Effect of NO• modulators on apoptosis induced by cisplatin treated for 48 h in HCT116 cells (p53+/+ and p53-/-). The results are shown as the mean ± SD (n=3). $^{*}p < 0.05$ versus control; $^{\#}p < 0.05$ versus cisplatin.

Figure 4 shows that apoptosis (by FACS analysis after staining with FITC-conjugated Annexin-V and propidium iodide) induced by cisplatin alone or in combination with 1400W, Vinyl-L-NIO, gallotannin and c-PTIO in HCT116 (p53+/+ and p53-/-) cells. Approximately 37.9 and 36.8% of HCT116 (p53+/+ and p53-/-) cells (4- and 3.5-fold over control level), respectively, were apoptotic after cisplatin treatment and co-treatment with NO• modulators was more frequent than cisplatin alone ($p < 0.05$) (Figure 4). A stronger apoptotic response was induced in HCT116p53+/+ cells than in HCT116p53-/- cells (Figure 4).

Similar results were obtained in other cancer cell lines with different p53 status (Figure 5). By FACS analysis of cell DNA content, there was a remarkable accumulation of subploid cells, sub-G1 peak, in HCT116 (p53+/+ and p53-/-) cells after treatment with cisplatin alone or in combination with NO• modulators for 48 h when compared with the untreated group (Figure 5A). Since sub-G1 peaks include early and late apoptotic cells, but also a part of necrotic cells, such large sub-G1 fractions provide strong evidence for cytotoxicity induced by with cisplatin alone or in combination with NO• modulators resulting in the decrease of the number of viable cells. Furthermore, the stage at which growth inhibition induced by with cisplatin alone or in combination with NO• modulators occurs in the HCT116 (p53+/+ and p53-/-) cell cycle progression was determined, with cellular distribution in the different phases the treatment.



B

	Apoptotic cells (sub-G1)	Non-apoptotic cells		
		G0/G1	S	G2/M
HCT116 (p53+/+)				
Ctrl	8.3 ± 0.48	74.4 ± 1.10	9.4 ± 1.03	7.4 ± 0.49
Cisplatin	10.1 ± 0.11	74.0 ± 0.49	6.7 ± 0.16	8.7 ± 0.20
+ 1400W	11.3 ± 0.49	59.9 ± 1.23*	11.9 ± 2.28	16.2 ± 0.54*
+ Vinyl-L-NIO	22.2 ± 0.69*	16.8 ± 0.51*	18.6 ± 0.47*	42.0 ± 0.57*
+ Gallotannin	26.4 ± 1.47*	43.3 ± 1.40*	18.8 ± 0.67*	10.9 ± 0.61
+ c-PTIO	30.8 ± 1.24*	18.0 ± 1.49*	14.3 ± 0.54	35.7 ± 2.26*
HCT116 (p53-/-)				
Ctrl	9.5 ± 0.74	73.4 ± 0.01	8.8 ± 0.51	7.9 ± 0.08
Cisplatin	10.7 ± 1.65	72.4 ± 1.32*	7.7 ± 0.05	8.8 ± 0.48
+ 1400W	10.5 ± 0.25	62.4 ± 1.39	11.8 ± 0.89	14.7 ± 0.25*
+ Vinyl-L-NIO	24.5 ± 1.71*	23.4 ± 0.45*	14.6 ± 0.83*	36.9 ± 1.67*
+ Gallotannin	27.8 ± 1.00*	43.8 ± 2.69*	19.0 ± 0.79*	9.0 ± 0.99
+ c-PTIO	37.8 ± 1.44*	20.1 ± 1.30*	12.0 ± 0.16	28.9 ± 1.69*

Fig. 5. Effect of NO• modulators on cisplatin-induced cell cycle distribution in HCT116 cells (p53+/+ and p53-/-). (A) Cells were treated with cisplatin and/or NO• modulators for 48 h, stained with PI, and analyzed for sub-G1 and cell cycle using flow cytometry. Representative flow cytometry patterns are shown. (B) Cellular distribution (as percentage) in different phases of the cell cycle (sub-G1, G0/G1, S and G2/M) after treatment with cisplatin and/or NO• modulators is shown. Apoptotic nuclei were identified as a subploid DNA peak and distinguished from cell debris on the basis of forward light scatter and PI fluorescence. Results are presented as mean ± SD of three assays. $^{*}p < 0.05$ versus control; $^{\#}p < 0.05$ versus cisplatin.

Figure 5B shows that co-exposure of cisplatin and NO• modulators resulted in a progressive and sustained accumulation of cells in the S and G2/M phases. Further, the percentage of S and G2/M phases cells increased, while those in the G1 phase decreased after co-treatment with A cisplatin and NO• modulators ($p < 0.05$), suggesting that they promotes cell growth inhibition by inducing S and G2/M phase arrests in colon cancer cells (Figure 5B).

X-linked inhibitor of apoptosis (X-IAP) is a potent endogenous inhibitor of apoptosis. It is expressed at relatively low levels in most normal cells, whereas in many cancers including colorectal cancer, it is generally over-expressed (Holcik et al., 2001; Tong et al., 2005; Qiao et al., 2009). Down-regulation of XIAP leads to a sensitization of cancer cells to apoptosis (Inoue et al., 2004; Marienfeld et al., 2004; Qiao et al., 2009). To further confirm that endogenous NO• depletion enhanced cisplatin induced apoptosis in colon cancer cells, we carried out semi-quantitative RT-PCR of X-IAP. Cisplatin treatment resulted in a reduction in expression of X-IAP and it was less expressed when co-treated with NO• modulators in both HCT116p53+/+ cells and HCT116p53-/- cells (Figure 6). The expression of X-IAP in HCT116p53+/+ cells is much lower than in HCT116p53-/- cells (Figure 6B), suggesting the high expression of X-IAP in HCT116p53-/- cells may act as a contributing factor to cisplatin resistance by co-treatment with NO• modulators.

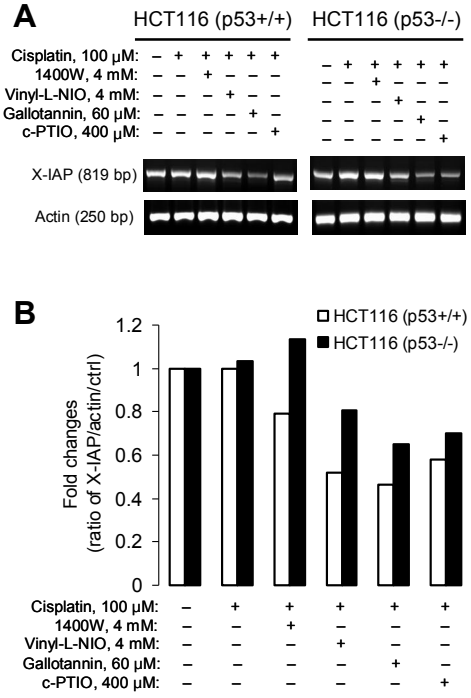


Fig. 6. RNA expression pattern of X-IAP after treatment with cisplatin and/or NO• modulators for 48 h. (A) Semi-quantitative PCR was performed using primer specific to X-IAP or a actin control on 1 μg total RNA prepared from cells treated with cisplatin and/or NO• modulators for 48 h. (B) Band intensities were calculated by densitometric analysis and normalized to actin levels.

Taken together, our results indicate that the depletion of endogenous NO• in melanoma cell lines leads to increased sensitivity to cisplatin, suggesting that NO• is required for p53 activation, which acts as a protective factor against apoptosis in colon cancer cells. Further research will be required to define more specifically the mechanisms through which they act.

Corresponding Author:
Dr. Min Young Kim
Laboratory of Pharmacology and Toxicology
Faculty of Biotechnology
College of Applied Life Sciences
Jeju National University
Jeju 690-756, Republic of Korea
E-mail: jeffmkim@jejunu.ac.kr

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