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An approach to enhancing the phototoxicity of a novel hypocrellin congener to MGC803 cells

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Abstract

A novel photosensitizer, 2-butylamino-2-demethoxy-hypocrellin A (2-BA-2-DMHA) has been found to cause high phototoxicity to MGC803 cells mainly via apoptosis. To increase the efficiency of 2-BA-2-DMHA-mediated photodynamic therapy (PDT) to MGC803 cells, we transferred the retrovirus-mediated wild-type p53 gene to MGC803 cells. As a result, we found that wild-type p53 infected MGC803 cells expressed exogenous p53 mRNA and a high level of p53 protein. Overexpression of the gene made the cells more sensitive to 2-BA-2-DMHA-mediated PDT. At a concentration of 0.25 μM 2-BA-2-DMHA with a red light of 10 J cm^{-2} ($\lambda = 600$–700 nm), the wild-type p53 infected MGC803 cell survival was 4 times lower than that associated with the parent and the empty vector infected MGC 803 cell survival. In addition, apoptosis appeared earlier in wild-type p53 infected MGC803 cells.

Keywords: MGC803 cells; Hypocrellin congener; Photodynamic therapy; Photosensitizer

1. Introduction

It is known that photodynamic therapy (PDT) is a promising cancer-treatment technology [1]. PDT combines the best of radiotherapy and chemotherapy, has low systemic toxicity, and is repeatable. However, Photofrin® is the only photosensitizer that has been approved by the US FDA so far for PDT [2]. To overcome its shortcomings such as a complex chemical composition, low extinction coefficient at 630 nm and prolonged cutaneous phototoxicity [3], scientists have been searching for more effective compounds for PDT. In this regard, hypocrellins, owing to their ability to produce high quantum yields of singlet oxygen and side-directed chemical modification, have been selected as potential photosensitizers for PDT [4–7].

Many derivatives of the parent hypocrellins A and B (HA and HB; Fig. 1) have been synthesized, and their photophysical properties, mechanism of action [8,9] and biological properties [10–13] have been studied. Among them, 2-butylamino-2-demethoxy-hypocrellin A (2-BA-2-DMHA) (Fig. 1), which exhibits stronger red-light absorption than

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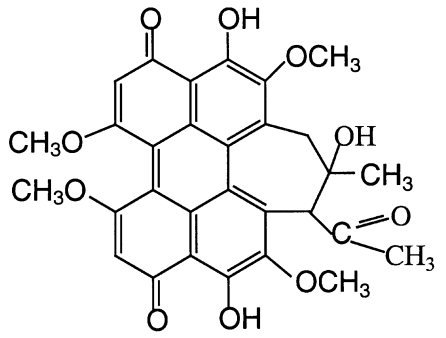
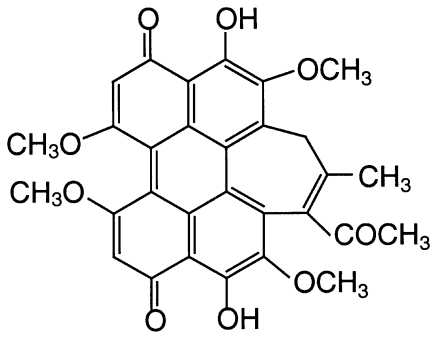


HA



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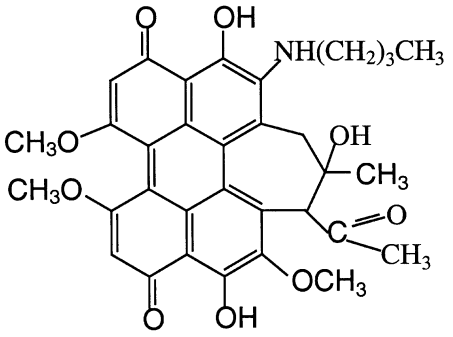

2-BA-2-DMHA

Fig. 1. The chemical structures of HA, FHB and 2-BA-2-DMHA.

its parent compound HA, has been shown to have a 50-fold higher photopotentiation factor than HA at a dose of 4 J cm^{-2} of red light on human gastric adenocarcinoma MGC803 cells [13]. The study also showed that its phototoxicity to MGC803 cells mainly proceeded via apoptosis [13]. These results suggest that 2-BA-2-DMHA is a potential photosensitizer.

With an increasing number of cancer patients receiving PDT worldwide, increasing the sensitivity of malignant cells to the effects of PDT has the potential to significantly improve the cure rate of many malignancies. Fisher and co-workers found that the introduction of the wild-type p53 gene made HL60 cells more sensitive to Photofrin®-mediated PDT [14]. The p53 gene is a critical tumor suppressor gene and HL60 cells are human promyelocytic leukemia cells. The p53 gene normally controls cell proliferation by causing cell-cycle arrest and apoptosis [15] and when it is mutated the control function is lost. The results of several studies have shown that transfection with wild-type p53 can directly drive cells into apoptosis and/or growth arrest when p53 is over-expressed, suggesting a gene therapy approach for tumor cells lacking normal p53 function [16–20]. Therefore, an approach to increasing the phototoxicity of 2-BA-2-DMHA to MGC803 cells, we transferred wild-type p53 gene to MGC803 cells and examined the transfected cells' sensitivity to 2-BA-2-DMHA-mediated PDT.

2. Experimental

2.1. Chemicals

2-BA-2-DMHA (2-butylamino-2-demethoxy-hypocrellin A) was synthesized and purified using a published method [13]. Geneticin (G418), polybrene, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) were obtained from Sigma (St. Louis, MO, USA). Lipofectin reagent, RPMI-1640 medium, Tris(hydroxymethyl) aminomethane (Tris), fetal bovine serum (FBS), ethidium bromide (EB) and phenylmethylsulfonyl fluoride (PMSF) were purchased from Gibco-BRL (Grand Island, NY, USA). Sodium dodecyl sulfate (SDS) was obtained from Merk and Company (Rahway, NJ, USA).

2.2. Cell culture

Human gastric adenocarcinoma cells, MGC803 [21], packaging cell line, PA317 and NIH3T3 cells were cultured in RPMI-1640 medium containing 5% FBS at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. The cells were maintained at exponential growth in monolayer cultures with regular medium changes.

2.3. Retrovirus vectors harboring p53 gene and p53-expressed MGC803 cells

A human 1.8 kb p53 cDNA fragment from pC53-SN3 was subcloned into the BamHI site of retrovirus vector, pLXSN [22]. The resultant revirus vector was termed pLXp53N. As a control, the retrovirus vector, pLXSN itself (without p53 cDNA insert) was designated as pLXNeoN. The DNAs extracted from plasmids pLXp53N and pLXNeoN were transfected into PA317 packaging cells using lipofectin reagents according to the manufacturer's instructions. After selection with 600 μg ml^{-1} G418, the drug-resistant PA317 cells were cloned. The viral titer of the supernatant was tested by infecting NIH3T3 cells. The cell-free culture supernatants of G418-resistant PA317 cells were obtained as retrovirus stocks. MGC803 cells were incubated with the culture supernatants in the presence of 8 μg ml^{-1} polybrene (Sigma) and one-half of the medium was replaced daily for 3 days. The cells were washed and maintained with medium containing serum for a day and then the drug selection with 400 μg ml^{-1} G418 was started. G418-resistant clones were examined for p53 expression with reverse transcription polymerase chain reaction (RT-PCR) and western blot.

The parental MGC803 cells were designated as MGC803-S. Transfectant MGC803 cells expressing exogenous wild-type p53 were designated as MGC803-p53. MGC803 cells transfected with the empty vector LXNeoN were designated as MGC803-Neo.

2.4. RT-PCR analysis

Total RNA was isolated from the three cell lines using the acid–phenol–guanidinium thiocyanate procedure [23] and used as templates. Reverse transcription was performed at 42 °C for 60 min and at 95 °C for 5 min using 1.0 μg of RNA per reaction. PCR was performed in a volume of 50 μl. The specific primers used for the packaging signal region/p53 exon 9 of LXp53N were a 5′ primer (5′-TATCCAGCCCTCACTCCTTC-3) and a 3′ primer (5′-CACGGATCTGAAGGGT-GAAA-3′). The primers used for GAPDH were GAPDH-S (5′-CGGAGTCAACGGATTTGGTG GTAT-3′) and GAPDH-AS (5′-AGCCTTCTC-CATGGTGGTGAAGAC-3′). The amplification reaction involved denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min, and elongation at 72 °C for 2 min for 30 cycles. PCR products were resolved on 1% agarose gels and visualized by ethidium bromide staining.

2.5. Western blot analysis

Cells were trypsinized, pelleted, rinsed with ice-cold phosphate-buffered saline (PBS) and suspended in 1×SDS polyacrylamide gel electrophoresis buffer (Tris–EDTA buffer containing 2% SDS, 10% glycerol and 100 μg ml^{-1} PMSF) and boiled for 10 min. Equal amounts of the proteins (80 μg) were electrophoresed and transferred to a nitrocellulose filter (Bio-Rad). The levels of p53 expression were measured using p53 (DO-1) mouse monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

2.6. Photosensitizer uptake

The cellular uptake of photosensitizer was performed by modification of a previously reported procedure [24]. Cells incubated with 2-BA-2-DMHA for 4 h were rinsed three times with PBS to completely remove excess dye and then detached, counted and centrifuged. The photosensitizer was extracted from cells by dissolving in 2.5 ml DMSO. After centrifugation (1500 *g*, 15 min), fluorescence in the supernatant was measured using a Hitachi 850 fluorescence spectrophotometer (Japan) at an excitation wavelength of 335 nm and an emission wavelength of 400 nm. The fluorescence peaks were recorded and then the relative fluorescence intensity was calculated. The experiments were repeated four times.

2.7. Cell treatment

Cells were incubated with 2-BA-2-DMHA and irradiated by red light using the method described previously [13]. In this regard, cells incubated with 2-BA-2-DMHA in RPMI-1640 medium (Gibco; without phenol red and serum) were trypsinized, washed and resuspended in the same medium at a concentration of 3×10^5 cells ml^{-1}. At that point, the cells were exposed to different doses of red light (more than 90% at 600–700 mm) at a power output of 33 mW cm^{-2}.

Cell survival was measured 20 h after photosensitization using the MTT assay [13]. In summary, control and treated cells were transferred into flat-bottomed 96-well plates (1×10^4 cells ml^{-1}) and MTT (in PBS) was added to give a final concentration of 1 mg ml^{-1}. The cells were incubated at 37 °C for 4 h and then cleavage of MTT (proportional to number of viable cells) was determined by absorbance measurements at 595 nm using a Bio-Rad model 3550 microplate reader (Richmond, CA, USA). Samples were evaluated in 12 replicates and each experiment was repeated at least twice. Data for the survival of PDT-treated cells were normalized against cells incubated with photosensitizer alone.

The toxicity of 2-BA-2-DMHA in the absence of light was assessed separately, following a similar procedure for cell exposure to graded doses of the dye for 4 h. Precautions were taken to avoid exposure of the cells to light including red light.

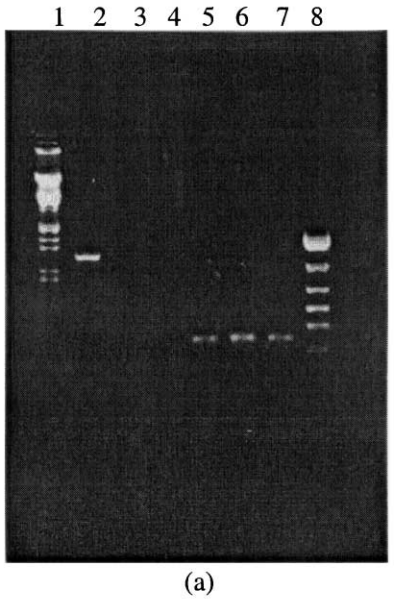

Fig. 2. (a) Reverse-transcription PCR analysis. Lane 1, marker (λDNA + Hind III + EcoR I); lanes 2–4, exogenous p53 mRNA expression in MGC803-p53, MGC803-S, MGC803-Neo cell lines, respectively; lanes 5–7, GAPDH mRNA expression in the three cell lines; lane 8, PCR marker (1543, 994, 697, 515, 377 and 237 bp). Note the exogenous p53 mRNA expression only in MGC803-p53 cells. (b) Analysis by Western blot of p53 expression in cell lysates using the DO-I mouse antihuman p53 monoclonal antibody. Lane 1, MGC803-S cells; lane 2, MGC803-Neo cells; lane 3, MGC803-p53 cells.

2.8. Assay of apoptosis

Apoptosis was detected by the presence of internucleosomal fragmentation of DNA on gel electrophoresis. Cells were collected at the 1, 3, and 12 h stages after photosensitization, and DNA was isolated and analyzed by a method described previously [13].

3. Results

3.1. Retrovirus-mediated p53 mRNA and protein expression

RT-PCR analysis using a sense primer for packaging signal region (PSR) near multiple cloning site of LXp53N and an antisense primer located within p53 extron 9 was performed to examine whether the transduced p53 gene was expressed in MGC803 cells. A PSR/p53 segment was detected in MGC803 cells transduced with the wild-type

p53 gene, whereas it was not present in MGC803-S and MGC803-Neo cells (Fig. 2a). Western blot evaluation revealed that the expression of p53 protein in MGC803-p53 cells was much higher than that in MGC803-S and MGC803-Neo cells (Fig. 2b). These results suggested that the retrovirus-mediated gene transfer and expression were highly efficient in human gastric cancer cells.

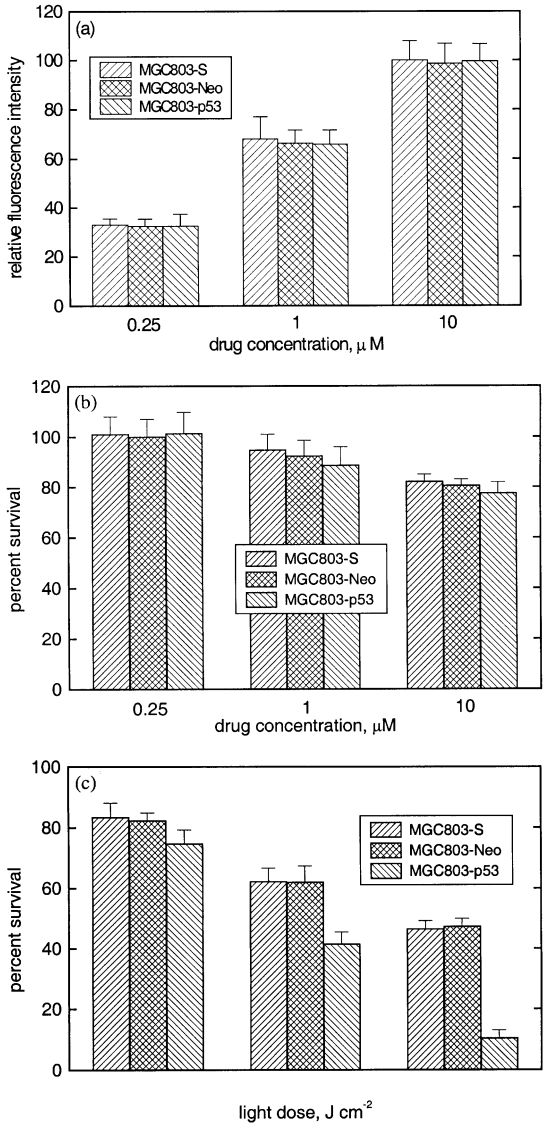

Fig. 3. (a) Uptake of 2-BA-2-DMHA in the cell lines (each bar represents the mean±standard error of four separate experiments); (b) and (c) cytotoxicity and phototoxicity (0.25 μM 2-BA-2-DMHA plus different doses of light) of 2-BA-2-DMHA (the data plotted are the mean±standard error of at least two experiments).

3.2. Effect of wild-type p53 on 2-BA-2-DMHA phototoxicity

The results summarised in Fig. 3a show that uptake of the photosensitizer (2-BA-2MHA) was equivalent for the three cell lines, while the results in Fig. 3b show that the cytotoxicity of this dye to the three cell lines in the absence of light was similar at the same dye concentrations. Survival curves for the three MGC803 cell lines exposed to 2-BA-2-DMHA photosensitization are shown in Fig. 3c. The MTT measurements demonstrated that MGC803-p53 cells were significantly more sensitive to the treatment than MGC803-S and MGC803-Neo cells. At a dye concentration of 0.25 μM and a dose of 10 J cm^{-2} red light, the phototoxicity of 2-BA-2-DMHA to MGC803-p53 cells was about 4 times higher than that to MGC803-S and MGC803-Neo cells.

3.3. DNA fragmentation

Internucleosome DNA fragmentation, a typical characteristic of apoptosis, was detected in MGC803-p53 cells as early as at 1–3 h following 0.25 μM 2-BA-2-DMHA plus 10 J cm^{-2} red light and increased with the incubation time (Fig. 4, lanes 9–11). However, at the same PDT dose, typical DNA ladder fragmentation was observed for up to 12 h after photosensitization in the other two cell lines. This pattern was not observed when cells were exposed to either photosensitizer alone or red light alone. The induction of apoptosis following PDT in MGC803-S cells was the same as that in MGC803-Neo cells (data not shown).

4. Discussion

The results of this study have demonstrated that retrovirus vector can effectively mediate wild-type p53 gene transfer and expression in MGC803 cells and that overexpression of the gene resulted in an enhanced sensitivity of the cells to 2-BA-2-DMHA-mediated photosensitization. It is well known that

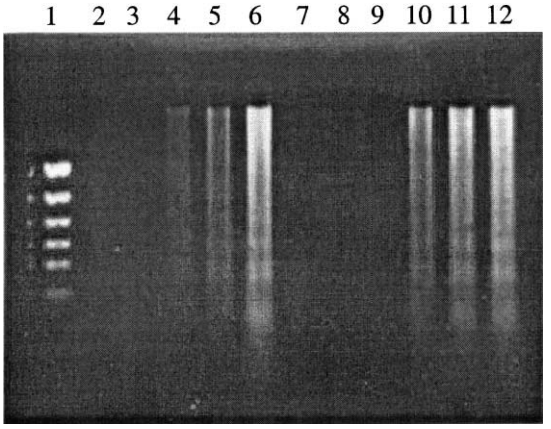

Fig. 4. Gel electrophoresis analysis of DNA isolated from 13 000 g supernatants of MGC803-Neo (lanes 2–6) and MGC803-p53 (lanes 7–12) cells. Lane 1, PCR marker (1543, 994, 697, 515, 377 and 237 bp); lanes 2 and 8, light only (10 J cm^{-2}); lanes 3 and 9, 2-BA-2-DMHA incubation alone (0.25 μM, 1 h); lanes 4–6 and 10–12, fragmented DNA isolated at 1, 3 and 12 h after MGC803-Neo and MGC803-p53 cells were treated with 0.25 μM 2-BA-2-DMHA plus 10 J cm^{-2} red light; lane 7, control (serum-free for 1 h, no dye, no light).

one of the most rapidly developing strategies for cancer gene therapy involves restoring the function of the wild-type p53 tumor suppressor gene in tumors using gene transfer approaches [25]. The use of recombinant retrovirus vectors is attractive for this purpose because of their capacity for highly efficient infection, long-term stable gene expression and non-toxic integration into the genome of a wide range of cell types [26]. Many studies have shown that transfection with the wild-type p53 gene can sensitize cancer cells to chemotherapy [18,27–29] and radiotherapy [30–32]. However, there are also results indicating that there is no direct or inverse correlation between p53 status and tumor responsiveness to chemotherapy [33] or radiation [34,35]. Therefore, whether transfection of p53 gene can sensitize cancer cells to the treatment may depend on both the specific cell type and therapy. In this study, the delivery of exogenous wild-type p53 to MGC803 cells increased its sensitivity to 2-BA-2-DMHA PDT may be associated with the combinations of the cells and the photosensitizer employed.

To examine the molecular mechanism responsible for increased photosensitivity in MGC803 cells infected with wild-type p53, we performed DNA fragmentation assay and observed that apoptosis in MGC803-p53 cells occurred much earlier than in the other two cell lines. This indicated that expression of the exogenous wild-type p53 gene potentiated the induction of apoptosis in the cells, and suggested that the introduction of wild-type p53 and potentiating the induction of apoptosis may be an effective strategy to enhance the effects of PDT.

A remaining question is—why does wild-type p53 gene increase photosensitivity by potentiating apoptosis? The mechanism for increased p53 photosensitization may be related to the induction of reactive oxygen species (ROS). It is known that PDT-mediated ROS is the main cause of cell death [1,2,14] and that ROS is a powerful inducer of apoptosis [36]. The results of two studies have suggested that ROS may mediate p53-dependent apoptosis [37,38]. Both studies employed adenovirus-mediated gene transfer of wild-type p53. Overexpression of p53 resulted in a significant increase in ROS levels. On the other hand, p53-mediated apoptosis was inhibited by an antioxidant. In the present study, it is possible MGC803 cells infected with wild-type p53 maintained a high ROS level from an overexpression of the exogenous p53 gene. Thus following PDT, the MGC803-p53 cells reached the threshold of apoptosis [39] more quickly than MGC803-S and MGC803-Neo cells and then became more sensitive to the treatment. Although the precise mechanisms associated with increased p53 photosensitivity requires further investigation, our results provide further support for the use of the p53 gene in combination with PDT in the clinical cancer treatments.

5. Conclusions

Retrovirus vector can effectively transfer and express p53 protein in MGC803 cells and an overexpression of the gene enhances the photosensitivity of 2-butylamino-2-demethoxy-hypocrellin A-based PDT by strongly potentiating apoptosis induction in the cells. Therefore, gene therapy using retrovirus-mediated wild-type p53 gene transfer and expression in combination with

PDT may provide a more effective treatment for human gastric cancer.

Acknowledgements

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