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p53-Reactivating small molecules induce apoptosis and enhance chemotherapeutic cytotoxicity in head and neck squamous cell carcinoma

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SUMMARY

We evaluate whether p53-reactivating (p53RA) small molecules induce p53-dependent apoptosis in head and neck squamous cell carcinoma (HNSCC), a question that has not been previously addressed in head and neck cancer. PRIMA-1, CP-31398, RITA, and nutlin-3 were tested in four human HNSCC cell lines differing in *TP53* status. Cell growth, viability, cell cycle progression, and apoptosis after treatment with p53RA small molecules individually or in combination with chemotherapeutic agents were assessed. Prominent p53 reactivation was observed in mutant *TP53*-bearing tumor cell lines treated with PRIMA-1 or CP-31398, and in wild-type *TP53*-bearing cell lines treated with nutlin-3. Cell-cycle arrest and apoptosis induced by p53RA small molecules were associated with upregulation of p21 and BAX, and cleavage of caspase-3. Nutlin-3 showed maximal growth suppression in tumor cells showing MDM2-dependent p53 degradation. High-dose treatment with p53RA small molecules also induced apoptosis in cell lines independent of p53 or MDM2 expression. In combination therapy, p53RA small molecules enhanced the antitumor activity of cisplatin, 5-fluorouracil, paclitaxel, and erlotinib against HNSCC cells. The p53RA small molecules effectively restored p53 tumor suppressive function in HNSCCs with mutant or wild-type *TP53*. The p53RA agents may be clinically useful against HNSCC, in combination with chemotherapeutic drugs.

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Keywords

p53; Reactivation; Head and neck squamous cell carcinoma; Apoptosis; Small molecules

Introduction

The p53 tumor suppressor serves as a key guardian of the genome by protecting cells from malignant transformation.¹ Upon exposure to cellular stress, p53 is stabilized and induces cell-cycle arrest or programmed cell death (apoptosis).^{2,3} Many of these effects reflect the transactivation of a number of genes by p53, acting as a transcription factor, but p53 also activates mitochondrial-dependent apoptotic pathways that are independent of p53 transcriptional activity.⁴ Alterations in the p53 gene, mostly missense mutations, are found in approximately half of all human cancers, and other factors that cause dysregulation of the p53 pathway are seen in most remaining malignancies.⁵ *TP53* mutations may be associated with an aggressive phenotype and poor prognosis, and some p53 mutants counteract the effects of anticancer agents that attack tumors.^{6,7} The tumor-suppressive function of wild-type p53 can be also inferred from the observations that spontaneous cancers occur at a young age in *TP53* knockout mice and in patients with Li-Fraumeni syndrome, characterized by *TP53* germline mutations.^{8,9}

The high prevalence of p53 pathway inactivation in human malignancies has led to the development of therapeutic strategies based on restoring wild-type p53 function. Because sustained p53 inactivation is required for the maintenance of the aggressive tumor phenotype, restoration of p53 function leads to senescence and tumor regression.^{10,11} In both experimental and clinical trials, reconstitution of wild-type p53 function through gene therapy or p53-targeting small molecules has been shown to inhibit tumor growth.^{12,13} CP-31398, PRIMA-1, MIRA-1, and ellipticine restore the transcriptional transactivation function of p53 and induce cell death preferentially in mutant *TP53*-carrying tumors.^{12,14,15} RITA, nutlins, and HLI98 restore the tumor-suppressive function of p53 by inhibiting MDM2-mediated p53 degradation in wild-type *TP53*-bearing tumors.^{12,16,17} Pharmacologic restoration of the p53 pathway induces cell-cycle arrest and massive apoptosis of tumors without causing adverse effects on normal cells. Thus, reconstitution of the p53 pathway is becoming one of the most exciting novel therapeutic strategies against cancer.

p53-reactivating (p53RA) small molecules have been tested in patients with some types of cancers, but not in those with head-and-neck squamous cell carcinoma (HNSCC).^{12,14–17} HNSCC is one of the most common human cancers; being diagnosed in more than half-a-million patients worldwide each year, and the overall cure and survival rates of such patients have not substantially changed over the last three decades.¹⁸ In addition to playing a role in tumorigenesis, alterations in tumor suppressor genes or signaling pathways may also be associated with therapeutic resistance,^{6,19} which contributes to the failure of conventional chemotherapy. Thus, use of p53RA agents in HNSCC patients may yield improvements in therapeutic efficacy compared to conventional treatments. Accordingly, the aim of this study was to investigate whether p53RA small molecules induce cell-cycle arrest and apoptosis in human HNSCC cell lines, and to examine the therapeutic efficacy of these small molecules in HNSCC cells when they were combined with currently used chemotherapeutic agents.

Materials and methods

Cell culture and reagents

Four human HNSCC cell lines, JHU-O28, JHU-O29, UMSCC-22A and Fadu, were used in this study. The cell lines were maintained in RPMI 1640, DMEM, or MEM supplemented

with 10% fetal bovine serum at 37°C in a humidified atmosphere containing 5% CO₂. Human oral keratinocytes (OKF6/TERT1), immortalized by forced expression of telomerase but resembling the characteristics of primary oral keratinocyte,²⁰ were cultivated in keratinocyte serum-free medium (Gibco/Invitrogen) supplemented with bovine pituitary extract (25 µg/mL), recombinant epidermal growth factor (2.5 ng/mL) and calcium chloride (0.4 mM). Human dermal fibroblasts (HDF) were cultivated in medium 106 (Gibco/Invitrogen) supplemented with low serum growth supplement (Gibco/Invitrogen).

Cells were treated by adding the small molecules PRIMA-1 (Cayman Chemical), CP-31398 (Tocris Bioscience), nutlin-3 or RITA (Cayman) directly to cell culture media. p53RAs were dissolved in DMSO and stored at -20°C prior to use. The cells were also treated with the chemotherapeutic agents *cis*-platinum (II) diamine dichloride (CDDP; Sigma-Aldrich), 2,4-dihydroxy-5-fluoropyrimidine (5-FU; Sigma), paclitaxel (Sigma), or erlotinib hydrochloride salt (LC Laboratories) alone or in combination with small molecules. Controls were treated with an equivalent amount of DMSO.

Direct sequencing of TP53 mutations

Genomic DNA was extracted from untreated cells and DNA sequences within exons 2–11 of the *TP53* gene were amplified by polymerase chain reaction (PCR) according to a protocol currently used at the International Agency for Research on Cancer (IARC) (<http://www-p53.iarc.fr/>). The resulting PCR products were sequenced by the Genewiz DNA Sequencing Service Center. The locations and types of mutation were determined and confirmed by a second PCR reaction followed by resequencing.

MTT chemosensitivity and cell viability assays

The cell lines were seeded at $3\text{--}5 \times 10^3$ cells/well in 96-well plates, incubated overnight, and then treated with different concentrations of p53RA small molecules and/or chemotherapeutic agents. At 96 h, 10 µL of MTT reagent (3-[4,5-dimethyl-2-thiazolyl]-2,5-diphenyl-2H-tetrazolium bromide) was added to each well. After a 4-h incubation, 150 µL of solubilization buffer was added, and cells were incubated at 37°C in the dark for 2 h. The absorbance in each well was measured at 570 nm in a SpectraMax M2 microplate reader (Molecular Devices). The concentration of added agent that induced a 50% reduction in absorbance relative to controls was defined as the 50% inhibitory dose (ID₅₀). Cell viability was examined using trypan blue exclusion, and cell counts were repeated in triplicate.

Cell cycle and apoptosis assays

The cells were cultured in the presence of 2.5–10 µM p53RA small molecules, 1 µM cisplatin, or an equivalent amount of DMSO (control). After 24–48 h, the cells were harvested, washed with PBS, fixed overnight in ice-cold ethanol, and stained for 30 min with propidium iodide solution (Sigma) at 37°C. DNA content was measured using a FACSCalibur flow cytometer (BD Bioscience). For apoptosis assays, cells treated for 48 h were harvested and washed in ice-cold PBS, resuspended in binding buffer, and stained sequentially with Annexin V-FITC and propidium iodide using an Annexin V-FITC apoptosis detection kit (BD Bioscience), according to the manufacturer's instructions. Data were analyzed using Cell Quest Software (BD Bioscience).

Western blot analysis

Cells were lysed with radioimmunoprecipitation assay (RIPA) buffer (Upstate Biotechnology). A total of 50 µg protein was loaded onto 4%–12% NuPAGE® Novex® Bis-Tris precast gels (Invitrogen), transferred to nitrocellulose polyvinylidene difluoride membranes (Amersham Pharmacia), and immunoblotted with primary antibodies. The

antibodies used were anti-p53 Ab-5 (DO-7), anti-p21^{WAF1} Ab-11 (NeoMarkers); anti-MDM2 2A10 (Calbiochem); anti-Bax (Santa Cruz Biotechnology); anti-Bcl-xL, anti-Ser46-phospho-p53, anti-cleaved caspase-3 (Cell Signaling Technology); and anti- β -actin (Sigma).

Real-time quantitative reverse transcription-PCR

Cells were treated with p53RA small molecules and harvested after 24 h. Total RNA was extracted from cells using QIAzol lysis reagent and an RNeasy Mini kit (Qiagen). cDNA was synthesized using a QuantiTect[®] Reverse Transcription kit (Qiagen), according to the manufacturer's instructions. Real-time RT-PCR was performed using SYBR Green Mix (Qiagen) in a 7900HT Fast Real-time PCR System (Applied Biosystems). p53, p21, MDM2, Bax, PUMA, NOXA and GAPDH mRNA were amplified using previously described primers.²¹ Relative target mRNA levels were determined using the $2^{-(\Delta C_t)}$ method, and were expressed as the ratio to GAPDH Mrna.^{21,22}

Clonogenic assay

Cells were treated with 5–10 μ M p53RA small molecules or an equivalent amount of DMSO for 72 h, harvested, and then plated in triplicate culture dishes at 20 cells/cm². The cells were then cultured in drug-free medium for 10–14 days to allow colonies to form. Colonies were counted after staining with 0.01% crystal violet (Sigma), and the number of colonies in each drug-treatment group was expressed as a percentage of that in DMSO-treated controls.

Immunofluorescence

Cells were seeded on Lab-Tek[™] chamber slides (NUNC) at an initial density of $2.5-5 \times 10^3$ cells/cm². The following day, cells were treated with 5–10 μ M p53RA small molecules, CDDP, or DMSO for 24 h. The cells were then fixed in 4% paraformaldehyde, permeabilized with 0.25% Triton X-100, washed with PBS, and incubated with anti-p53 antibody (NeoMarkers) overnight. The next day, cells were washed with PBS, then incubated with Alexa Fluor 488 goat anti-mouse secondary antibody (Invitrogen) and counterstained with DAPI (Sigma).

Statistical analysis

Values were expressed as mean $\pm$ SD. A two-tailed Mann-Whitney test was used for comparisons of means between different treatment groups. A *P*-value < 0.05 was accepted as a significant difference. Synergistic action of two drugs was considered when growth suppression by the combined treatment was greater than the sum of growth inhibition induced by each drug alone, determined using the Chou-Talalay method.^{23,24}

Results

TP53 mutation status and p53 expression in HNSCC cell lines

p53 protein was detected in UMSCC-22A, JHU-O28, and JHU-O29 cells by Western blotting, and the level was increased by CDDP treatment, particularly in JHU-O28 cells, which expressed wild-type *TP53* (Fig. 1A and 1B). Direct sequencing revealed the presence of TP53 mutations in three cell lines: a homozygous Y220C missense mutation in UMSCC-22A cells, a single nucleotide deletion of codon 108 in JHU-O29 cells, and a heterozygous R243L missense mutation in Fadu cells (Fig. 1C).

p53RA small molecules inhibit growth of HNSCC cell lines and induce accumulation of p53 protein

Incubation of cells with the p53RA small molecules PRIMA-1 or CP-31398 for 96 h caused growth inhibition, especially in cell lines with *TP53* mutations (UMSCC-22A, JHU-O29, and Fadu), whereas nutlin-3 inhibited growth in JHU-O28, containing wild-type *TP53* (Fig. 2A). The *TP53*-mutant Fadu cell line also showed a good response to RITA treatment. The same results were obtained in clonogenic assays (Fig. 2B). However, none of p53RA small molecules significantly inhibited the growth of OKF6/TERT1 or HDF cell lines (Fig. 2C). By Western blotting, p53 expression levels increased markedly after treatment of JHU-O28 cells with nutlin-3 (Fig. 2D). MDM2 expression was detected only in JHU-O28 cells and was significantly increased after RITA or nutlin-3 treatment.

p53RA small molecules induce cell-cycle arrest and apoptosis in HNSCC cell lines

Treatment of cells for 48 h with the p53RA small molecules that induced prominent growth inhibition (Fig. 2A) caused cell-cycle arrest (Fig. 3A). PRIMA-1 and RITA caused G2 arrest in UMSCC-22A and Fadu cells, respectively, and nutlin-3 caused G1 arrest in JHU-O28 cells. The S-phase fraction significantly decreased from 12% to 3% upon nutlin-3 treatment. Treatment of cells for 48 h with 5–10 μ M p53RA small molecules increased Annexin-V-positive (apoptotic) cells by up to 30% in HNSCC cell lines (Fig. 3B).

p53RA small molecules lead to activation of p53-dependent apoptotic pathways

Treatment of the JHU-O28 cell line with nutlin-3 induced a dose-dependent increase in p53 protein expression, resulting in upregulation of p21^{WAF1} and activation of the intrinsic apoptotic pathway, as evidenced by increased Bax expression and caspase-3 cleavage in both Western blotting and real-time quantitative RT-PCR analyses (Fig. 4A and 4B). Upregulation of p21^{WAF1} and activation of apoptotic pathways were also observed in UMSCC-22A cells treated with PRIMA-1 in the absence of a prominent increase in overall p53 level. MDM2 expression was also elevated in JHU-O28 cells by nutlin-3 treatment. Accumulation of p53 in the nucleus was directly related to the duration of exposure to p53RA small molecules (Fig. 4C). RITA induced substantial cell death in Fadu cells but was much less cytotoxic toward JHU-O28 cells (Fig. 4D). After treatment with RITA for 24 h, phospho-p53 (Ser46), p21, and Bax levels increased in a dose-dependent manner in Fadu cells, with no significant increase in p53 levels.

p53RA small molecules enhance the cytotoxicity of chemotherapeutic agents active against HNSCC cell lines

Concurrent treatment with p53RA small molecules and cisplatin resulted in a synergistic antitumor effect (Fig. 5A and 5B). Most chemotherapeutic agents showed a similar synergistic effect when used in combination treatment, particularly when admixed with small molecules that showed prominent growth inhibition (Fig. 5C). With the use of the Chou-Talalay method, the combination index values were < 1.0 (Figs. 5A and 5C), indicating synergistic effect. Western blot analyses showed that p21^{WAF1} was upregulated and apoptotic pathways were activated to a greater extent by combined treatment of p53RA small molecules and chemotherapeutic agents than by treatment with PRIMA-1 (UMSCC-22A cells) or nutlin-3 (JHU-O28 cells) alone (Fig. 5D). With combined treatment, p53 accumulation was increased in the nucleus (Fig. 5E), and the levels of p21, Bax, PUMA, and NOXA mRNAs were also significantly elevated compared to the concentrations seen in cells treated with a single agent (Fig. 5F). In addition, induction of cell-cycle arrest and apoptosis in HNSCC cells were augmented by combined treatment (Fig. 5G and 5H).

Discussion

We showed that four p53RA small molecules, PRIMA-1, CP-31398, RITA, and nutlin-3, effectively restored p53 function to induce cell-cycle arrest and apoptosis in four selected HNSCC cell lines with mutant or wild-type *TP53*. The induced p53 accumulated in the nucleus, resulting in p53-dependent transactivation and increased expression of the cyclin-dependent kinase inhibitor p21. This is consistent with previous reports that p53RA small molecules reconstitute the DNA binding capability of wild-type p53, restoring the ability of p53 to transactivate downstream genes.^{14–17} The *Gadd45* and *14-3-3 σ* genes are also known to be effectors of p53-dependent cell-cycle arrest.²⁵ In the present study, p53RA small molecules led to accumulation of cells in the G2 phase as well as the G1 phase. Previous reports have shown that cell-cycle arrest caused by p53RA small molecules may occur through a p21-independent mechanism, which predominantly induces G1 arrest through suppression of Rb phosphorylation,²⁶ whereas G2 arrest is achieved by suppression of CDC2 and cyclin B activities after activation of *Gadd45* and *14-3-3 σ* .²⁵

Treatment with p53RA small molecules effectively induced apoptosis of HNSCCs. The accumulation of functional p53 led to both transcription-dependent and -independent apoptosis. p53RA small molecules activated transcription of the proapoptotic genes *BAX*, *PUMA* (*BBC3*), and *NOXA* (*PMAIP1*), the protein products of which are known to promote apoptosis through displacement of the anti-apoptotic proteins Bcl-2 or Bcl-xL, and recruitment of the apoptotic proteins BID or BAK.²⁷ The changes in Bax and Bcl-xL expression observed in our study support the potential activation of the p53-dependent apoptotic pathway by treatment with p53RA small molecules. p53 reactivation by small molecules has also been reported to trigger transcription-dependent apoptosis by the extrinsic pathway involving TRAIL or FasL^{28,29}; whether this also occurs in HNSCC is a question that will require further study. Moreover, p53 activation also induces apoptosis of cancer cells in the absence of transcription.³⁰ Following stabilization, p53 accumulates in the cytoplasm as well as the nucleus, becoming localized to the mitochondria where the protein directly induces mitochondrial membrane permeability and apoptosis.³¹ That p53 might act through this mechanism in HNSCC cells may be inferred from our data showing that Bax and caspase-3 activation was induced as rapidly as 4 h after treatment with p53RA small molecules. Because massive apoptosis occurred during a later phase after pharmacologic activation of p53, however, it is possible that a second wave of apoptosis caused by transcriptional upregulation of proapoptotic genes combined with an early apoptotic wave may be required to fully trigger the pronounced death of cancer cells observed here and reported previously.^{21,32}

Our study tested four p53RA small molecules in HNSCC cells. The small molecules showed different responses that generally depended on the mutational status of *TP53*. PRIMA-1 and CP-31398 caused prominent growth inhibition in mutant *TP53*-bearing tumors, and nutlin-3 did so in wild-type *TP53*-bearing tumors, as previously reported.^{14–17,32} High-dose treatment with mutant or wild-type p53RA small molecules also induced growth inhibition and enhanced the antitumor efficacy of chemotherapeutic agents in cancer cells independent of p53 or MDM2 expression. E2F-1 transcriptional activity and enhanced p73 function may explain apoptosis induction by nutlin-3 in cancer cells with mutant p53.^{33,34} We found that PRIMA-1 and CP-31398 induced apoptosis of HNSCC cells with wild-type *TP53* to some degree, although further studies are required to elucidate the underlying mechanism.

Our study also showed that *TP53*-mutant Fadu cells showed a good response to RITA treatment. This may be explained, in part, by the observed increase in Ser-46-phosphorylated p53, which would be predicted to increase p53-dependent transcriptional activation of apoptosis-inducing genes.³⁵ A recent report supports our data that RITA

restored transcriptional transactivation function of p53 so as to suppress growth and induce apoptosis in human cancer cell lines with mutant p53 protein.³⁶ JHU-O28 cell line with wild-type *TP53* was not sensitive to RITA, lacking the increase of p53 and Ser-46-phosphorylated p53 levels, which is required to elucidate the mechanism. In addition, none of the p53RA induced p53 expression were observed in JHU-O29 but inhibitory effects and p21 increase did occur. This may also suggest p53-independent induction of cell cycle arrest and apoptosis by p53RA small molecules, which should be elucidated by further studies.

We also evaluated the combined effects of p53-targeting small molecules and anticancer drugs, and found that all p53RA small molecules synergized with the four chemotherapeutic agents tested: CDDP, a DNA alkylator; 5-FU, an antimetabolite that inhibits DNA/RNA synthesis; paclitaxel, a mitotic inhibitor that blocks cytoskeleton polymerization; and erlotinib, a tyrosine kinase inhibitor. These drugs have become mainstays in chemotherapy against HNSCC in the time since the introduction of the concept of organ preservation.¹⁸ *In vitro* and *in vivo* studies have shown that p53RA small molecules are less toxic to normal cells than to cancer cells and have no significant adverse or genotoxic effects.^{37,38} The ability of these small molecules to synergize with conventional chemotherapeutic agents has been well-established by previous reports,^{21,32,39,40} and they may help reduce the dose of chemotherapeutic agents required in clinical settings, thereby minimizing potential side-effects in cancer patients. *In vivo* studies are required to elucidate the clinical potential of these drugs.

In conclusion, our data suggest that p53RA small molecules restore p53 pathways in HNSCCs with mutant or wild-type *TP53*, resulting in cell-cycle arrest and apoptosis. These small molecules enhance the cytotoxic effects of chemotherapeutic agents that are currently used against HNSCC. Thus, targeting of p53 might be a promising therapeutic option for patients with HNSCC.

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Figure 1. p53 expression and mutation status in the HNSCC cell lines used in this study. **A**, Western blot analysis of p53 expression before and after stimulation with CDDP. β-actin was used as a loading control. **B**, immunofluorescence staining of p53 in untreated cells. DAPI-stained nuclei and intracellular p53 are shown. **C**, TP53 mutation status determined by direct sequencing of exons 2–11.

Figure 2.

Differential response of HNSCC cell lines to p53RA small molecules. **A**, growth-inhibitory effects examined by MTT assays after incubating cells for 96 h with increasing doses of small molecules. Graphs were drawn from mean values $\pm$ SD from three different experiments. **B**, colony forming assays. The number of colonies was counted 10–14 days after cells treated with the indicated concentrations of the agents for 72 h were plated in drug-free media. Error bars represent SD. **C**, growth suppression tested by MTT assays after incubating OKF6/TERT1 and HDF for 96 h with 5–10 μ M p53RA small molecules. **D**, Western blot analysis of p53, MDM2, and p21^{WAF1} after treatment with p53RA agents for 24 h. Normalized ratios of p53 or p21^{waf1}: β -actin to control (Ct) were determined by

densitometry. P, PRIMA-1 (5 μ M); C, CP-31398 (5 μ M); R, RITA (10 μ M); N, nutlin-3 (10 μ M).

Figure 3.

Cell cycle and apoptosis assays after treatment with p53RA small molecules. **A**, cell-cycle changes examined by PI staining of cell lines after treatment for 24 h. **B**, Annexin-V binding fraction representing an apoptotic population of cells induced by treatment for 48 h. The figures are the representative of three independent experiments.

Figure 4. Activation of the p53 pathway by treatment with p53RA small molecules. **A**, Western blot analysis after treatment of two selected cell lines with different concentrations of small molecules for different times. **B**, changes in mRNA expression measured by real-time quantitative RT-PCR, shown as fold-reduction relative to DMSO-treated cells. *P*-values versus untreated cells were calculated using a two-tailed Mann-Whitney test. **C**, immunofluorescence staining of p53 after PRIMA-1 or nutlin-3 treatment for 8 or 24 h. Accumulation of p53 was seen in nuclei (counterstained with DAPI). **D**, Western blot and fluoromicroscopic examinations after treatment with RITA for 24 h. Cell viability was examined by trypan blue exclusion assay after treatment with RITA or nutlin-3 for 96 h.

Figure 5.

Combined effects of p53RA small molecules and chemotherapeutic agents on HNSCC cell lines. **A**, MTT assays of cells incubated for 96 h with increasing concentrations of CDDP in combination with 2.5 μ M PRIMA-1 or nutlin-3. **B**, phase-contrast images of two cell lines treated with p53RA or CDDP alone or in combination for 48 h. **C**, growth inhibition relative to control, assayed by MTT after treatment with individual p53RA and chemotherapeutic agents alone or in combination for 96 h. **D–F**, Western blot, immunofluorescence (p53), and real-time quantitative RT-PCR analyses of the expression of p53 pathway and proapoptotic genes in two selected cell lines after individual or combined treatment for 24 h. **G**, cell-cycle analysis of three cell lines treated with p53RA agents and CDDP, alone and in combination,

for 48 h. The tabulated data are the representative of three independent experiments. ***H***, apoptosis induction in JHU-O28 cell was increased by combined treatment with nutlin-3 and CDDP for 48 h compared with either agent alone.