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MDM2 inhibitor MI-319 in combination with cisplatin is an effective treatment for pancreatic cancer independent of p53 function

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ABSTRACT

Small molecule inhibitors (SMIs) of murine double minute 2 (MDM2) are known to restore the apoptotic and cell cycle regulatory functions of p53 by disrupting the MDM2–p53 interaction. In principle, these SMIs are not effective against tumours with mutation in the tumour suppressor p53 (mut-p53), which is known to be present in approximately 50% of all cancers. In this study we are reporting, for the first time, that MI-319 in combination with cisplatin induced cell growth inhibition and apoptosis in pancreatic cancer (PC) cells irrespective of their p53 mutational status. MI-319–cisplatin combination synergistically suppressed cell growth (MTT Combination Index [CI] < 1) and colony formation (clonogenic assay) and induced apoptosis. Western blot analysis and siRNA silencing studies in mutant as well as p53 null cells highlighted a mechanism involving p73 which is also known to be under the regulation of MDM2, and unlike p53, it is rarely mutated in PC. Down-regulating MDM2 using siRNA enhanced p73 reactivation and increased cell death. Further, the combination effectively reduced tumour growth in both wt-p53 and mut-p53 tumour xenograft models (50% Capan-2 animals were tumour free). Consistent with our *in vitro* results, remnant tumour tissue analysis showed up-regulation of p73 and the cell cycle regulator p21. In conclusion, this study highlights a new role of MDM2 inhibitors in combination with cisplatin, and thus warrants further clinical investigation in human pancreatic tumours containing both wt-p53 and mut-p53.

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1. Introduction

Pancreatic cancer (PC) is a deadly disease and is considered to be among the most intractable malignancies as it is refractory to the conventional chemotherapeutics and radiation.[1] Among the various genetic alterations in PC, mutation in the tumour suppressor p53 (mut-p53) gene has been reported to be about 50%.[2] In the remaining 50%, the p53 is normal (wild type), but its function is inhibited by MDM2 (murine double minute 2) protein whose key role is to promote ubiquitination and proteasomal-dependent degradation of p53.[3] Therapies that are based on restoring p53 function by blocking MDM2 using small molecule inhibitors (SMIs) have by far been effective in inducing growth arrest and apoptosis in cells in culture as well as in different animal model system via the activation of wt-p53 pathway.[4–6] Our laboratory has been working on a new class of MDM2 inhibitors (MI-319 and MI-219) and assessing its effects on PC showing induction of cell growth inhibition, apoptosis and tumour growth arrest. However, the therapeutic applicability of blocking MDM2 by such inhibitors including Nutlin-3 has been focused on its activity in wt-p53 tumours and very modest information is available on the role of other MDM2-regulated proteins such as the role of p73 especially in PC. Interestingly, recent evidences suggest that all p53 family proteins may cooperate in preventing tumour formation and this is proven by the observation that p63- and p73-deficient cells are resistant to p53-induced apoptosis.[7–9]

P73 appears to be an important protein, which is also under the influence of MDM2 and shares a high degree of sequence homology to p53.[10] Although p73 has been implicated in developmental biology, there is ample evidence suggesting that, at least in part, the function of p73 may closely resemble that of p53.[11,12] Indeed, like p53, p73 induces G_1 cell growth arrest, activates the transcription of some endogenous p53 target genes such as *p21Waf1/Cip1*, *mdm2*, *bax*, *cyclin G*[13–15] and induces apoptosis irrespective of p53 status.[1,4] Most significantly, p73 has been shown to be a key determinant of cellular sensitivity to anticancer therapeutics, and is widely induced by chemotherapeutic agents in a variety of tumour cell lines particularly in tumours lacking p53.[16] Several studies have shown that p73 is activated by DNA-damaging agents, including anthracyclines, topoisomerase I inhibitors and cisplatin.[17–20] Importantly, not all cells demonstrate the activation of p73 in response to each of these DNA-damaging agents, and thus the p73-dependent response to drugs is likely dependent on the cellular context including the presence or absence of functional p53. Among chemotherapeutic agents, cisplatin, docetaxel (Taxotere) and doxorubicin (Adriamycin) have been frequently used for the treatment of cancers including prostate, breast, lung and pancreatic cancers, alone or in combination with other agents.[21–25] Several clinical trials have reported that these agents, used in combination with other drugs, show improved outcomes in objective response rates and survival in pancreatic cancer (El-Reyes and Philip 2003).[26] Since both p53 and p73 have been implicated in cell response to cisplatin, the aim of the present study was to explore role of p73 in MDM2 inhibitor-cisplatin mediated anti-tumour effects in PC cells lacking functional p53 than in PC cells having wt-p53.

2. Materials and methods

2.1. Cell culture, experimental reagents and chemicals

Human PC cell lines Capan-2, BxPC-3, Colo-357 and Panc-28 were purchased from ATCC. The cell lines have been tested and authenticated in core facility Applied Genomics Technology Center at Wayne State University on 13th March 2009. The method used for testing was short tandem repeat (STR) profiling using the PowerPlex® 16 System from Promega (Madison, WI). Primary antibodies for p53, p73 and p21 were purchased from Cell Signaling. All secondary antibodies were obtained from Sigma (Saint Louis). MI-219 and MI-319 were synthesized by using our previously published methods.[27,28]

2.2. Cell growth inhibition studies by MTT assay

The cells Capan-2, Colo-357, BxPC-3 and Panc-28 (3×10^3) were seeded in a 96-well culture plate and treated with MI-319 (0 or 15 μM) or cisplatin (1 μM) or combination of both for 72 h and MTT assay was done as described earlier.[29] The results were plotted as mean ± SD of three separate experiments having six determinations per experiment for each experimental condition. Clonogenic assay for cell survival on Colo-357, HPAC and Capan-2 was performed according to the previously described methods.[30]

2.3. Trypan blue exclusion test

Panc-28, Colo-357 and Capan-2 cells were treated with either MI-319 (15 μM), cisplatin (1 μM) or their combination for 24 h. On completion of incubation, viability was assessed after adding 50 μL trypan blue solution (0.4% in PBS) in culture medium.

2.4. siRNA and transfections

The p73 siRNA, $p21^{WAF1}$ siRNA, MDM2 siRNA and control siRNA were obtained from Cell Signaling. Colo-357 cells were transfected with respective siRNAs for 5 h using LipofectAMINE 2000 as described in the manufacturers protocol (Cell Signaling).

2.5. Quantification of apoptosis by Annexin V FITC flow cytometry and ELISA

The cell apoptosis in Capan-2, Colo-357, BxPC-3 and Panc-28 post MI-319 (15 μM) alone; cisplatin (1 μM) alone or their combination treatment (for 72 h) was determined using Annexin V FITC apoptosis kit (Biovision Research Products) and ELISA detection kit (Roche, Palo Alto, CA) according to manufacturer's protocol.

2.6. Western blot analysis

Panc-28, Colo-357, Capan-2 and BxPC-3 cells were treated with either MI-319 (15 μM) or cisplatin (1 μM) or their combination for 20 h followed by extraction of protein for Western blot analysis. Procedure for cells lyses, protein concentration