Anti-tumor Activities of 3-Hydroxy-3-methylglutaryl Coenzyme A (HMG-CoA) Reductase Inhibitors and Bisphosphonates in Pancreatic Cell Lines Which Show Poor Responses to Gemcitabine

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Received May 21, 2019; accepted October 9, 2019

Few therapeutic options exist for gemcitabine-resistant pancreatic cancer. In this study, we investigated the anti-cancer effects of 3-hydroxy-3-methylglutaryl (HMG)-CoA reductase inhibitors and bisphosphonates in pancreatic cancer cell lines (SUIT-2 and MIA PaCa-2) which show poor responses to gemcitabine, established through long-term culture in nutrient-deprived or gemcitabine-containing media. Under the nutrient-deprived condition, IC_{50}s for statins and bisphosphonates decreased and those for gemcitabine increased compared with those under normal conditions. In cells cultured long-term with gemcitabine, although IC_{50}s for gemcitabine increased, those for statins and bisphosphonates either slightly increased or remained unchanged. Thus, these drugs may be effective against pancreatic cancer cells which show poor responses to gemcitabine.

Key words statin; bisphosphonate; pancreatic cancer

INTRODUCTION

Pancreatic cancer has a very poor prognosis. Although gemcitabine-based chemotherapy is widely used for the treatment of pancreatic cancer, few therapeutic options exist in case the tumor develops gemcitabine resistance. Cancer cells may develop resistance to chemotherapy under nutrient-deprived conditions such as in the interior of large tumors. Previous studies on cultured cell lines have reported that pancreatic carcinomas cultured under nutrient-deprived conditions, such as low glucose or low amino acids, show resistance to chemotherapeutic agents, including gemcitabine.1,2) Furthermore, it has been reported that long-term exposure to gemcitabine confers gemcitabine resistance to pancreatic cancer cell lines.3,4) Our laboratory previously reported that fluvastatin, which is a 3-hydroxy-3-methylglutaryl (HMG)-CoA reductase inhibitor, and/or zoledronate, which is a bisphosphonate, both induce anti-proliferative effects in cultured pancreatic cancer cells.5)

In this study, we investigated whether statins and bisphosphonates possess anti-cancer effects against pancreatic cancer cell lines which show poor responses to gemcitabine, established either through long-term exposure to gemcitabine or by culturing under nutrient-deprived conditions.

MATERIALS AND METHODS

Cell Cultures Human pancreatic cancer cell lines MIA PaCa-2 and SUIT-2 were a kind gift from Dr. Soichi Takiguchi of National Kyushu Cancer Center (Fukuoka, Japan). For experiments on growth in a nutrient-deprived condition, cells were grown in either a nutrition-rich medium (NRM) or a nutrition-deprived medium (NDM) in a humidified atmosphere of 5% CO_2 at a constant temperature of 37°C for a few weeks. The NRM comprised RPMI-1640 media (Sigma-Aldrich Co. LLC., MO, U.S.A.) (containing amino acids and glucose) supplemented with 10% fetal bovine serum (FBS), penicillin, and streptomycin. The NDM comprised 60% reductions of amino acids, glucose, and FBS from the composition of the NRM. For experiments on growth in high-gemcitabine (Comb-Blocks Inc., CA, U.S.A.) conditions, cell lines show poor responses to gemcitabine were generated through long-term culture in gemcitabine-containing NRM. The concentration of gemcitabine was gradually increased by 10nM per week from 10nM to achieve a final concentration of 100nM for MIA PaCa-2 cells and 200nM for SUIT-2 cells. Standard NRM without gemcitabine was selected for healthy control cell lines (normal cell lines).

Cell Viabilities and IC_{50}s The cells were seeded into 96-well plates (Thermo Fisher Scientific Inc., MA, U.S.A.) at a density of 2.0 × 10^3 cells/well and used for experiments on the following day. The cells were exposed to each drug for 72 h, and cell viabilities were assessed through the mitochondrial activity represented by the reduction of WST-8 [2-(2-meth-oxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disul-fophenyl)-2H-tetrazolium, monosodium salt] to formazan. To perform these tests, WST-8 assay solutions (Cell Counting Kit-8; Dojindo Laboratory, Kumamoto, Japan) were added to the cell cultures, and the cultures were further incubated for 1 h at 37°C in humidified air supplemented with 5% CO_2. The extent of formazan dye formation was determined by measuring the absorbance at 450 nm using a microplate reader (iMark™ Microplate Reader; Bio-Rad Laboratories, Inc., CA, U.S.A.). IC_{50} values were calculated using approximated sigmoidal curves.6)

Statistical Analyses Data are shown as mean ± standard error of the mean (S.E.M.). Statistical analysis was performed using Student’s t-test (Statview; Abacus Concepts, CA, U.S.A.) to determine differences between the groups. A probability level of p < 0.05 was considered as statistically significant.

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RESULTS

Chemotherapeutic agents including gemcitabine; oxaliplatin and paclitaxel; statins (simvastatin, atorvastatin, rosuvastatin, fluvastatin, and pitavastatin), except pravastatin; and bisphosphonates (zoledronate and alendronate) all demonstrated dose-dependent decrease in the cell viabilities of SUIT-2 cells cultured in both the NRM and NDM (Figs. 1A–H, J, K). However, pravastatin did not affect cell viability under either condition (Fig. 1I). Cells cultured in NDM containing gemcitabine (1 nM) and paclitaxel (1 and 10 nM) exhibited significantly higher cell viabilities than those cultured in NRM (Figs. 1A, C). Conversely, statins, except pravastatin, and bisphosphonates caused lower viabilities in cells cultured in NRM (Fig. 1G, K). However, pravastatin did not affect cell viability under either condition (Fig. 1I). Cells cultured in NDM containing gemcitabine (1 nM) and paclitaxel; statins (simvastatin, atorvastatin, rosuvastatin, fluvastatin, and pitavastatin), except pravastatin; and bisphosphonates were associated with higher cell viabilities in cells chronic-cultured with gemcitabine than in normal SUIT-2 cells (Table 1). Conversely, some statins and bisphosphonates were associated with slightly higher cell viabilities in the cells chronic-cultured with gemcitabine (Fig. 2A). IC_{50} values for gemcitabine was 4.67-fold higher in the cells chronic-cultured with gemcitabine than in normal SUIT-2 cells (Table 1). These increases in IC_{50} values were less pronounced than those induced by gemcitabine. Furthermore, no significant differences in IC_{50} values of bisphosphonates were observed between the normal SUIT-2 cells and those chronic-cultured with gemcitabine (Table 1). Finally, no significant differences in IC_{50} values of statins or bisphospho-

![Image](image-url)

Fig. 1. Cell Viabilities of SUIT-2 Cells Cultured in a Nutrient-Rich Medium (NRM) and Those Cultured in a Nutrient-Deprived Medium (NDM)

Cells were incubated with gemcitabine (A), oxaliplatin (B), paclitaxel (C), simvastatin (D), atorvastatin (E), rosuvastatin (F), fluvastatin (G), pitavastatin (H), pravastatin (I), zoledronate (J), and alendronate (K) for 72 h. Cell viabilities were measured using the WST-8 method. Open and closed circles mean the cell viabilities of cell lines cultured in NRM and NDM, respectively. Results are expressed as mean ± S.E.M. (n = 6–12). *p < 0.05, **p < 0.01 compared with NRM.

Table 1. Differences in IC_{50}s for HMG-CoA Reductase Inhibitors or Bisphosphonates between Cells Cultured in a Nutrient-Rich Medium (NRM) and Those Cultured in a Nutrient-Deprived Medium (NDM), or between Normal Cells and Those Chronic-Cultured with Gemcitabine

<table>
<thead>
<tr>
<th>Chemotherapy agents</th>
<th>SUIT-2</th>
<th>MIA PaCa-2</th>
<th>SUIT-2</th>
<th>MIA PaCa-2</th>
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<tbody>
<tr>
<td></td>
<td>Nutrient-rich medium (NRM)</td>
<td>Nutrient-deprived medium (NDM)</td>
<td>Nutrient-rich medium (NRM)</td>
<td>Nutrient-deprived medium (NDM)</td>
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<tr>
<td>Gemcitabine (nM)</td>
<td>2.25 ± 0.29</td>
<td>4.58 ± 0.36**</td>
<td>4.64 ± 0.39</td>
<td>8.90 ± 0.59**</td>
</tr>
<tr>
<td>Oxaliplatin (μM)</td>
<td>9.04 ± 0.74</td>
<td>8.37 ± 0.66</td>
<td>20.8 ± 2.7</td>
<td>3.64 ± 0.96**</td>
</tr>
<tr>
<td>Paclitaxel (nM)</td>
<td>7.92 ± 1.03</td>
<td>11.6 ± 1.1*</td>
<td>0.518 ± 0.024</td>
<td>0.778 ± 0.085*</td>
</tr>
<tr>
<td>HMG-CoA reductase inhibitors</td>
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<tr>
<td>Simvastatin (μM)</td>
<td>7.88 ± 0.48</td>
<td>2.01 ± 0.49**</td>
<td>0.947 ± 0.040</td>
<td>0.211 ± 0.092**</td>
</tr>
<tr>
<td>Atorvastatin (μM)</td>
<td>11.4 ± 0.7</td>
<td>8.41 ± 1.12*</td>
<td>1.31 ± 0.07</td>
<td>0.802 ± 0.154**</td>
</tr>
<tr>
<td>Rosuvastatin (μM)</td>
<td>79.6 ± 5.2</td>
<td>67.2 ± 12.0</td>
<td>5.04 ± 0.37</td>
<td>3.77 ± 0.25*</td>
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<tr>
<td>Fluvastatin (μM)</td>
<td>9.09 ± 0.46</td>
<td>2.41 ± 0.46**</td>
<td>2.39 ± 0.40</td>
<td>0.508 ± 0.031*</td>
</tr>
<tr>
<td>Pitavastatin (μM)</td>
<td>1.80 ± 0.13</td>
<td>0.965 ± 0.045**</td>
<td>0.250 ± 0.009</td>
<td>0.245 ± 0.034</td>
</tr>
<tr>
<td>Pravastatin (μM)</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
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<tr>
<td>Bisphosphonates</td>
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<tr>
<td>Zoledronate (μM)</td>
<td>9.96 ± 1.25</td>
<td>2.28 ± 0.41**</td>
<td>3.86 ± 0.96</td>
<td>1.14 ± 0.15*</td>
</tr>
<tr>
<td>Alendronate (μM)</td>
<td>18.0 ± 2.1</td>
<td>5.94 ± 0.22</td>
<td>26.8 ± 1.9</td>
<td>1.83 ± 0.48**</td>
</tr>
</tbody>
</table>

Cells were incubated with each drugs for 72h. IC_{50}s are showed as mean ± S.E.M. (n = 6–12). *p < 0.05, **p < 0.01 compared with NRM or normal cell line.
Although IC\textsubscript{50} values of statins were mildly elevated were nearly equal to those observed in normal MIA PaCa-2 cells, which had MIA PaCa-2 cells. In addition, the anti-cancer effects of and bisphosphonates was displayed by both SUIT-2 and therapy.

in the interior of large tumors, or after long-term gemcitabine containing medium, consistent with previous reports. \textsuperscript{1–4) Such conditions are believed to mimic chemotherapy resistance in hypoxic and nutrient-deprived conditions. \textsuperscript{8,9) The lack of equilibrative nucleoside transporter 1 (ENT1) and deoxycytidine kinase (dCK) and increase in the levels of ribonucleotide reductase subunits M1 (RRM1) and M2 (RRM2) are involved in the development of gemcitabine resistance. \textsuperscript{10–13) Furthermore, a clinical study reported that the expression of these four key genes influences the prognosis of patients with pancreatic cancer undergoing adjuvant chemotherapies.\textsuperscript{4) ENT1 is a cellular transporter involved in the intracellular incorporation of gemcitabine. In contrast, lipophilic statins can penetrate the cell membranes \textit{via} passive diffusion.\textsuperscript{15) Therefore, it is considered that statins provide reliable efficacy against pancreatic cancer cells even if the cells down-regulate the expression of ENT1 in the process of acquiring gemcitabine resistance. The remaining factors—dCK, RRM1, and RRM2—are all specific to the action mechanisms and metabolic pathways of gemcitabine. Our institution previously reported that statins and bisphosphonates induce anti-proliferative effects \textit{via} the downregulation of prenylation of RhoA and Ras in the mevalonate pathway in normal pancreatic cancer cell lines.\textsuperscript{5) Because these pathways are independent of gemcitabine-resistance related factors (dCK, RRM1, and RRM2), statins and bisphosphonates display anti-cancer effects also against the cell lines which show poor responses to gemcitabine. Furthermore, RhoA and Ras are not specific to pancreatic cancers. Therefore, HMG-CoA reductase inhibitors and bisphosphonates might be effective for other cancers.

In this study, we had not performed any experiments about the effects of combinations of HMG-CoA reductase inhibitors and the other drugs because we wanted to check the effects of these drugs when used alone respectively. Previous studies had reported the additive effects of HMG-CoA reductase inhibitors, bisphosphonates and gemcitabine for pancreatic cancer in cultured cells and clinical study.\textsuperscript{5,6) Thus, it is possible that combinations of HMG-CoA reductase inhibitors, bisphosphonates and gemcitabine show additive effects for pancreatic cancer cell line which show poor responses to gemcitabine. In conclusion, our results suggest that HMG-CoA reductase inhibitors and bisphosphonates have anti-cancer activities against pancreatic cancer cells which show poor responses to gemcitabine, established through long-term culture in either nutrient-deprived or gemcitabine-containing conditions. These effects were generally equitable to, or greater than, those observed in normal cancer cells. Recently, a meta-analysis has also indicated that statins improve the survival time of pa-
tients with pancreatic cancer. Therefore, statins and bisphosphonates may be effective in the treatment of pancreatic cancer, particularly in chemotherapy-resistant cases.

**Conflict of Interest** The authors declare no conflict of interest.

**REFERENCES**


