Article

Repurposing Niclosamide for Targeting Pancreatic Cancer by Inhibiting Hh/Gli Non-Canonical Axis of Gsk3β

Jyoti B. Kaushal, Rakesh Bhatia, Ranjana K. Kanchan, Pratima Raut, Surya Mallapragada, Quan P. Ly, Surinder K. Batra and Satyanarayana Rachagani

Supplementary Materials:


Figure S1. Inhibitory effect of Nic on poorly and well differentiated PC cell lines. Concentration (1, 5, 10 and 20 µM) and time-dependent (24 and 48 h) effect of Nic treatment on viability of poorly differentiated (PANC-1, MIAPaCa-2) and well differentiated (Capan-1) pancreatic cell lines was determined by MTT assay. Values are expressed as mean ± SEM (n=6), p values: ***p < 0.001, *p < 0.05 vs control (24 h); ###p < 0.001 vs control (48 h).

Academic Editor: Murray Korc

Received: 24 April 2021
Accepted: 17 June 2021
Published: 22 June 2021

Publisher’s Note: MDPI stays neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Copyright: © 2021 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (http://creativecommons.org/licenses/by/4.0/).
**Figure S2.** Nic-mediated inhibition of migration potential in PC cell lines. (A) Nic-treated COLO 357, SW1990, and T3M4 cells were analyzed for their migration potential. A total of 1×10⁵ cells were seeded into top chamber of the trans-well with serum-free media, whereas, bottom chamber with serum containing media, and after 24 h, migrated cells were stained and analyzed. (B) Representative western blots showing the expression of protein markers involved in migration and EMT transition such as E-cadherin, N-cadherin, claudin in PC cell lines (COLO 357 and SW1990) upon treated with Nic (5µM and 10µM).

**Figure S3.** The efficacy of Nic on pancreatic cancer-associated fibroblasts. (A) The effect of Nic on viability of cancer-associated fibroblast cell lines (09-11, 09-17, 10-32, 10-15, 10-03CFs) and normal fibroblast (09-26N) were determined by MTT assay. Values are expressed as mean ± SEM(n=6), p values: ***p < 0.001, **p < 0.001, *p < 0.05 vs control (24 h); #&p < 0.00, #kp < 0.001, #kp < 0.05 vs control (48 h) (B) Representative western blot image showing the expression of α-SMA and FAPα in 10-32 CFs cells treated with Nic (10 µM) for 24 h.
Figure S4. Analysis of PARP expression in PC cell lines upon Nic treatment. Densitometric quantitation of western blot results shown as percentage protein expression of full-length PARP, cleaved PARP, and the ratio of cleaved to full-length PARP in COLO 357 and SW1990 cells treated with Nic (10 µM) for various time periods i.e., 1, 3, 12, 24, and 48 h. Values are expressed as mean ± SEM (n = 3), p values: ***p < 0.001, **p < 0.001, *p < 0.05, ns p >0.05 vs control.

Figure S5. Effect of Nic on the Cleaved caspase-3 expression in PC cell lines. Representative western blot images showing the expression of Cleaved caspase-3 in COLO 357 and SW1990 PC cell lines treated with Nic (10 µM) for 24 h and 48 h and observed time dependent increase of cleaved caspase-3 expression in PC cell lines.
Figure S6. Nic exhibits growth suppression in poorly differentiated PC cell lines and pancreatic cancer-associated fibroblasts (CAFs) by inducing apoptosis. (A) Poorly differentiated PC cell lines (PANC-1 and AsPC-1) and (B) pancreatic cancer associated fibroblast cell line (10-32 CAFs) were treated with Nic (5 and 10 µM) for 24 h, and induction of apoptosis was determined by flow cytometric analysis of annexin-V Cy-5/PI- dual stained cells (AV+/PI– intact cells; AV+/PI+– nonviable/necrotic cells; AV+/PI+ and AV+/PI+– apoptotic cells) in PC cell lines (left panel). Quantitative analysis of these micrographs was shown as mean ± SEM (n = 3), p values: ***p < 0.001, **p < 0.01 vs live control cells; ###p < 0.001 vs apoptotic control cells (right panel).
Figure S7. Nic stimulates the expression of a lipilized form of LC3B in PC cell lines. Densitometric quantitation of western blotting images was shown as percentage protein expression of LC3I, LC3 II, and LCII/I in COLO 357 and SW1990 cells treated with Nic (10 µM) for various time periods i.e., 1, 3, 12, 24, and 48 h. Values are expressed as mean ± SEM (n = 3), p values: ***p < 0.001, **p < 0.001, *p < 0.05, ns p > 0.05 vs control.

Figure S8. Effect of functional blockage autophagy via spautin-1 on Nic- mediated growth suppression via Calcein AM assay. COLO 357 and SW1990 cells were pretreated with spautin-1 for 2 h, followed by Nic (10 µM) for 24, 48, and 72 h and cell viability was assessed by Calcein-AM assay Values are expressed as mean ± SEM (n = 3), p values: ***p < 0.001, **p < 0.001, *p < 0.05 vs control (24 h); p values: $***p < 0.001, $**p < 0.001, $p < 0.05 vs control (48 h); p values: $$$p < 0.001, $$p < 0.001, $p < 0.05 vs control (72 h).
Figure S9. Demonstration of the effect of Nic on the β-catenin signaling molecules in normal pancreatic cell line (HPNE) and colorectal cancer cell line (LS-180). Representative western blot images showing the expression of β-catenin (active), p-Gsk3β, and Gsk3β in normal pancreatic cell line HPNE and colorectal cell line LS180 treated with Nic (5 µM or 10 µM) for 24 h. Membrane was stripped and reprobed with β-actin for internal loading control.

Figure 10. Demonstration of the effect of Nic on the expression of Hh signaling ligand (Shh) in PC cell lines. Representative western blot images showing the expression of Shh in PC cells treated with Nic (10 µM) for different time points. β-actin used as an internal loading control.
Figure 2

(B) COLO357

- Cleaved PARP
- Cleaved Caspase 9
- β-actin
- BCL2

(F) SW1990

- Drp1
- Cleaved PARP
- Cleaved Caspase 9
- β-actin
- BCL2

Figure 3

(B) COLO357

- LC3/II
- β-actin

(D) Left panel

- Beclin-1
- LAMP 2
- SQSTM1/p62
- β-actin

(D) Right panel

- SW1990
- Beclin-1
- LAMP 2
- SQSTM1/p62
- β-actin
**Figure 3**  
(E) Left panel  
COLO357  
SW1990  

**Figure 4**  
(E)  
COLO357  
SW1990  

(F) Left panel  
COLO357  
SW1990  

(F) Right panel  
COLO357  
SW1990
Figure 4 (G)  

SW1990

**Figure 5 (A)**

GSK3 β

Non-phospho (Active) β-Catenin (Ser33/37/Thr41)

p-GSK3 β (s-9)

β-actin
Figure 5
(B) – Left Panel
COLO357

Figure 5
(B) – Right Panel
SW1990

Figure 5
(D) – Left Panel
COLO357

Figure 5
(D) – Right Panel
SW1990

(Active) β-Catenin
(Ser33/37/Thr41)

(Active) β-Catenin
(Ser33/37/Thr41)

Total β-catenin

Total β-catenin

β-actin

β-actin

Lamin B1

Lamin B1

GAPDH

GAPDH

Gsk3β

Gsk3β

p-Gsk3β (s-9)

p-Gsk3β (s-9)
Figure S11. The whole western blot images of Figure 2, Figure 3, Figure 4, Figure 5, Figure 6, Figure 7
### Table S1. List of primary antibodies.

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Primary antibody (Ab)</th>
<th>Cat# No.</th>
<th>Manufacturer</th>
<th>Primary Ab Dilution used</th>
<th>Application</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>cleaved PARP</td>
<td>9542</td>
<td>Cell Signaling Technology</td>
<td>1:1000</td>
<td>WB</td>
</tr>
<tr>
<td>2</td>
<td>cleaved caspase 9</td>
<td>20750</td>
<td>Cell Signaling Technology</td>
<td>1:1000</td>
<td>WB</td>
</tr>
<tr>
<td>3</td>
<td>LC3A1/BII</td>
<td>4108</td>
<td>Cell Signaling Technology</td>
<td>1:2000</td>
<td>WB</td>
</tr>
<tr>
<td>4</td>
<td>LC3</td>
<td>AP1802a</td>
<td>Abgent</td>
<td>1:200</td>
<td>IF</td>
</tr>
<tr>
<td>5</td>
<td>Beclin-1</td>
<td>sc-11427</td>
<td>Santa Cruz Biotechnology</td>
<td>1:2000</td>
<td>WB</td>
</tr>
<tr>
<td>6</td>
<td>LAMP2</td>
<td>PA1-655</td>
<td>Thermo Fisher Scientific</td>
<td>1:2000</td>
<td>WB</td>
</tr>
<tr>
<td>7</td>
<td>SQSTM1/p62 Antibody</td>
<td>5114</td>
<td>Cell Signaling Technology</td>
<td>1:1000</td>
<td>WB</td>
</tr>
<tr>
<td>8</td>
<td>BCL2</td>
<td>PA5-27094</td>
<td>Thermo Fisher Scientific</td>
<td>1:1000</td>
<td>WB</td>
</tr>
<tr>
<td>9</td>
<td>β-Catenin</td>
<td>9562</td>
<td>Cell Signaling Technology</td>
<td>1:1000</td>
<td>WB</td>
</tr>
<tr>
<td>10</td>
<td>Non-phospho (Active) β-Catenin (Ser33/37/Thr41)</td>
<td>8814</td>
<td>Cell Signaling Technology</td>
<td>1:1000; 1:200</td>
<td>WB; IF</td>
</tr>
<tr>
<td>11</td>
<td>p27 kip1</td>
<td>2552S</td>
<td>Cell Signaling Technology</td>
<td>1: 1000</td>
<td>WB</td>
</tr>
<tr>
<td>12</td>
<td>Cyclin D1</td>
<td>sc-8396</td>
<td>Santa Cruz Biotechnology</td>
<td>1:1000</td>
<td>WB</td>
</tr>
<tr>
<td>13</td>
<td>p21 waf1/cip1</td>
<td>2947S</td>
<td>Cell Signaling Technology</td>
<td>1:1000</td>
<td>WB</td>
</tr>
<tr>
<td>14</td>
<td>p- Gsk3β (Ser9)</td>
<td>9322</td>
<td>Cell Signaling Technology</td>
<td>1:1000; 1:100</td>
<td>WB; IF</td>
</tr>
<tr>
<td>15</td>
<td>Gsk3β</td>
<td>12456</td>
<td>Cell Signaling Technology</td>
<td>1:1000</td>
<td>WB</td>
</tr>
<tr>
<td>16</td>
<td>p-P70 S6K1 (Thr 421/Ser 424)</td>
<td>9204</td>
<td>Cell Signaling Technology</td>
<td>1:1000</td>
<td>WB</td>
</tr>
<tr>
<td>17</td>
<td>p-4EBP1(Ser-65)</td>
<td>9451</td>
<td>Santa Cruz Biotechnology</td>
<td>1:1000</td>
<td>WB</td>
</tr>
<tr>
<td>18</td>
<td>Gli3</td>
<td>19949-1-AP</td>
<td>Thermo Fisher Scientific</td>
<td>1:1000; 1:100</td>
<td>WB; IF</td>
</tr>
<tr>
<td>19</td>
<td>Gli1</td>
<td>sc-515781</td>
<td>Santa Cruz Biotechnology</td>
<td>1:1000; 1:100</td>
<td>WB; IF</td>
</tr>
<tr>
<td>20</td>
<td>Sufu</td>
<td>PA5-29952</td>
<td>Thermo Fisher Scientific</td>
<td>1:2000</td>
<td>WB</td>
</tr>
<tr>
<td>21</td>
<td>Shh</td>
<td>sc-373779</td>
<td>Santa Cruz Biotechnology</td>
<td>1:1000</td>
<td>WB</td>
</tr>
<tr>
<td>22</td>
<td>E-cadherin</td>
<td>3195</td>
<td>Cell Signaling Technology</td>
<td>1:1000</td>
<td>IF</td>
</tr>
<tr>
<td>23</td>
<td>N-cadherin</td>
<td>ab76011</td>
<td>abcam</td>
<td>1:1000</td>
<td>WB</td>
</tr>
<tr>
<td>24</td>
<td>Claudin-1</td>
<td>ab15098</td>
<td>abcam</td>
<td>1:1000</td>
<td>WB</td>
</tr>
<tr>
<td>25</td>
<td>Lamin B1</td>
<td>12586</td>
<td>Cell Signaling Technology</td>
<td>1:1000</td>
<td>WB</td>
</tr>
<tr>
<td>26</td>
<td>GAPDH</td>
<td>5174</td>
<td>Cell Signaling Technology</td>
<td>1:1000</td>
<td>WB</td>
</tr>
<tr>
<td>27</td>
<td>Cleaved Caspase-3</td>
<td>9664</td>
<td>Cell Signaling Technology</td>
<td>1:1000</td>
<td>WB</td>
</tr>
<tr>
<td>28</td>
<td>α-SMA</td>
<td>56856</td>
<td>Cell Signaling Technology</td>
<td>1:1000</td>
<td>WB</td>
</tr>
<tr>
<td>29</td>
<td>FAP-I</td>
<td>ab 53066</td>
<td>abcam</td>
<td>1:1000</td>
<td>WB</td>
</tr>
<tr>
<td>30</td>
<td>β-actin</td>
<td>sc-47778</td>
<td>Santa Cruz Biotechnology</td>
<td>1:5000</td>
<td>WB</td>
</tr>
</tbody>
</table>

### Table S2. List of secondary antibodies.

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Secondary antibody (Ab)</th>
<th>Cat# No.</th>
<th>Manufacturer</th>
<th>Secondary Ab Dilution used</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Goat anti-rabbit IgG-HRP</td>
<td>31460</td>
<td>Invitrogen</td>
<td>1:5000</td>
</tr>
<tr>
<td>2</td>
<td>Goat anti-mouse IgG-HRP</td>
<td>31430</td>
<td>Invitrogen</td>
<td>1:5000</td>
</tr>
<tr>
<td>----</td>
<td>------------------------</td>
<td>-------</td>
<td>------------</td>
<td>--------</td>
</tr>
<tr>
<td>3</td>
<td>Goat anti-Rabbit IgG (H+L) Secondary Antibody, Alexa Fluor 568</td>
<td>A-11011</td>
<td>Invitrogen</td>
<td>1:200; 1:500</td>
</tr>
<tr>
<td>4</td>
<td>Goat anti-Mouse IgG (H+L) Secondary Antibody, Alexa Fluor 568</td>
<td>A-11004</td>
<td>Invitrogen</td>
<td>1:200; 1:500</td>
</tr>
<tr>
<td>5</td>
<td>Goat anti-Rabbit IgG (H+L) Secondary Antibody, Alexa Fluor 488</td>
<td>A-11034</td>
<td>Invitrogen</td>
<td>1:200</td>
</tr>
</tbody>
</table>