The Ibr-7 Derivative of Ibrutinib Exhibits Radiosensitivity in Pancreatic Cancer Cells via Targeting EGFR

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Abstract

**Background:** Radiotherapy is one of the main therapeutic methods for pancreatic cancer, but radiation resistance limits the clinical application. As a result, novel therapeutic agents to improve the radiosensitivity is urgent. This study aimed to investigate the effect of Ibr-7 (the derivative of ibrutinib) on radiosensitivity of human pancreatic cancer cells.

**Methods:** The effect of Ibr-7 on pancreatic cancer cell's proliferation were detected by CCK-8 assay. Radiosensitivity was assessed by clonogenic formation assay. Cell cycle, cell apoptosis were analyzed by flow cytometry. DNA damage was detected by immunofluorescence analysis. The expression of p-EGFR, EGFR were determined by western blot.

**Results:** Ibr-7 showed anti-proliferative effect in PANC-1 and Capan2 cells in a dose- and time-dependent manner. Ibr-7 (2 µmol/L) enhanced the effect of radiation in PANC-1 and Capan2 cells. Further findings showed that this combination enhanced G2/M phase arrest and increased cell apoptosis. Additional molecular mechanism studies revealed that the expression of p-EGFR was decreased by Ibr-7 alone or combined with radiation. Overexpression of EGFR reversed the cell apoptosis induced by Ibr-7 combined with radiation. Moreover, the expression of γ-H2AX was significantly decreased in Ibr-7 combined with radiation group.

**Conclusions:** Our study indicated that the potential application of Ibr-7 as a highly effective radiosensitizer for the treatment of pancreatic cancer cells.

Background

Pancreatic cancer is a highly invasive and fatal disease, with a low 5-year survival rate of only 5%[1]. It is diagnosed in the medium or terminal stage, and 80% of patients are ineligible for surgery. The treatment options for them are chemotherapy and radiation therapy[2, 3]. Approximately 70% of pancreatic cancer patients receive radiotherapy, either alone or in combination with other treatments[4, 5]. However, pancreatic cancer cells inevitably develop radioresistance, which is one of the main cause for poor prognosis of patients in clinical treatment[6–8]. Recent progress in radiation combination with chemotherapy has raised concerns. Exploring new targeted agents to improve the radiosensitivity of pancreatic cancer is urgent.

Ibrutinib is a small molecule inhibitor that targets Bruton's tyrosine kinase[9]. It exhibited potent anti-tumor effect against various cancers, including lymphoma and solid tumors (such as non-small cell lung cancer and pancreatic cancer)[10–14]. However, in our previous research, it was demonstrated that ibrutinib’s anti-tumor effect was limited to blood cancer cells [15]. The clinical trials of ibrutinib in solid tumors are facing dilemma. Based on these, we synthesized a series of ibrutinib derivatives, of which Ibr-7 exhibited great anti-tumor toward NSCLC and pancreatic cancer[15, 16]. Besides, in our previous research ibrutinib (10 µmol/L) has been shown to enhance the effects of radiation therapy[17]. Therefore, towards pancreatic cancer cells.
In the present study, we sought to evaluate the radiosensitizing effect of Ibr-7 in pancreatic cancer cell lines. Our results suggested that Ibr-7 sensitized pancreatic cancer cell lines PANC-1, Capan2. Further study indicated that Ibr-7 induced G2/M cell cycle arrest and cell apoptosis. Ibr-7 efficiently inhibited the p-EGFR, just as ibrutinib. However, as distinct from ibrutinib, Ibr-7 did not suppress the phosphorylation of the AKT/mTOR signaling pathway. These results may offer a effective strategy for enhancing the effect of radiation therapy, and it will provide revealing insights into the development of ibrutinib derivatives.

Materials And Methods

Cell lines and reagents

Human pancreatic cancer cell line PANC-1, Capan2 cell lines were purchased from the Stem Cell Bank of Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). PNAC-1, Capan2 cells were cultured in DMEM or 1640 medium supplemented with 10% FBS in a atmosphere of 5% CO2 at 37°C. Ibrutinib (PCI-32765, 99.2%) was purchased from Selleck, USA. Ibr-7Ibr-7 (Lot: 20161216, > 95%) were provided by Hangzhou Hezheng Pharmaceutical Co., Ltd. Ibrutinib or Ibr-7 were dissolved in DMSO (Sigma-Aldrich, St Louis, Missouri, USA) and stored at -20°C.

Antibodies and reagents

Anti-PARP (46D11, 9532), anti-cleaved caspase3 (CST, D175R, 9661S), anti-caspase3 (CST, 9662S), anti-EGFR (4267s), anti-pEGFR (Tyr1068; 3777S) were purchased from Cell Signaling Technology (Danvers, MA, USA). β-actin (J0914) was purchased from Santa Cruz biotechnology (Dallas, TX, USA). PVDF(0.45 μm,Millpore CAT. NO: IPVH00010); 5 × Loading biosharp, BL502b); Goat anti-mouse IgG, Peroxidase Conjugated, H + L (BL001A) were purchased from biosharpand Goat anti-rabbit IgG, Peroxidase Conjugated, H + L (BL003A). ECL HRP substrates for western blot purchased from CYANAGEN (WESTAR nC2.0, lot HG13A-CC).

Cell viability assay

Cell proliferation was measured by Cell Counting Kit-8 (CCK-8, Bestbio, Shanghai, China) assay. Cells were cultured in 96-well plates with 3–5 × 10^3/well. Cells were treated with Ibr-7 (1.56-25 μmol/L) or Ibrutinib (1.56-50 μmol/L) for 24, 48 or 72 h. The cells were measured using CCK-8 solutions at 450 nm by A SpectraMax M2e (Molecular Devices, San Jose, CA, USA). Cell viability was calculated for each well and the 50% growth inhibition (IC50) was determined. Assays were performed on at least three independent experiments.

Clonogenic assay

Cell viability was measured by Cell Counting Kit-8 (CCK-8, Bestbio, Shanghai, China) assay. Cells were cultured in 96-well plates with 3–5 × 10^3/well. Cells were treated with Ibr-7 (1.56-25 μmol/L) or Ibrutinib (1.56-50 μmol/L) for 24, 48 or 72 h. The cells were measured using CCK-8 solutions at 450 nm by A SpectraMax M2e (Molecular Devices, San Jose, CA, USA). Cell viability was calculated for each well and the 50% growth inhibition (IC50) was determined. Assays were performed on at least three independent experiments.
The pancreatic cancer cells were plated in six-well plates and pretreated with Ibr-7 or DMSO as control for 24 h. Then the cells were exposed to the indicated doses (0, 2, 4, 6 Gy) of radiation respectively. The cells were then incubated with DMEM or RPMI-1640 medium supplementing 10% FBS at 37°C in a 5% CO2 atmosphere. After 14 days, the colonies were stained with 0.1% crystal violet (Sigma-Aldrich) in absolute methanol for 30 min. Colonies more than 50 cells were counted. The survival fraction (SF) was calculated as described previously\[18]\). SF = mean number of colonies / (plating efficiency × number of cells inoculated) in treated groups, the plating efficiency= (mean number of colonies / number of cells inoculated) in untreated groups. A multi-target click mathematical model was applied to simulate the cell survival fraction. We then calculated the the sensitization enhancement ratio (SER) as the dose (2 Gy) for the radiation divided by the dose (2 Gy) for radiation plus Ibr-7 (SER = SFIR/SFIR + Ibr-7). The mean lethal dose of cells (D0), extrapolation number (N) values, the quasithreshold dose (Dq) were also calculated according to the curve. Error bars were calculated as SD via the results of three independent experiments.

**Cell cycle assay**

PANC-1 and Capan2 cells were divided into four groups: The control group (DMSO), the Ibr-7 (2 µM) group, the radiation (2 Gy) group, The combination group. 5 × 10^5 cells were plated into 6-well plates, exposed to Ibr-7 for 24 h, then the cells exposed to 6-MV X-ray. After another 24 h, the cells were trypsinized and fixed with precooled 70% ethanol at 20°C overnight. Then the cells were added with PI (supplemented with RNase A) and incubated for 30 min at room temperature. Then the treated cells were detected by flow cytometry (Becton Dickinson, Franklin Lakes, NJ, USA). Error bars were calculated as SD via the results of three independent experiments.

**Apoptosis assay**

Cells were divided into four groups as mentioned above. Cells were plated into 6-well plates and pretreated with Ibr-7 for 24 h, then the cells were exposed at 2 Gy of radiation. After another 24 h, the cells were trypsinized and washed with PBS for twice. Then cell suspension was stained with 5 µL Annexin V-FITC and 5 µL PI solution and analyzed by flow cytometry (Becton Dickinson, Franklin Lakes, NJ, USA). Error bars were calculated as SD via the results of three independent experiments.

**Immunofluorescence**

3 × 10^4 cells were plated on coverslips in 24-well plates and pretreated with DMSO or Ibr-7 for 24 h. Then the cells were exposed to radiation (6 Gy). The cells were then incubated for 24 h and collected. The cells were washed in PBS for 3 times and fixed with 4% paraformaldehyde for 30 min. After permeabilizing cells with 0.5% Triton-100 for 15 min, the cells were blocked with 1% BSA, then incubated with anti-γ-H2AX antibody overnight at 4 °C. The cells were incubated with goat anti-rabbit IgG (FITC-labeled)
antibody (Beyotime) for 60 min, followed by incubation with DAPI for 5 min. Finally, the cells were photographed with immunofluorescence microscopy.

**Western blot analysis**

Cultured cells were collected and washed with PBS thrice. Total proteins extraction was conducted using RIPA lysing buffer containing phosphatase inhibitors and protease according to manufacturer instructions. A total of 20–40 µg of proteins were submitted to 10% SDS/PAGE and transferred to PVDF membrane. The membranes were then blocked with 5% skim milk for 1 h in room temperature. The membranes were incubated with primary antibodies against EGFR, p-EGFR and β-actin respectively overnight at 4 °C, and then incubated with secondary antibodies for 1 h at room temperature. The membranes were washed with TBST 3 times, the proteins were visualized by adding ECL.

**Plasmid transfection**

The pcDNA3.1-EGFR plasmid and empty vector were purchased from GenePharma. The plasmid was transfected into PANC-1 and Capan2 cells by jetPRIME according to the manufacturer’s protocol.

**Statistical analysis**

The results came from at least three independent experiments. All quantitative values are given as mean ± SD (standard deviation). Student’s t-test was used to determine the significance and statistically significant was defined when $p < 0.05$. Graphs were performed using GraphPad Software.

**Results**

**Ibr-7 induced cell growth inhibition in pancreatic cancer cells**

Ibr-7 is a newly synthesized derivative of ibrutinib that showed anti-tumor activity against various cancer cells compared with its the parental compound[15]. To determine the anti-proliferation effect of Ibr-7 in pancreatic cancer cells, both two pancreatic cancer cells were treated with increasing doses of ibrutinib or Ibr-7 (1.56-25 µmol/L) for 24 h, 48 h or 72 h respectively and changes in cell proliferation were examined by CCK-8 assay. Our data demonstrated that cell viability was markedly reduced by Ibr-7 in both dose- and time-dependent ways. The IC50 values of Ibr-7 at 48 h of treatment were 1.7 µmol/L, 2.5 µmol/L, while the IC50 values of ibrutinib were 20.8 µmol/L and 29.6 µmol/L for PANC-1 and Capan2 respectively. For both two cells, the IC50 values of Ibr-7 treatments were approximately one-tenth of ibrutinib’s IC50, indicating more effective anti-tumor activity of Ibr-7 compared to ibrutinib.
Ibr-7 pretreatment enhanced the radiosensitivity of pancreatic cancer cells

Based on Ibr-7’s effect in pancreatic cancer cells, we further investigated the radiosensitizing effect of Ibr-7 by clonegenic survival assay. Cells were seeded into 6-well plates and treated with 2 µmol/L Ibr-7, and then exposed to IR (2, 4, or 6 Gy). The survival curve was derived from the single-hit multi-target model \[ Y = 1 - \left( 1 - e^{-k*x} \right)^N \]. The sensitization enhancement ratio (SER) was measured according to the model. As shown in Fig. 2, pretreatment with Ibr-7 enhanced radiosensitivity of both PANC-1 (SER=1.63) and Capan2 cells (SER = 1.59). These results suggest that Ibr-7 enhanced radiosensitivity in pancreatic cancer cells. These results suggested that pretreatment with Ibr-7 enhanced radiosensitivity in pancreatic cancer cells.

Ibr-7 promoted G2/M cell cycle arrest of pancreatic cancer cells after radiation

To identify whether Ibr-7 could alter radiation induced cell cycle distribution, PI staining assay in PANC-1 and Capan2 cells was evaluated. PANC-1 and Capan2 cells were treated with 2 µmol/L Ibr-7 for 24 h, then irradiated for 6 Gy, the cells were collected after 24 h. As shown in Fig. 3, pretreatment with Ibr-7 showed little effect (16.12% ± 0.88% vs 8.32% ± 0.48%) while exposed to 6 Gy radiation in PANC-1 cells compared with controls in G2/M phase. Same results were detected in Capan2 cells (11.43% ± 2.07% vs 11.54% ± 1.87%). Cell exposed to radiation alone accumulated G2/M phase arrest compared with control. Markedly, compared with IR group, significant increase in G2/M were found in combination group in both PANC-1 (41.93% ± 1.07% vs 27.17% ± 0.04%, *p < 0.05) and Capan2 cell lines (46.6% ± 3.5% vs 23.92 ± 0.22%, *p < 0.05). Therefore, Ibr-7 increased radiation induced G2/M arrest in pancreatic cancer cells.

Ibr-7 increased IR-induced apoptosis in pancreatic cancer cells

To further investigate the potential mechanisms of radiation enhancement by Ibr-7, we next conducted the apoptosis assay by Annexin V-conjugated FITC and propidium iodide (PI) staining. The total apoptosis rate was calculated. The results indicated that Ibr-7 (11.67 ± 0.84%) and radiation (25.56 ± 1.07%) could increase apoptosis at 48 h and ibrutinib combined with radiation (50.2 ± 1.33%) increased the apoptosis ratio significantly (Fig. 4A, C), compared with the control group (5.57 ± 0.30%) in PANC-1 cells. The results were the same in Capan2 cells (31.37 ± 4.36% vs 5.93 ± 0.52%). It indicates that Ibr-7 enhances apoptosis which is induced by radiation.
Ibr-7 combined with radiation increases the DNA damage in pancreatic cancer cells

Since the major impact of radiation in cells is to induce DNA double strand breaks (DSBs) and stimulate DNA damage repair. Meanwhile, γ-H2AX is a rapid and sensitive cellular biomarker of the DSBs[19]. γ-H2AX (phosphorylated at C-terminal serine residue 139) forms nuclear foci in the region of the nascent DSBs, and subsequently endures de-phosphorylation after the repair of DSBs. Therefore, the numbers of γ-H2AX foci are used as a standard of the relative amount of DSBs and repair. To determine whether Ibr-7 could enhance radiation induced DNA damage, we conducted immunofluorescence to evaluate foci of γ-H2AX. As shown in Fig. 5, Ibr-7 significantly increased the number of γ-H2AX foci per cell at 24 h following 6 Gy radiation than that in radiation alone (12.0 ± 2.0 compared to 6.67 ± 1.53 in PANC-1, and 10.0 ± 1.0 compared to 6.0 ± 1.0 in Capan2, *p < 0.05). These results suggested that Ibr-7 combined radiation increased DNA damage.

Enhanced radiosensitivity of pancreatic cancer cells by Ibr-7 is p-EGFR dependent

Down-regulation of p-EGFR was considered to play a major role in the effect of ibrutinib. We therefore asked whether p-EGFR was the major target of ibrutinib derivative Ibr-7 in PANC-1 and Capan2 cells after radiation. As we expected, the expression of p-EGFR was decreased markedly in response to Ibr-7 alone or together with radiation (Fig. 6A), indicating the involvement of EGFR in Ibr-7 combined with radiation in PANC-1 and Capan2 cells.

Based on these results, we further analyze whether the downregulation of p-EGFR was required for apoptosis caused by Ibr-7 combined with radiation, we transfected pcDNA3.1-EGFR plasmid and detected the apoptosis. The overexpression of EGFR significantly decreased apoptosis in PANC-1 (22.00 ± 5.09 compared to 41.30 ± 2.12) and Capan2 cells (12.60 ± 3.96 compared to 29.95 ± 2.47) under the combination treatment (Fig. 6B, C). Together, it was suggested that the inhibition of p-EGFR might, conceivably, contribute to the radiosensitivity of Ibr-7.

| Cell line | Ibrutinib (μmol/L) | Ibr-7 (μmol/L) |
|-----------|--------------------|---------------|
| PANC-1    | 20.8               | 1.7           |
| Capan2    | 29.6               | 2.5           |

Table 1
The IC50 of Ibr-7 and ibrutinib in PANC-1 and Capan2 cells
### Discussion

Our findings demonstrate that Ibr-7 have the strong ability to enhance the radiosensitivity of pancreatic cancer cells. These effects of Ibr-7 is attributable to activate G2/M arrest and induce apoptosis, which resulted from the decrease of the expression of p-EGFR.

Ibrutinib has shown anti-tumor effects towards chronic lymhocytic leukemia[20–23]. It also shown growth inhibitory effect in solid cancers, but the results is limited[24–27]. Based on these, a series of derivatives are obtained. Ibr-7 is a promising candidate and exhibites great anti-tumor toward NSCLC and pancreatic cancer[15, 16]. Meanwhile, ibrutinib (10 µmol/L) has been shown radiosensitivity of pancreatic cancer cell lines in our previous study[17]. In this study, the radio-sensitivity of Ibr-7 towards pancreatic cancer cells were evaluated. The IC50 values of Ibr-7 against PANC-1 and Capan2 cells were 1–2 µmol/L. at a concentration of 2 µmol/L, Ibr-7 exhibited a stronger radiosensitivity in pancreatic cancer cells.

To explore the underlying molecular mechanisms responsible for effect of Ibr-7, we first analyzed the molecular structure of Ibr-7 and the relationship between ibrutinib and Ibr-7. Due to the similar structure of BTK, ibrutinib forms complex with BTK and then inhibits the BTK activity. Data also showed ibrutinib inhibits the expression of EGFR, HER2/neu, Her4/ErbB4. As the derivative of ibrutinib, Ibr-7 could also inhibit EGFR in NSCLC [28]. Whether Ibr-7 could exchange the expression of EGFR in pancreatic cancer cells is still unknown. In our study, Ibr-7 inhibited the expression of EGFR in pancreatic cancer cell lines as we expected. EGFR inhibition could increase the radiosensitivity in various tumors. Inhibition of EGFR/HER2 enhances radiosensitivity in pancreatic cancer[29, 30]. Targeting of EGFR and β1 integrin receptor showed efficient radiosensation in head and neck cancers.....based on these results, we

| K     | N       | D0/Gy | Dq/Gy | SF2      | SERSF2 |
|-------|---------|-------|-------|----------|--------|
| PANC-1 |         |       |       |          |        |
| IR    | 1.134   | 9.097 | 0.88  | 1.94     | 63.05±0.08 | -      |
| IR+Ibr| 1.492   | 9.432 | 0.67  | 1.50     | 38.69±0.02*| 1.63   |
| Capan2 |         |       |       |          |        |
| IR    | 1.044   | 13.89 | 0.96  | 2.53     | 84.20±0.02 | -      |
| IR+Ibr| 1.105   | 6.462 | 0.90  | 1.68     | 52.85±0.01***| 1.59 |

K, a passivation constant, derived directly from the fitting equation; N, extrapolation number, derived directly from the fitting equation; D0, mean lethal dose; Dq, quasithreshold dose; SF2, survival fraction (2 Gy); SER, sensitization enhancement ratio.

Table 2
The radiosensitization activity of PANC-1 and Capan2 cells treated with or without Ibr-7 (*p<0.05, ***p<0.001).
EGFR decreased the apoptosis induced by Ibr-7 and radiation, which demonstrated the important role of EGFR in radiosensitivity of Ibr-7.

AKT signaling pathway showed efficiency in many tumor progress. Ibrutinib (10 µmol/L) was capable of decreasing the p-AKT (S473) and its downstream genes, including mTOR, p-p70s6. However, Ibr-7 (2 µmol/L) did not showed significantly decrease the phosphorylation of AKT, mTOR and p70s6 (data not shown). As a result, the intrinsic mechanism remains to be further studied.

Radiation therapy leads to DNA lesions, DSBs (DNA double strand breaks) are regarded as the major impact of radiation[31]. The radiosensitivity largely depends on the ability to repair radiation-induced DNA damage. γ-H2AX (the phosphorylated form of H2AX) induced by radiation is involved in the event of DNA damage, and showed closely interrelated with DSBs, which served as a sensitive and classic cellular indicator of DSBs[32, 33]. The number of γ-H2AX indicated the relative amount of DSBs. In our study, we have found that Ibr-7 enhanced the foci of γ-H2AX induced by radiation. In response to DNA damage, the cell cycle progression is arrested. Our study found that Ibr-7 prolonged radiation induced G2/M phase arrest, which is consistent with the higher level of γ-H2AX at 24 h in Ibr-7 + radiation group that in radiation group alone. Based on these results, it indicated that Ibr-7 may affect the expression of p-EGFR, leading to DNA damage in pancreatic cancer cells in response to radiation.

In conclusion, this study showed that the Ibr-7 sensitized pancreatic cancer cells to radiation in vitro, and that the effect was likely attributable, at least in part, to the induction of DNA damage, subsequently stimulation of G2/M phase arrest and cell apoptosis that result from the downregulation of p-EGFR. To our knowledge, this is the first evidence showing that the radiosensitivity of Ibr-7 in pancreatic cancer cells. Our preclinical results not only suggested an effective strategy to improve the radiosensitivity in pancreatic cancer, but also provide a meaningful insights into the investigation of Ibr-7 in cancer treatment.

Conclusions

In summary, Ibr-7 enhanced radiation sensitivity in pancreatic cancer cells by promoting cell cycle arrest at G2/M phase, inducing cell apoptosis, and increasing DSBs induced by radiation. The mechanism investigation revealed that decrease of p-EGFR played an important role in its radiosensitizing effects. This study indicate that Ibr-7 may be a novel potential radiosensitizer for pancreatic cancer.

Declarations

Ethics approval and consent to participate

Not applicable
Availability of data and materials

All data generated or analyzed during this study are included in this published article and its Additional files.

Competing interests

The authors declare that they have no competing interests

Authors' contributions

LNM designed the study, prepared, edited and reviewed the manuscript.

TBQ, WF and YYY performed experimental studies. ZB, LQY did literature research, gave comments and reviewed the manuscript. TBQ designed the study and wrote the manuscript. All authors read and approved the final manuscript.

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Not applicable

Conflict of interest

The authors declare that there are no conflicts of interest.

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References

1. Miller KD, Nogueira L, Mariotto AB, Rowland JH, Yabroff KR, Alfano CM, Jemal A, Kramer JL, Siegel RL: Cancer treatment and survivorship statistics, 2019. CA: A Cancer Journal for Clinicians 2019,
2. Chu LC, Goggins MG, Fishman EK: **Diagnosis and Detection of Pancreatic Cancer.** *Cancer journal* 2017, **23**(6):333-342.

3. Arasaradnam RP, Wicaksono A, O’Brien H, Kocher HM, Covington JA, Crnogorac-Jurcevic T: **Noninvasive Diagnosis of Pancreatic Cancer Through Detection of Volatile Organic Compounds in Urine.** *Gastroenterology* 2018, **154**(3):485-487 e481.

4. Xu X, Xiao Y, Hong B, Hao B, Qian Y: **Combined detection of CA19-9 and B7-H4 in the diagnosis and prognosis of pancreatic cancer.** *Cancer biomarkers : section A of Disease markers* 2019, **25**(3):251-257.

5. Zhou H, Zhu Y, Wei F, Shao Y, Pan J, Wang G, Xu K, Cheng Y: **Significance of MUC2 gene methylation detection in pancreatic cancer diagnosis.** *Pancreatology : official journal of the International Association of Pancreatology* 2019, **19**(8):1049-1053.

6. Seshacharyulu P, Baine MJ, Soucek JJ, Menning M, Kaur S, Yan Y, Ouellette MM, Jain M, Lin C, Batra SK: **Biological determinants of radioresistance and their remediation in pancreatic cancer.** *Biochimica et biophysica acta Reviews on cancer* 2017, **1868**(1):69-92.

7. Nguyen AM, Zhou J, Sicairos B, Sonney S, Du Y: **Upregulation of CD73 Confers Acquired Radioresistance and is Required for Maintaining Irradiation-selected Pancreatic Cancer Cells in a Mesenchymal State.** *Molecular & cellular proteomics : MCP* 2020, **19**(2):375-389.

8. Jiang YH, You KY, Bi ZF, Li LT, Mo HQ, Liu YM: [The relationship between the radioresistance of pancreatic cancer cell SW1990 and the induction of the Epithelial-Mesenchymal Transition: an in vitro study]. *Zhonghua yi xue za zhi* 2018, **98**(12):939-943.

9. Deeks ED: **Ibrutinib: A Review in Chronic Lymphocytic Leukaemia.** *Drugs* 2017, **77**(2):225-236.

10. Gao W, Wang M, Wang L, Lu H, Wu S, Dai B, Ou Z, Zhang L, Heymach JV, Gold KA et al: **Selective antitumor activity of ibrutinib in EGFR-mutant non-small cell lung cancer cells.** *Journal of the National Cancer Institute* 2014, **106**(9).

11. Kim ES, Dhillon S: **Ibrutinib: a review of its use in patients with mantle cell lymphoma or chronic lymphocytic leukaemia.** *Drugs* 2015, **75**(7):769-776.

12. Hershkovitz-Rokah O, Pulver D, Lenz G, Shpilberg O: **Ibrutinib resistance in mantle cell lymphoma: clinical, molecular and treatment aspects.** *British journal of haematology* 2018, **181**(3):306-319.

13. de Porto AP, Liu Z, de Beer R, Florquin S, de Boer OJ, Hendriks RW, van der Poll T, de Vos AF: **Btk inhibitor ibrutinib reduces inflammatory myeloid cell responses in the lung during murine pneumococcal pneumonia.** *Molecular medicine* 2019, **25**(1):3.

14. Byrd JC, Hillmen P, O’Brien S, Barrientos JC, Reddy NM, Coutre S, Tam CS, Mulligan SP, Jaeger U, Barr PM et al: **Long-term follow-up of the RESONATE phase 3 trial of ibrutinib vs ofatumumab.** *Blood* 2019, **133**(19):2031-2042.

15. Zhang B, Wang L, Zhang Q, Yan Y, Jiang H, Hu R, Zhou X, Liu X, Peng J, Lin N: **The Ibr-7 derivative of ibrutinib exhibits enhanced cytotoxicity against non-small cell lung cancer cells via targeting of mTORC1/S6 signaling.** *Molecular oncology* 2019, **13**(4):946-958.
16. Yan Youyou ZB, Zhang Qi, Zhou Dongmei, Lin Nengming: Study on the Inhibitory Effects and Mechanism of New Small Molecular Kinase Inhibitors Ibr-7 on Human Pancreatic Cancer Capan-2 Cells (in Chinese). China Pharmacy 2019, 30(4):499-506.

17. Tan B, Huang Y, Zhang B, Lin N: The effect of ibrutinib on radiosensitivity in pancreatic cancer cells by targeting EGFR/AKT/mTOR signaling pathway. Biomedicine & pharmacotherapy = Biomedecine & pharmacotherapie 2020, 128:110133.

18. Shin YK, Park JS, Kim HS, Jun HJ, Kim GE, Suh CO, Yun YS, Pyo H: Radiosensitivity enhancement by celecoxib, a cyclooxygenase (COX)-2 selective inhibitor, via COX-2-dependent cell cycle regulation on human cancer cells expressing differential COX-2 levels. Cancer research 2005, 65(20):9501-9509.

19. Rogakou EP, Pilch DR, Orr AH, Ivanova VS, Bonner WM: DNA double-stranded breaks induce histone H2AX phosphorylation on serine 139. The Journal of biological chemistry 1998, 273(10):5858-5868.

20. Treon SP, Castillo JJ, Skarbnik AP, Soumerai JD, Ghobrial IM, Guerrera ML, Meid K, Yang G: The BTK inhibitor ibrutinib may protect against pulmonary injury in COVID-19-infected patients. Blood 2020, 135(21):1912-1915.

21. Mosna K, Ladicka M, Drgona L, Vranovska M, Hojsikova I, Tomasova R, Danihel L, Jr., Kyselovic J, Babal P: Ibrutinib treatment of mantle cell lymphoma complicated by progressive multifocal leukoencephalopathy. International journal of clinical pharmacology and therapeutics 2020, 58(6):343-350.

22. Lucas F, Larkin K, Gregory CT, Orwick S, Doong TJ, Lozanski A, Lozanski G, Misra S, Ngankeu A, Ozer HG et al: Novel BCL2 mutations in venetoclax-resistant, ibrutinib-resistant CLL patients with BTK/PLCG2 mutations. Blood 2020.

23. Kater AP, Melenhorst JJ: CAR-T and ibrutinib vs CLL: sequential or simultaneous? Blood 2020, 135(19):1611-1612.

24. Qiu Q, Li C, Song Y, Shi T, Luo X, Zhang H, Hu L, Yan X, Zheng H, Liu M et al: Targeted delivery of ibrutinib to tumor-associated macrophages by sialic acid-stearic acid conjugate modified nanocomplexes for cancer immunotherapy. Acta biomaterialia 2019, 92:184-195.

25. Prabaharan CB, Yang AB, Chidambaram D, Rajamanickam K, Napper S, Sakharkar MK: Ibrutinib as a potential therapeutic option for HER2 overexpressing breast cancer - the role of STAT3 and p21. Investigational new drugs 2019.

26. Chen J, Kinoshita T, Sukbuntheng J, Chang BY, Elias L: Ibrutinib Inhibits ERBB Receptor Tyrosine Kinases and HER2-Amplified Breast Cancer Cell Growth. Molecular cancer therapeutics 2016, 15(12):2835-2844.

27. Hong D, Rasco D, Veeder M, Luke JJ, Chandler J, Balmanoukian A, George TJ, Munster P, Berlin JD, Gutierrez M et al: A Phase 1b/2 Study of the Bruton Tyrosine Kinase Inhibitor Ibrutinib and the PD-L1 Inhibitor Durvalumab in Patients with Pretreated Solid Tumors. Oncology 2019, 97(2):102-111.

28. Wei L, Su YK, Lin CM, Chao TY, Huang SP, Huynh TT, Jan HJ, Wang-Peng J, Chiou JF, Wu AT et al: Preclinical investigation of ibrutinib, a Bruton's kinase tyrosine (Btk) inhibitor, in suppressing glioma invasion and angiogenesis. Acta neurochirurgica (Vienna) 2016, 7(43):69961-69975.
29. Anakura M, Nachankar A, Kobayashi D, Amornwichet N, Hirota Y, Shibata A, Oike T, Nakano T: Radiosensitivity Differences between EGFR Mutant and Wild-Type Lung Cancer Cells are Larger at Lower Doses. International journal of molecular sciences 2019, 20(15).

30. Poschau M, Dickreuter E, Singh-Muller J, Zscheppang K, Eke I, Liersch T, Cordes N: EGFR and beta1-integrin targeting differentially affect colorectal carcinoma cell radiosensitivity and invasion. Radiotherapy and oncology : journal of the European Society for Therapeutic Radiology and Oncology 2015, 116(3):510-516.

31. Lord CJ, Ashworth A: The DNA damage response and cancer therapy. Nature 2012, 481(7381):287-294.

32. Lobrich M, Shibata A, Beucher A, Fisher A, Ensminger M, Goodarzi AA, Barton O, Jeggo PA: gammaH2AX foci analysis for monitoring DNA double-strand break repair: strengths, limitations and optimization. Cell cycle 2010, 9(4):662-669.

33. Redon CE, Nakamura AJ, Zhang YW, Ji JJ, Bonner WM, Kinders RJ, Parchment RE, Doroshow JH, Pommier Y: Histone gammaH2AX and poly(ADP-ribose) as clinical pharmacodynamic biomarkers. Clinical cancer research : an official journal of the American Association for Cancer Research 2010, 16(18):4532-4542

Figures

Figure 1

Ibr-7 showed potent anti-proliferative activity against pancreatic cancer cells in dose- and time-dependent manner. CCK8 assay was performed to detect the inhibitory activity of Ibr-7 on the proliferation on pancreatic cancer cell line PANC-1, Capan2 cell lines for 24, 48 or 72 hours in vitro. Three independent experiments were performed and data were presented as mean ± SD (standard deviation).
Ibr-7 sensitized pancreatic cancer cells to radiation. Clonogenic cell survival assays were performed for PANC-1 and Capan2 cells that were treated with Ibr-7 (2 μmol/L) or DMSO for 24 h, then were exposed to different doses of γ-radiation. A multi-target click mathematical model simulated the cell survival fraction curve. For all assays. Results shown are the mean ± SD of 3 independent experiments. Significance was determined by Student’s t-test (*p<0.05 compared with combination group).
Figure 3

Ibr-7 promoted radiation induced G2/M phase arrest in pancreatic cancer cells. (A) Representative histograms showing cell cycle of PANC-1 and Capan2 cells in Ctrl, Ibr-7, Radiation and Combination groups. Cells were treated with Ibr-7 (2 μmol/L) for 24 h, exposed to 6 Gy of radiation and stained with PI after another 24 h. (B) The percentages of cell cycle phase in each group were quantified. Results shown are the mean ± SD of 3 independent experiments. Significance was determined by Student’s t-test (*p<0.05 compared with combination group).
Figure 4

Ibr-7 induced cell apoptosis in irradiated PANC-1 and Capan2 cells. (A) Representative histograms showing cell apoptosis of PANC-1 and Capan2 cells in Ctrl, Ibr-7, Radiation and Combination groups respectively. Cells were treated with Ibr-7 (2 μmol/L) for 24 h, exposed to 6 Gy of radiation and stained with Annexin V-FITC and PI after another 24 h. (B) The percentages of cell apoptosis in each group were quantified. Results shown are the mean ± SD of 3 independent experiments. Significance was determined by Student’s t-test (*p<0.05 compared with combination group).
Figure 5

Ibr-7 increased radiation induced DNA double-strand breaks. (A) The representative images of γ-H2AX foci immunofluorescence staining in PANC-1 and Capan2 cells treated with 6 Gy radiation with or without Ibr-7 at 24 h after radiation. (B) Cells with γ-H2AX foci were counted and quantified. Error bars indicated SEM. Significance was determined by Student’s t-test (**p<0.01, ***p<0.001 compared with combination group).
Figure 6

Involvement of p-EGFR in ibr-7 sensitive radiation induced apoptosis in pancreatic cancer cells. (A) Cells were pretreated with ibr-7 for 24 h and then exposed to radiation for another 24 h. Western blot analysis was performed to detect the expression of p-EGFR, EGFR, β-actin was used as a loading control. (B) Both PANC-1 and Capan2 cells were transfected with EGFR plasmid and empty vector. Cells were collected after 24 h transfection and the expression of EGFR were detected by western blot. (C) The ratio of p-EGFR/EGFR was quantified by densitometry based on immunoblot images. (D) The ratio of EGFR/β-actin
was quantified by densitometry based on immunoblot images. (E-F) The ratio of apoptosis in PANC-1 and Capan2 cells that had been transfected with EGFR plasmid or empty vector, then pretreated with Ibr-7 and exposed to radiation. The percentages of cell apoptosis were quantified. Results shown are the mean ± SD of 3 independent experiments. Significance was determined by Student's t-test (*p<0.05, **p<0.01, ***p<0.001).