Combination of 5-Fluorouracil with Epigenetic Modifiers Induces Radiosensitization, Somatostatin Receptor 2 Expression, and Radioligand Binding in Neuroendocrine Tumor Cells In Vitro

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Peptide receptor radionuclide therapy in advanced neuroendocrine tumors (NETs) demonstrates a limited objective response rate. The therapeutic efficacy might be further increased by peptide receptor chemoradionuclide therapy. In this preclinical study, we explored the effects of 5-fluorouracil plus the DNA methyltransferase inhibitor decitabine or the histone deacetylase inhibitor tacedinaline on NET cells in vitro. **Methods:** Human NET cell lines BON1 and QGP1 were treated with 5-fluorouracil alone or in combination with decitabine or tacedinaline, respectively. Radiosensitivity was tested in combination with γ-irradiation at doses of 0, 2, 4, or 6 Gy by colony formation assay. Somatostatin receptor type 2 (SSTR2) expression and ^{68}Ga-DOTATOC uptake by human NET cell lines were investigated by Western blot analysis and by a radioligand binding assay. **Results:** Treatment with 5-fluorouracil alone or in combination with decitabine or tacedinaline reduced tumor cell viability and induced apoptosis, enhanced radiosensitivity in BON1 and QGP1 cells, induced SSTR2 expression, and resulted in increased radioligand binding of ^{68}Ga-DOTATOC in NET cells. **Conclusion:** This preclinical study demonstrated that 5-fluorouracil alone or in combination with decitabine or tacedinaline caused radiosensitization of tumor cells, upregulation of SSTR2 expression in tumor cells, and increased radioligand binding of ^{68}Ga-DOTATOC to these tumor cells. These preclinical in vitro findings indicate that 5-fluorouracil in combination with epigenetic modifiers might be a putative strategy to improve the treatment efficacy of peptide receptor chemoradionuclide therapy in NET.

Key Words: neuroendocrine tumor (NET); peptide receptor radionuclide therapy (PRRT); peptide receptor chemoradionuclide therapy (PRCRT); 5-fluorouracil (5-FU); decitabine; tacedinaline

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Neuroendocrine tumors (NETs) frequently form distant metastases and require systemic treatment (*1*). An established treatment option in advanced NETs is peptide receptor radionuclide therapy (PRRT) with ^{177}Lu-DOTATATE (*1–3*).

PRRT in patients with NETs demonstrates a high disease control rate (*1–4*) and improves progression-free and overall survival (*1–4*). However, PRRT yields an objective response rate of only 20%–30%, and complete remissions are observed in only 2%–7% (*1–4*). Novel therapeutic strategies are needed to further improve PRRT efficacy in NETs.

One strategy to enhance PRRT efficacy is the use of radiosensitizers in peptide receptor chemoradionuclide therapy (PRCRT) (*3,5–8*). Clinical studies have suggested that PRCRT using chemotherapy with 5-fluorouracil, capecitabine or capecitabine and temozolomide causes an improved objective tumor response and disease control rate (*3,5–8*).

A prerequisite for the efficacy of PRRT in NETs is an abundance of somatostatin receptor type 2 (SSTR2) expression (*3*). Epigenetic regulation of gene expression (*9*) can be modified by histone deacetylase (HDAC) inhibitors and DNA methyl transferase (DNMT) inhibitors (*10*). The role of epigenetics has been investigated in NETs (*11*), and preclinical studies have revealed that DNMT inhibitors such as decitabine and HDAC inhibitors such as valproic acid or tacedinaline can upregulate the expression of SSTR2 and enhance somatostatin ligand binding to tumor cells (*12,13*). In these studies, decitabine and tacedinaline was demonstrated to exert synergistic effects (*12*).

In this study, we now investigated the effects of 5-fluorouracil alone and in combination with decitabine or tacedinaline on cell viability, radiosensitization, SSTR2 expression, and binding of ^{68}Ga-DOTATOC in different human NET cell lines.

MATERIALS AND METHODS

Cell Lines and Treatments

The human pancreatic NET BON1 cell line (*14*) was kindly provided by Prof. Rüdiger Göke, University of Marburg. The pancreatic islet tumor QGP1 cell line (*14*) was originally obtained from the Japanese Collection of Research Bioresources Cell Bank. The human, bronchopulmonary neuroendocrine NCI-H727 cell line (*14*) was obtained from the American Type Culture Collection. The human midgut carcinoid

cell line GOT1 (*14,15*) was kindly provided by Prof. Ola Nilsson, Sahlgrenska University Hospital. All cell lines were certified for identity by short-tandem repeat (STR) typing, performed by the German Biologic Resource Centre DSMZ. All cell lines used in this study were cultured and incubated as previously described (*16*).

5-FU, decitabine, and tacedinaline were purchased from Selleckchem, dissolved in dimethyl sulfoxide, and stored at −20°C. ^{68}Ga-DOTATOC was prepared using SomaKit TOC purchased from Advanced Accelerator Applications SA. ^{68}Ga was obtained by elution of a GalliaPharm ^{68}Ge/^{68}Ga generator by Eckert and Ziegler.

Cell Viability Assay

A cell viability assay was performed as described previously (*16*). NET cells were treated for up to 144 h before being subjected to the Cell Titer Blue cell viability assay (Promega). Cells were incubated with Cell Titer Blue solution, and fluorescence was measured at 560/590 nm using a GLOMAX plate reader (Promega). All experiments were performed in triplicates and repeated at least 3 times. The data were summarized as the percentage of control of the mean (±SEM).

Colony Formation Assay

The NET cells were seeded and treated for 24 h with 5-fluorouracil, decitabine, or tacedinaline at inhibitory concentration (IC) 10 or IC20 for each drug before being irradiated at doses of 0–6 Gy using a XStrahl RS225 radiation cabinet (XStrahl Inc.) and further cultured for 14 d (BON1 cells) or 21 d (QGP1 cells). After that, cells were fixed with 70% ethanol and stained with 0.3% methylene blue. Colonies with more than 50 cells were counted, and the surviving fractions (SFs) were calculated versus the plating efficiency. The survival curves were established by the linear-quadratic model, and the sensitization enhancement ratio (SER) was measured as follows: $SF = 1 - (1 - \exp[-D/D_0])^N$; and $SER = SER_{SF2}$ = control group SF2 value/treatment group SF2 value.

Immunofluorescent Staining for γH2AX and 53BP1

Immunofluorescence microscopy was performed using an Observer Z1 (Carl Zeiss). BON1 cells (40,000) were seeded into 24-well plates supplemented with coverslips and allowed to adhere for 24 h before being treated with either 500 nM decitabine or 1.0 μM 5-fluorouracil; dimethyl sulfoxide served as a control. After 24 h, drugs were removed by medium exchange and cells were irradiated at 2 Gy of dose. Cells were fixed in 3.7% isotonic formaldehyde at indicated time points and stained for phosphorylated histone H2AX (γH2AX, S139) and 53BP1. For microscopic analysis, 31 images were captured as z-stacks with an interstack distance of 250 nm. Deconvolution was performed using the ZEN software (Carl Zeiss, version 2.3). For kinetic analyses, the numbers of γH2AX foci found in 20 nuclei were counted by hand, and means ± SD were calculated for each condition of treatment.

Protein Extraction and Western Blot

Total cell protein extraction and Western blotting of 50 μg of total cell protein per lane were performed as previously described (*16*). The polyvinylidene difluoride membranes were first blocked in clear milk

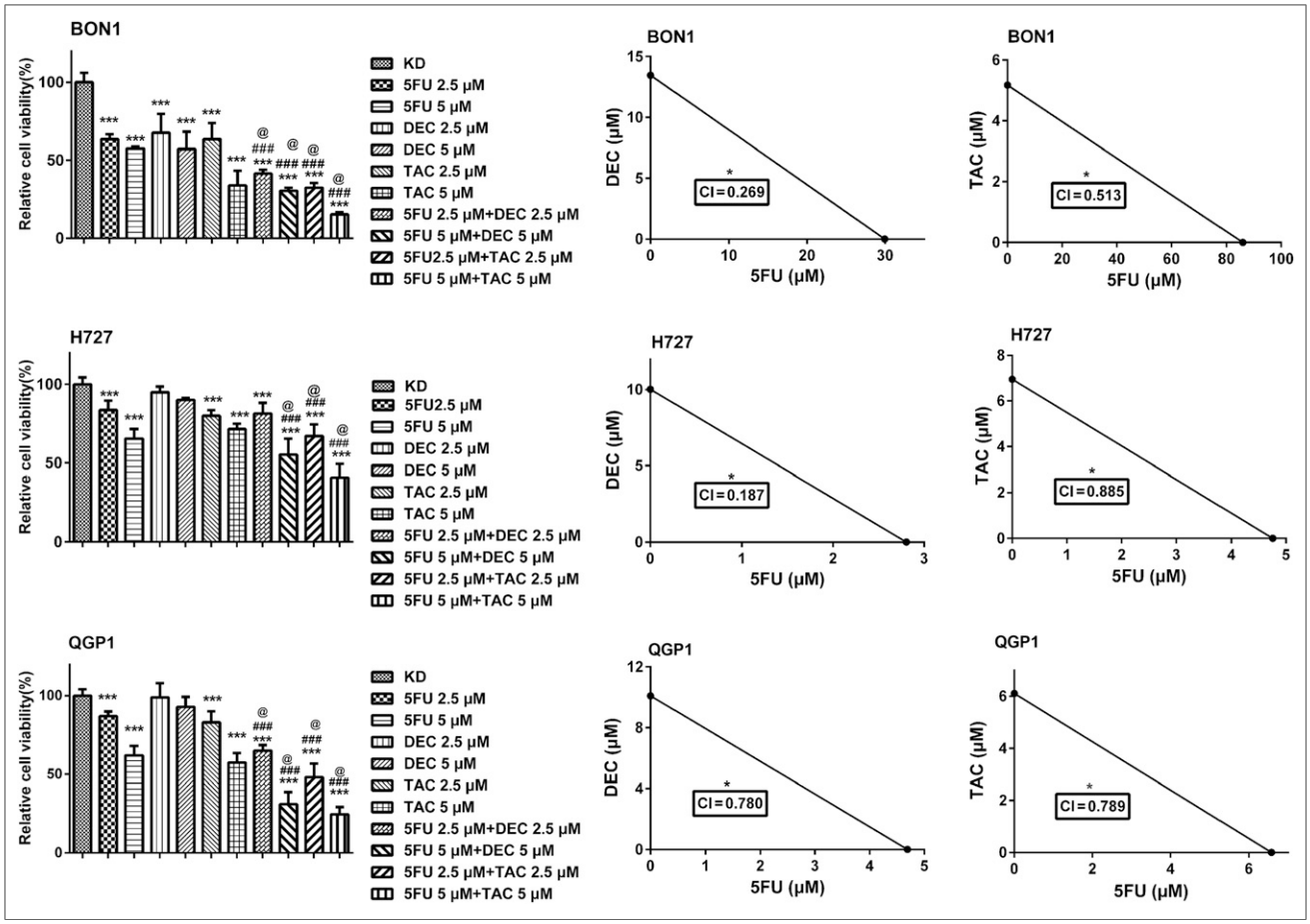

FIGURE 1. Effects of 5-fluorouracil (5-FU) in combination with decitabine (DEC) or tacedinaline (TAC) on NET cell viability. BON1, H727, and QGP1 cells were treated with different doses of 5-FU, DEC, or TAC for 144 h and then subjected to cell viability assay. $^{*}P < 0.05$ and $^{***}P < 0.001$ compared with that of controls. $^{\#\#\#}P < 0.05$ compared with 5-FU. $^{@}P < 0.05$ compared with monotreatment of DEC or TAC. Cell inhibition index (CI) and isobologram plots were calculated. KD = control DMSO.

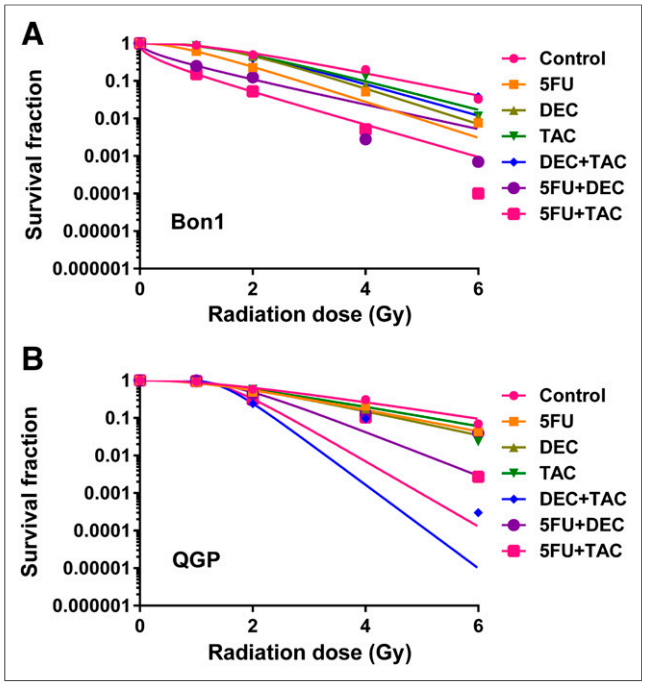

FIGURE 2. Effects of 5-fluorouracil (5-FU) in combination with decitabine (DEC) or tacedinaline (TAC) on NET cell colony formation after γ-irradiation. BON1 (A) and QGP1 (B) NET cells were grown and treated simultaneously with 5-FU and DEC or TAC plus γ-irradiation for 24 h and then subjected to colony formation assay.

blocking buffer (Pierce) and then incubated with a primary antibody against SSTR2 (Abcam), antibody against PARP, antibody against caspase-3 (all from Cell Signaling Technology), antibody against BCL2 (BD), and β-actin (Merck). Subsequently, the membranes were washed with TBST, incubated with horseradish peroxidase–conjugated goat antirabbit IgG or horse antimouse IgG (all from Cell Signaling Technology), and subsequently incubated with a chemiluminescence Western blotting detection system (Westar Supernova; Cyanagen). The chemiluminescence was detected by an imaging system (ECL Chemocam; Intas), and quantification was performed using the gel macros of ImageJ (National Institutes of Health).

Flow Cytometry

Flow cytometry was performed as previously described (*16*). Cell cycle phase distribution was analyzed using propidium iodide staining and flow cytometry (Accuri C6 Analysis; BD Biosciences). Cells were cultured in 6-well plates for 24 h. Subsequently, medium was replaced by fresh medium and cells were incubated with different compounds. After 72 h, cells were washed with phosphate-buffered saline and treated with 300 μL of trypsin for 5 min at 37°C. Cells were collected, washed twice, and resuspended in 350 μL of propidium iodide (Sigma-Aldrich).

^{68}Ga-DOTATOC Uptake Assay

The treated NET cells (~1 million NET cells per well) were incubated with the internalization medium (Dulbecco modified Eagle medium) at 37°C for 1 h and then added with 4 kBq (2.5 pmol) of ^{68}Ga-DOTATOC-labeled peptides before being further incubated for 0.5, 2, and 4 h. A 1,000-fold excess of nonlabeled DOTATATE was used for blocking to determine nonspecific internalization. At the end of incubation, the cells were treated with 1 M NaOH at 37°C for 10 min to detach the cells from the plates. The supernatant was collected, and radioactivity was measured using the γ-counter (PerkinElmer) and expressed as the percentage of measured to total added radioactivity and normalized to the protein levels.

Statistical Analysis

All experiments were performed in triplicate and repeated at least once, and the data were summarized as the mean ± SD (or SEM). Statistical analysis was performed using 1-way ANOVA followed by the Dunnett *t* test for multiple comparisons. A *P* value of less than 0.05 was considered statistically significant. Moreover, to assess the synergistic cytotoxic effects of the combined drug treatments on NET cells, we used CalcuSyn 2.1 software (Biosoft).

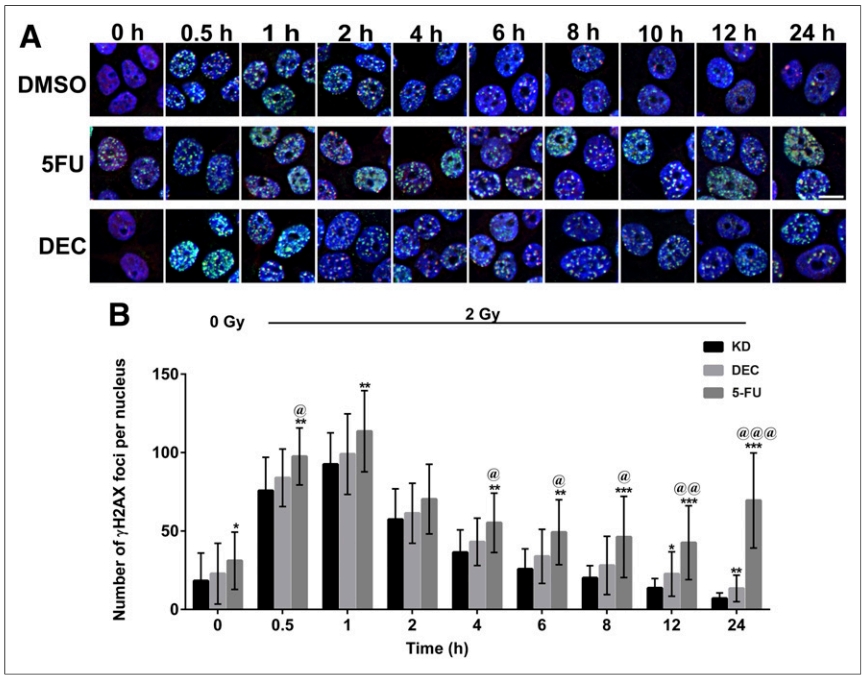

FIGURE 3. DNA double-strand-break repair was examined using γ-H2AX and 53BP1 DNA damage markers after administration of 5-fluorouracil (5-FU) or decitabine (DEC) with 2 Gy of γ-irradiation in BON1 cells. (A) Phospho-H2AX and 53BP1 immunofluorescence staining. Green = phospho-H2AX; red = 53BP1; blue = Hoechst 33342 (2 μg/mL). (B) Average number of foci for phosphoγ-H2AX after 2 Gy of γ-irradiation. Data represent mean ± SE foci of 3 independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ compared with that of controls. $^{@}P < 0.05$ (5-FU vs. DEC). KD = control DMSO.

RESULTS

Synergistic Effects of 5-Fluorouracil in Combination with Decitabine or Tacedinaline on NET Cell Growth and Apoptosis

Decitabine and tacedinaline were able to individually reduce NET cell growth in BON1, QGP1, and H727 cells in a dose-dependent manner (Supplemental Fig. 1; supplemental materials are available at http://jnm.snmjournals.org). The IC50 of decitabine and tacedinaline in BON1 cells was 15.19 and 3.58 μM, respectively. The IC50 of tacedinaline was 11.97 and 15.68 μM in QGP1 and H727 cells, respectively, whereas the IC50 of decitabine was not reached in these 2 cell lines within the investigated dose range.

Combined treatment with 5-fluorouracil plus decitabine or with 5-fluorouracil plus

tacedinaline (all drugs used at concentrations of 2.5 and 5.0 μM) caused synergistic reduction of cell survival in BON1, QGP1, and H727 cells (Fig. 1).

Flow cytometry analysis demonstrated a trend toward an increase in sub-G1 events during combined treatment with 5-fluorouracil plus decitabine or with 5-fluorouracil plus tacedinaline (Supplemental Fig. 2). Western blot analysis showed that treatment of BON1 and H727 cells with 5-fluorouracil, decitabine, or combined treatment with 5-fluorouracil plus decitabine or with 5-fluorouracil plus tacedinaline induced expression of the apoptosis-related protein-cleaved PARP and of cleaved caspase-3 levels (Supplemental Fig. 2). Finally, antiapoptotic Bcl-2 was reduced by 5-fluorouracil, decitabine, or 5-fluorouracil plus decitabine in BON1 and H727 cells (Supplemental Fig. 2).

Synergistic Effects of 5-Fluorouracil in Combination with Decitabine or Tacedinaline on Clonogenic Survival After γ-Irradiation

We next evaluated the effects of 5-fluorouracil in combination with decitabine or tacedinaline on the intrinsic radiosensitivity of NET cells. BON1 and QGP1 cells were treated with low doses of 5-fluorouracil (1.0 μM), decitabine (0.5 μM), or tacedinaline (2.5 μM) and subjected to colony formation assay. The SERs for 5-fluorouracil, decitabine, tacedinaline, decitabine in combination with tacedinaline, 5-fluorouracil in combination with decitabine, and 5-fluorouracil in combination with tacedinaline in BON1 cells were found to be 2.19, 1.10, 1.10, 1.27, 3.96, and 9.46, respectively, and 1.20, 1.12, 1.01, 2.42, 1.31, and 1.87, respectively, in QGP1 cells. Thus, the combination treatments of 5-fluorouracil plus tacedinaline and 5-fluorouracil plus decitabine were superior versus each single drug alone to reduce the clonogenic survival in NET cells after γ-irradiation (Fig. 2).

5-Fluorouracil and Decitabine Impair Efficient Repair of Irradiation-Induced DNA Double-Strand Breaks (DSBs)

The administration of 5-fluorouracil or decitabine in combination with γ-irradiation decreased the efficacy of DNA damage repair in these cells as revealed by γH2AX-/53BP1 staining and quantitative microscopic analysis (Fig. 3). Persistence of γH2AX-/53BP1 DNA damage foci even 24 h after irradiation with 2 Gy indicates a severe impairment in DNA damage repair efficacy (Fig. 3).

Synergistic Effects of 5-Fluorouracil in Combination with Decitabine or Tacedinaline on Induction of SSTR2 Expression

Expression of SSTR2 protein was induced by decitabine, tacedinaline, the combination of decitabine plus tacedinaline, and 5-fluorouracil alone or 5-fluorouracil in combination with decitabine or tacedinaline in BON1, GOT1, and H727 cells (Fig. 4).

The strongest upregulation of SSTR2 expression was observed in BON1 cells, where the combination of 5-fluorouracil plus decitabine was the most effective approach for upregulation of SSTR2 (8.56-fold increase; $P < 0.01$), followed by the combination of 5-fluorouracil plus tacedinaline (8.29-fold increase; $P < 0.01$) and the combination of decitabine plus tacedinaline (8.31-fold increase; $P < 0.01$), whereas single-drug treatments showed a less pronounced upregulation of SSTR2 expression (Fig. 4).

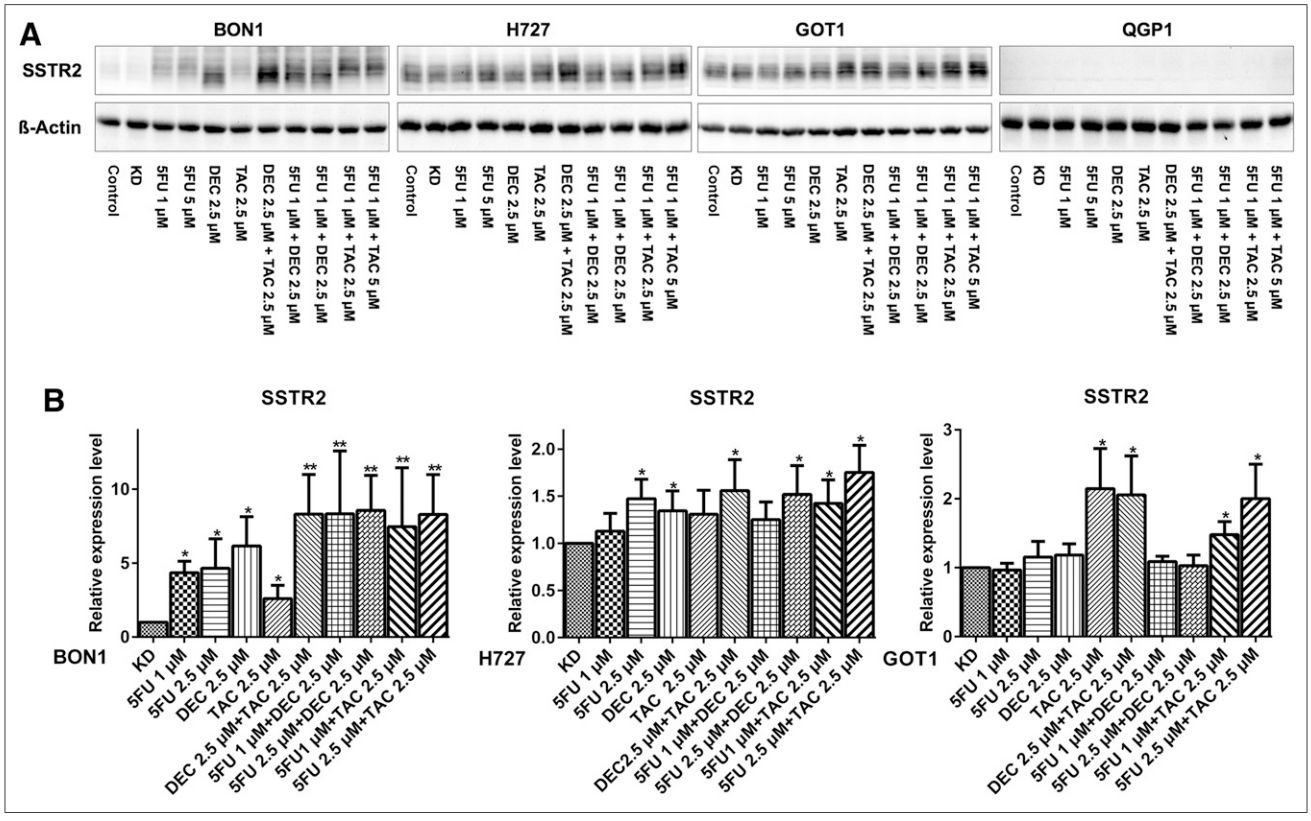

FIGURE 4. Effects of 5-fluorouracil (5-FU) in combination with decitabine (DEC) or tacedinaline (TAC) on SSTR2 expression. NET cells were grown and treated simultaneously with 5-FU and DEC or TAC for 72 h and then subjected to Western blot assay. (A) A representative Western blot is shown. (B) Relative protein expression levels were measured by densitometric analysis. Data are expressed as mean ± SEM. *$P < 0.05$ and **$P < 0.01$, compared with KD = control DMSO.

In H727 cells and especially in GOT1 cells, unstimulated baseline SSTR2 expression was higher than in BON1 cells (Fig. 4). However in H727 cells and in GOT1 cells, further upregulation of SSTR2 expression was maximally induced 2-fold (Fig. 4). In H727 cells, the highest effects were observed after combination of 5-fluorouracil with tacedinaline (1.75-fold increase; $P < 0.05$), combination of 5-fluorouracil plus decitabine (1.52-fold increase; $P < 0.05$), and combination of decitabine plus tacedinaline (1.56-fold increase; $P < 0.05$) (Fig. 4). In GOT1 cells, the combination of 5-fluorouracil with tacedinaline (2.0-fold increase; $P < 0.05$) and the combination of decitabine plus tacedinaline (2.06-fold increase; $P < 0.05$) were most effective, whereas 5-fluorouracil in combination with decitabine had only a minor effect (1.03-fold increase; $P > 0.05$) (Fig. 4). These data demonstrated that upregulation of SSTR2 after drug treatment might depend both on the applied tumor cell lines and on drug combinations.

Specific Radioligand Binding of ^{68}Ga-DOTATOC on NET Cells Is Induced by Combination of 5-Fluorouracil Plus Decitabine or 5-Fluorouracil Plus Tacedinaline

Next, we assessed whether the drug-induced upregulation of SSTR2 expression could also improve the specific radioligand binding of ^{68}Ga-DOTATOC on NET cells. Maximum 8- to 9-fold upregulation of SSTR2 expression was demonstrated in BON1 cells (Fig. 4). Correspondingly, 5-fluorouracil in combination with decitabine or tacedinaline induced a strong time-dependent increase in ^{68}Ga-DOTATOC intake by BON1 cells without reaching the plateau at their therapeutic dosages (Fig. 5). After 30 min of treatment, ^{68}Ga-DOTATOC showed 4.08% ± 0.34% and 2.25% ± 0.21% intake of the totally administered amounts, whereas at 4 h, the intake was increased to 19.06% ± 1.1% and 13.0% ± 0.43% in the 5-fluorouracil–plus–tacedinaline and 5-fluorouracil–plus–decitabine groups (vs. 2.96% ± 0.21% of the control), respectively ($P < 0.001$; Fig. 5). Competitive blocking experiments showed that the intake was specific (<1% intake was detected in all blockage groups). Upregulation of SSTR2 expression was only modest, at approximately 2-fold in H727 cells (Fig. 4). Correspondingly, in H727 cells, the combination of 5-fluorouracil plus decitabine or 5-fluorouracil plus tacedinaline revealed only a slight (~2-fold) increase in the intake of ^{68}Ga-DOTATOC (Fig. 5).

DISCUSSION

In this preclinical study in human NET cell lines in vitro, 5-fluorouracil in combination with the DNA methyltransferase (DNMT) inhibitor decitabine or the HDAC inhibitor tacedinaline demonstrated enhanced effects on inhibition of tumor cell proliferation and induction of apoptosis, radiosensitization, upregulation of SSTR2 expression, and upregulation of specific radioligand binding of ^{68}Ga-DOTATOC. These data suggest a putative role of 5-fluoropyrimidine–based chemotherapy in combination with epigenetic modifiers as a novel strategy to improve peptide receptor chemoradiotherapy (PRCRT) in NETs.

To improve the therapeutic efficacy of PRRT in NETs, different strategies have been investigated as follows: several clinical studies using fluoropyrimidine-based chemotherapy regimens for PRCRT in NET patients (*3,5–8*), preclinical studies using epigenetic modifiers as DNMT inhibitors/HDAC inhibitors to upregulate SSTR expression (*12,13*) and radioligand binding (*12,13*), preclinical studies using novel molecular target compounds for PRCRT (*17–19*), and variation of radiopharmaceuticals by the use of the α-emitters ^{215}Act or ^{213}Bis (*20*) or by the use of somatostatin antagonists (*21*).

In preclinical studies, Taelman et al. (*12*) and Veenstra et al. (*13*) reported that several DNMT inhibitors and HDAC inhibitors induce SSTR mRNA transcription and protein expression and enhance specific SSTR-mediated radioligand binding of NET cells. The combination of decitabine plus tacedinaline had additive effects on SSTR expression and radioligand binding in a mouse model (*12*). Our data are supportive and also demonstrate additive effects of a combination of decitabine plus tacedinaline on SSTR2 expression (Fig. 4).

As novel findings, we demonstrate 5-fluorouracil in combination with decitabine or tacedinaline to enhance antiproliferative effects (Fig. 1) and induction of apoptosis (Supplemental Fig. 2), radiosensitization (Figs. 2 and 3), upregulation of SSTR2 expression (Fig. 4), and radioligand binding to tumor cells (Fig. 5). These effects might possibly enhance the efficacy of PRCRT in NETs.

5-fluorouracil, DNMT inhibitors, and HDAC inhibitors radiosensitize cancer cells by suppressing DNA repair activity (*22–24*).

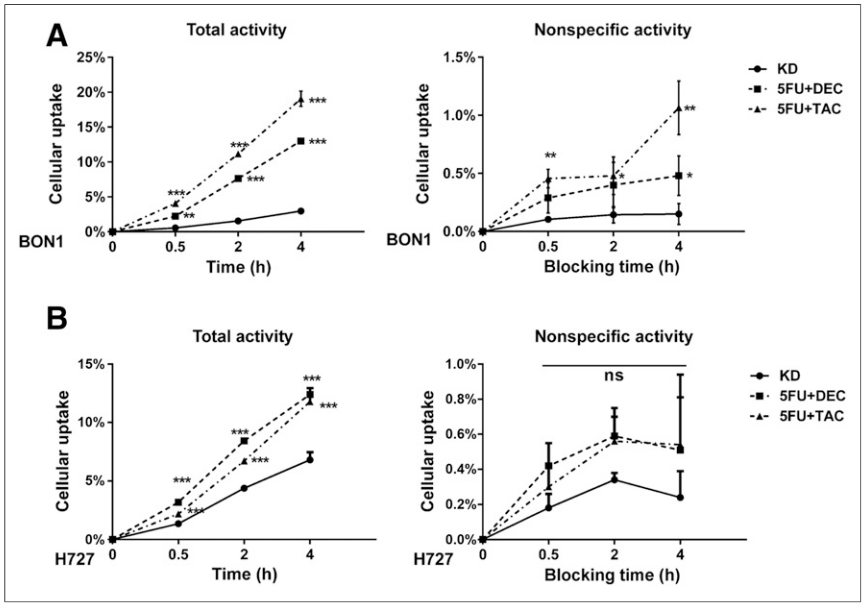

FIGURE 5. Effects of 5-fluorouracil (5-FU) in combination with decitabine (DEC) or tacedinaline (TAC) on NET cell intake of SSTR2. BON1 cells (A) and H727 cells (B) were treated simultaneously with 5-FU in combination with DEC or TAC plus ^{68}Ga-DOTATOC for 30 min, 2 h, and 4 h and then subjected to cell intake assay. Total internalized activity of DOTATOC and nonspecific uptake in NET cells blocked with nonlabeled DOTATATE are shown. Mean intake is expressed as mean ± SEM of triplicate experiments. KD = control DMSO.

Fluoropyrimidine-mediated radiosensitization is extensively used in chemoradiotherapy in solid cancers (*25,26*).

The DNMT inhibitors azacitidin and decitabine are approved in hematologic malignancies (*10,27,28*) and are under investigation in solid malignancies. The HDAC inhibitor tacedinaline has been investigated in clinical phase 1/2 trials. Currently, other HDAC inhibitors such as entinostat, mocetinostat, givinostat, resminostat, quisinostat, pracinostat, or tefinostat are in clinical trials (*10,29*).

DNMT inhibitors and HDAC inhibitors are extensively examined as epigenetic modifiers, radiosensitizers, chemosensitizers, and sensitizers to immunotherapy in oncology (*10,26,30*). There are numerous preclinical studies on the combination of epigenetic modifiers with fluoropyrimidine-based chemotherapy in various cancers (*26,30*). However, so far, there are only limited clinical phase I/II data available on the tolerability and toxicity of a fluoropyrimidine-based chemotherapy in combination with DNMT inhibitors or HDAC inhibitors (*30*).

Upregulation of SSTR expression by DNMT or HDAC inhibitors has been reported in human NET cell lines (*12,13*) but also in neuroendocrine prostate cancer LNCaP cells (*31*), human hepatocellular carcinoma (*32*), small cell lung cancer (*33*), and pancreatic adenocarcinoma (*34*) cell lines. The upstream promoter of the human SSTR2 is regulated by epigenetic modifiers such as DNMT inhibitors or HDAC inhibitors (*13,35*). However, to our knowledge, no data on the regulation of SSTR gene expression by 5-fluorouacil or other fluoropyrimidine agents in NETs have been previously reported. A major limitation of our study is the fact that in vitro radioligand binding has been investigated only by the PET imaging tracer ^{68}Ga-DOTATOC and not by a therapeutic radiotracer such as ^{177}Lu-DOTATATE. It might be questionable whether the theranostic results of the ^{68}Ga PET compound can be readily transferred into a therapeutic setting using ^{177}Lu. On the other hand, a high SUV of ^{68}Ga-DOTATOC PET has been reported to predict response probability in NET patients (*36–38*).

CONCLUSION

These preclinical data indicate that the combination of 5-fluorouracil plus epigenetic modifiers such as DNMT inhibitors or HDAC inhibitors might be a putative approach to enhance the efficacy of PRRT, as they combine radiosensitizing effects with the upregulation of SSTR2 expression and specific radioligand binding to the tumor cells. Further preclinical in vivo studies and clinical phase 1/2 trials will be needed to evaluate the proof of concept, tolerability, toxicity, and potential therapeutic efficacy combining the conventional chemotherapeutic drug 5-fluorouracil and epigenetic modifiers in PRCRT.

DISCLOSURE

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KEY POINTS

QUESTION: Novel strategies for peptide receptor chemoradionuclide therapy (PRCRT)—how to upregulate somatostatin receptor 2 (SSTR2) expression and cause radiosensitization in neuroendocrine tumor cells in vitro?

PERTINENT FINDINGS: In human neuroendocrine tumor cell lines in vitro, 5-fluorouracil alone or in combination with the epigenetic modifiers decitabine or tacedinaline caused significant effects on radiosensitization of tumor cells, upregulation of SSTR2 expression in tumor cells, and increased radioligand binding of ^{68}Ga-DOTATOC to these tumor cells.

IMPLICATIONS FOR PATIENT CARE: Further preclinical studies and clinical trials are requested.

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