

## Identification and Evaluation of Potential Anti-cancer Drugs on Human Neuroendocrine Tumor Cell Lines

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**Abstract.** The aim of this study was to investigate drug sensitivity in neuroendocrine tumor cell lines. Materials and Methods: In vitro drug sensitivity screening was performed using the fluorometric microculture cytotoxicity assay in one human pancreatic carcinoid and two human bronchial carcinoid cell lines. In addition, a normal human retinal pigment epithelial cell line was used for comparison. A total of 18 drugs with different mechanisms of action were tested. Results: The most active agents were brefeldin A, emetine, bortezomib and idarubicin, having  $IC_{50}$  values  $<1 \mu M$  in all four cell lines. In addition, the three tumor cell lines showed sensitivity for sanguinarine, Bay11-7085, mitoxantrone, doxorubicin,  $\beta$ -lapachone, NSC 95397 and CGP-74514A. Conclusion: The cell lines were sensitive for several drugs acting in different ways, covering a broad spectrum of mechanisms of action. Some of these compounds may possibly be used in clinical trials and show therapeutic effect in patients with neuroendocrine tumors.

Bronchial carcinoids are divided into typical and atypical. Typical bronchial carcinoids are more benign than atypical carcinoids, but both types are able to metastasize to regional lymph nodes or distantly to the liver, bones or brain (1, 2). Patients with typical carcinoids have an excellent prognosis and rarely die of the tumor. On the other hand, patients with atypical carcinoids have a higher rate of metastatic disease and survival is significantly reduced (3, 4). Our experience of patients with metastatic bronchial carcinoids indicated that there were poor response rates with the available treatments (5).

Medical treatment of metastatic endocrine pancreatic tumors includes different chemotherapy combinations and

biotherapy, such as with alpha-interferon and somatostatin analogs. Streptozocin combined with doxorubicin or 5-fluorouracil has generated partial remissions in 40-60% of the patients giving a median survival of two years in patients with advanced disease. Cisplatin plus etoposide has also demonstrated significant antitumor effect in patients with endocrine pancreatic tumors. Alpha-interferon causes significant tumor reduction in about 15% of patients with long duration, up to three years. Octreotide rarely leads to objective responses (6).

Even if an initial response is obtained in patients with malignant endocrine pancreatic tumors, as well as bronchial carcinoids treated with chemotherapy or biotherapy, resistance to treatment sooner or later occurs. There is, thus, a need for better treatments in patients with malignant neuroendocrine tumors.

Fluorometric microculture cytotoxicity assay (FMCA) is a short-term semi-automatic method based on the measurement of fluorescence of fluorescein from the conversion of fluorescein diacetate (FDA) by living cells. FMCA has been used in the past for testing drug sensitivity in fresh cells from patients with various diagnoses, as well as in human tumor cell lines (7, 8).

In the present study, we focused on finding more efficient treatments for patients with metastatic neuroendocrine tumors and used FMCA to test the activity of several drugs with various mechanisms of action in three neuroendocrine tumor cell lines, as well as in one normal epithelial cell line.

An annotated compound library consisting of 1,280 well-characterized compounds was screened in neuroendocrine tumor cell lines. The screening was used to identify compounds of interest for further evaluation and mechanism studies.

### Materials and Methods

*Screening of an annotated compound library.* One thousand, two hundred and eighty drugs obtained from the LOPAC<sub>1280</sub>™ library (Sigma Aldrich, St. Louis, MO, USA) were first evaluated. The

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Table I. The tested drugs, mechanisms of action and solvents.

Drug	Solvent	Mechanism of action
Apomorphine	Sterile water	Non-selective dopamine receptor agonist
Bay 11-70-85	DMSO	Inhibits cytokine induced IkBα (inhibitor of NF-κB) phosphorylation
Bortezomib	DMSO	Proteasome inhibitor-ubiquitin-proteasome pathway
Brefeldin A	Ethanol, 95%	Inhibitor of protein translocation from the endoplasmic reticulum (ER) to the Golgi apparatus
Camptothecin	DMSO	DNA topoisomerase I inhibitor
Cantharidin	DMSO	Protein phosphatase 2A inhibitor
CGP- 74514A	DMSO	Cyclin-dependent kinase-1 (Cdk1) inhibitor
Colchicine	Sterile water	Inhibitor of tubulin (prevents tubulin polymerization)
Doxorubicin	PBS	Inhibitor of DNA topoisomerase II
Emetine	Sterile water	Protein synthesis inhibitor at the level of translation
Idarubicin	Sterile water	Inhibitor of DNA metabolism
β-Lapachone	DMSO	Inhibition or activation of DNA topoisomerase and inhibition of NF-κB activity
Mitoxantrone	Ethanol, 95%	Inhibitor of DNA metabolism, DNA synthesis inhibitor
NSC 95397	DMSO	Selective, irreversible Cdc25 dual specificity phosphatase inhibitor
Ouabain	Sterile water	Inhibitor of Na <sup>+</sup> /K <sup>+</sup> -ATPase
Parthenolide	DMSO	Inhibits serotonin release from platelets
Sanguinarine chloride	Methanol	Inhibitor of Na <sup>+</sup> /K <sup>+</sup> -ATPase
Vincristin	PBS	Inhibitor of tubulin (inhibit microtubule assembly)

DMSO, dimethyl-sulphoxide; PBS, phosphate-buffered saline, pH 7.4.

library was screened at 10 μM in three tumor cell lines: atypical bronchial carcinoid (NCI-H720), typical bronchial carcinoid (NCI-H727) and pancreatic carcinoid (BON-1). Drugs with a survival index above 60% were eliminated from further studies. The survival index (SI) was defined as the fluorescence of experimental wells as a percentage of control wells, with blank values subtracted from both: SI = 100 x [(treated cells - blank)/(control cells - blank)]. Thus, a low SI value indicates a high cytotoxic effect. The primary screening resulted in 18 candidate drugs with SI-values of less than 60%. These drugs were chosen for further dose-response experiments in the three tumor cell lines. For comparison, a normal human retinal pigment epithelial cell line, hTERT-RPE1 was also studied. The activity of the compounds was determined by their IC<sub>50</sub> values. Compounds with IC<sub>50</sub> values <10 μM were selected as active.

**Preparation of compound library for primary screening.** The LOPAC<sub>1280</sub>™ library (Sigma-Aldrich) consists of 1,280 pharmacologically active compounds in 16 racks each containing 80 drugs dissolved in dimethyl sulphoxide (DMSO) to 10 mM. The drugs were transferred to 96-well plates and further diluted with phosphate-buffered saline (PBS) to obtain stock solutions of 100 μM from which four different 384-well plates for screening were prepared. In all steps, the Biomek 2000 pipetting station connected to a plate stacker Carousel (Beckman Coulter Inc., Fullerton, CA, USA) in a safety cabinet (Bigneat, Hampshire, UK) was used. Dose-response plates containing the drugs in duplicate were prepared at concentrations of 0.01 to 100 μM, or 0.001 to 10 μM, using the same robotic system. The plates were stored at -70°C until further use.

**Cell lines.** The human pancreatic carcinoid cell line, BON-1 wt (derived from a lymph node metastasis of a human pancreatic carcinoid tumor), was cultured in a 1:1 nutrient mixture of Dulbecco's Modified Eagle's Medium (DMEM) and Kaighn's modification medium (F12K) (Invitrogen, Sweden). The normal

Table II. In vitro sensitivity of the drugs with IC<sub>50</sub> values <10 μM in the tumor cell lines

Drug	Cell line IC <sub>50</sub> (μM)			
	NCI-H720	NCI-H727	BON-1	hTERT-RPE1
Brefeldin A	0.071	0.092	0.52	0.29
Emetine	0.094	0.15	0.11	0.40
Bortezomib	0.55	0.63	0.38	0.73
Idarubicin	0.71	0.87	0.99	0.26
Sanguinarine	0.57	1.0	1.8	1.5
Bay 11-7085	1.6	3.4	2.2	0.97
Mitoxantrone	1.6	3.5	2.1	0.69
Doxorubicin	1.4	5.4	3.4	2.7
β-Lapachone	2.1	2.7	3.0	3.2
CGP-74514A	2.2	3.2	1.9	13.0
NSC 95397	1.4	8.3	8.9	3.5

H720, atypical bronchial carcinoid cell line; H727, typical bronchial carcinoid cell line; BON-1, pancreatic carcinoid cell line; hTERT-RPE1, normal human retinal pigment epithelial cell line.

human retinal pigment epithelial cell line hTERT-RPE1 (a human RPE cell line that stably expresses human telomerase reverse transcriptase (hTERT)) was cultured in DMEM.

The human bronchial carcinoid cell line NCI-H727 (a more differentiated phenotype related to small cell lung cancer) and the human atypical bronchial carcinoid cell line NCI-H720 were obtained from ATCC (LGC Promochem, Sweden) and maintained in RPMI-1640 medium (Invitrogen, Sweden). All four cell lines were supplemented with 10% heat-inactivated fetal calf serum (FCS), 1% glutamine and 1% penicillin/streptomycin (Sigma Aldrich) and cultured in a 5% CO<sub>2</sub> humidified atmosphere at 37°C.

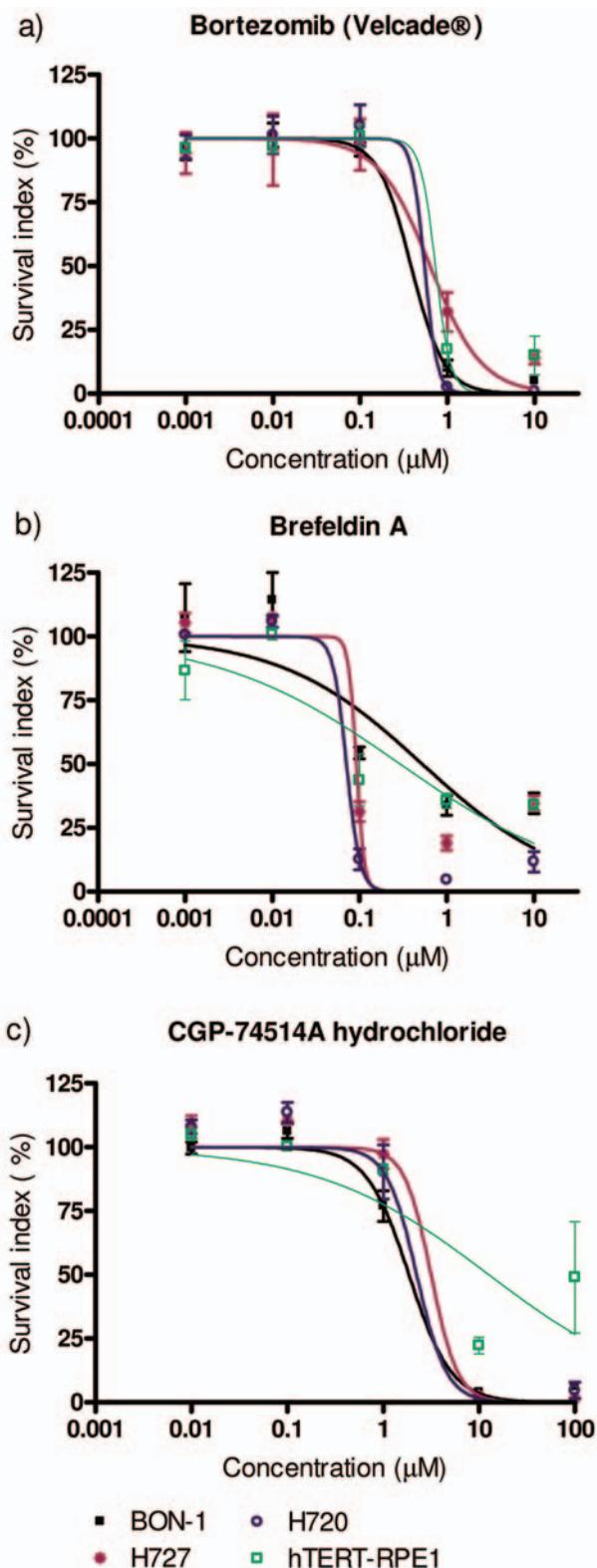


Figure 1. Effect on cell survival of (a) Bortezomib, (b) Brefeldin A and (c) CGP-74514A as a single drug in H720, H727, BON-1 and hTERT-RPE1 cell lines with continuous exposure for 72 h. Data are presented as mean value  $\pm$  SEM from three independent experiments.

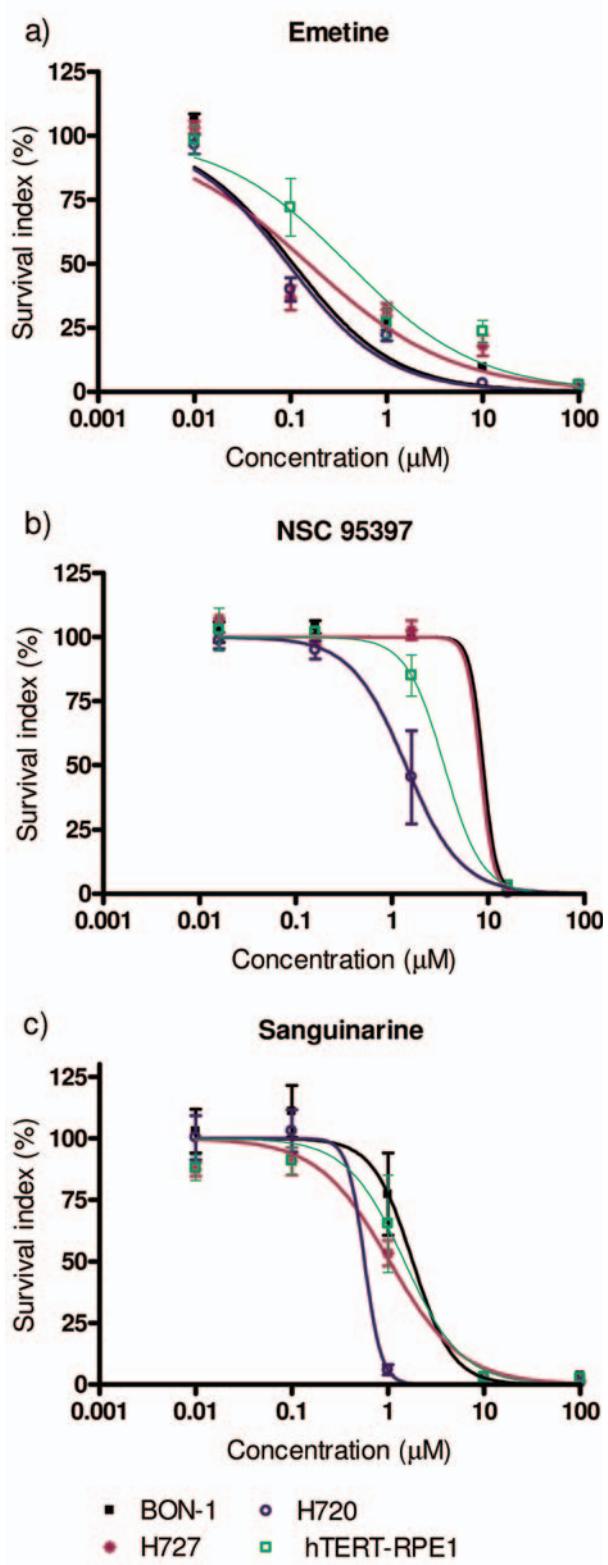


Figure 2. Effect on cell survival of (a) Emetine, (b) NSC 95397 and (c) Sanguinarine as a single drug in H720, H727, BON-1 and hTERT-RPE1 cell lines with continuous exposure for 72 h. Data are presented as mean value  $\pm$  SEM from three independent experiments.

**Reagents and drugs.** The 18 drugs selected from the initial screening, their mechanisms of action and the solvents they were made up in are shown in Table I. Drugs were purchased from Sigma-Aldrich. Doxorubicin was supplied by the local pharmacy (Uppsala, Sweden). The drugs were dissolved in PBS, DMSO, ethanol, methanol or sterile water to a stock concentration of 10 mM and further diluted with sterile water or PBS. All drugs were tested at five 10-fold dilutions ranging from 0.01 to 100  $\mu$ M, or 0.001 to 10  $\mu$ M, for the tumor cell lines and the epithelial cell line hTERT-RPE1, respectively.

**FMCA.** FMCA, described in detail previously (9), is based on the measurement of fluorescence generated from the hydrolysis of FDA to fluorescein by cells with intact plasma membranes. FDA (Sigma-Aldrich) was dissolved in DMSO to 0.5 mg/ml, kept frozen as a stock solution and protected from light. Cells were seeded in the drug-prepared 384-well plates using the pipetting robot Precision 2000 (Bio-Tek Instruments Inc., Winooski, VT, USA). The number of cells per well were 5,000. Two columns without drugs served as controls and one column with medium only served as blanks.

The plates were incubated for 72 h and then transferred to an integrated SAIGAN Core System for High Throughput Screening (Beckman Coulter Inc.) consisting of an ORCA robot (Beckman Coulter) with a CO<sub>2</sub> incubator (Cytomat 2C, Kendro, Sollentuna, Sweden), dispensor module (Multidrop 384, Titertek, Huntsville, AL, USA), washer module (ELx 405, Bio-Tek Instruments Inc.), delidding station, plate hotels, barcode reader (Beckman Coulter), liquid handler (Biomek 2000, Beckman Coulter) and a multipurpose reader (FLUOstar Optima, BMG Labtech GmbH, Offenburg, Germany). The plates were washed, FDA added and after 50-70 min of incubation, the fluorescence, which is proportional to the number of living cells, was measured. The cell survival was presented as SI. Quality criteria for successful assay required a fluorescence signal in control wells equal to or more than 5 times the mean blank value and a mean coefficient of variation (CV) in control wells of less than 30%. Only assays which met these criteria are included in the results reported here. IC<sub>50</sub> (50% inhibitory concentration) values were calculated from survival-concentration curves using non-linear regression analysis using Graph Pad Prism software (Graph Pad Software Inc., San Diego, CA, USA).

**Statistical analysis.** Statistical analysis was performed using the GraphPad Prism software. Comparison of activity in the cell lines was made with a two-way Anova test with Bonferroni's *post-hoc* test and an unpaired Student's *t*-test. The level of statistical significance was set to *p*<0.05.

## Results

When screening the annotated library at 10  $\mu$ M, 18 compounds resulted in a survival index of less than 60% in the tumor cell lines (not shown). In the further dose-response experiments, 11 out of 18 compounds were considered as active (*i.e.*, IC<sub>50</sub>< 10  $\mu$ M) and the IC<sub>50</sub>-values for these eleven drugs are shown in Table II; dose-response curves for six out of the eleven drugs are shown in Figures 1 and 2.

Brefeldin A, emetine, bortezomib and idarubicin were the most active agents *in vitro*, with IC<sub>50</sub> values <1  $\mu$ M in all four

cell lines, while sanguinarine had IC<sub>50</sub> values between 0.5  $\mu$ M and 2  $\mu$ M. In addition, Bay 11-7085, mitoxantrone, doxorubicin,  $\beta$ -lapachone, CGP-74514A and NSC 95397 had IC<sub>50</sub> values <10  $\mu$ M in all three tumor cell lines.

The atypical bronchial carcinoid cell line was more sensitive to doxorubicin (*p*<0.05), NSC 95397 (*p*<0.001) and sanguinarine (*p*<0.001) than the typical bronchial carcinoid cell line, and more sensitive to brefeldin A (*p*<0.001), doxorubicin (*p*<0.01), NSC 95397 (*p*<0.001) and sanguinarine (*p*<0.001) than the pancreatic carcinoid cell line. The atypical bronchial carcinoid cell line was also more sensitive to brefeldin A (*p*<0.01), CGP-74514A (*p*<0.01), emetine (*p*<0.001), NSC 95397 (*p*<0.001) and sanguinarine (*p*<0.001) than the normal human retinal pigment epithelial cell line. The typical bronchial carcinoid and pancreatic carcinoid cell lines were significantly more sensitive to emetine (*p*<0.001) and CGP-74514A (*p*<0.01) than the normal human retinal pigment epithelial cell line.

## Discussion

Our study demonstrated that Bay 11-7085, bortezomib, brefeldin A, CGP-74514A, doxorubicin, emetine, idarubicin,  $\beta$ -lapachone, mitoxantrone, NSC 95397 and sanguinarine showed antitumor effect in the human bronchial and pancreatic carcinoid cell lines *in vitro*. The most active agents were brefeldin A, emetine, bortezomib, idarubicin and sanguinarine, which all demonstrated IC<sub>50</sub> values <1  $\mu$ M in the two bronchial carcinoid cell lines. Since bronchial carcinoids are frequently resistant to conventional chemotherapy, these five agents are interesting candidates for further studies, either alone or, since they have different mechanisms of action, in various combinations.

Bortezomib (Velcade<sup>®</sup>), a proteasome inhibitor, has shown activity in early clinical trials among patients with Non-Hodgkin's lymphoma and multiple myeloma. In a phase II trial, 50% of patients with recurrent myeloma who received 1.3 mg/m<sup>2</sup> Bortezomib responded with complete inhibition of myeloma cell growth (10). Our study with bortezomib showed 50% cell death at low concentrations of pancreatic and bronchial carcinoid cells, indicating potent activity in endocrine tumors. The lack of a therapeutic window for bortezomib (*i.e.*, same IC<sub>50</sub> for normal and tumor cells) in our study could be attributed to our choice of a normal cell line, but could also reflect the observed clinical side-effects.

Doxorubicin has, despite its cardiotoxic effect (11-13), an important role in the treatment of neuroendocrine tumors. Mitoxantrone, another anthracycline, was about equally effective as doxorubicin in all three tumor cell lines studied. Medical treatment of patients with metastatic neuroendocrine tumors is usually palliative. If our results are valid in clinical conditions, doxorubicin may be substituted with mitoxantrone, making long term treatment possible due to lower

cardiotoxicity (14, 15). Mitoxantrone is, thus, an interesting drug for clinical studies in neuroendocrine tumor patients.

Emetine and CGP-75414A were more effective in the tumor cell lines than in the retinal pigment endothelial cell line. In addition brefeldin A, NSC 95397 and sanguinarine were more effective in the atypical carcinoid cell line than in retinal pigment epithelial cell line. This may implicate a clinical antitumor effect with less toxicity to normal tissues. On the other hand, part of the antitumor effect of various agents in patients may possibly be related to antiangiogenesis due to the effect on endothelial cells of the tumor vasculature.

There is a need for new therapies in patients with neuroendocrine tumors. The cost of bringing new drugs to the clinic is considerable and it is necessary to reduce the time and cost of their development. Although FMCA is a better predictor of drug resistance than drug sensitivity, this method may predict objective tumor responses in breast cancer patients (16), long-term outcome in childhood leukemia and individual cytotoxic drug sensitivity in tumors such as non-Hodgkin's lymphoma, B-cell chronic lymphocytic leukaemia and ovarian carcinoma. There has been a lack of good methods to predict drug sensitivity in neuroendocrine tumor patients. Our results indicate that *in vitro* screening of annotated compound libraries may be used for identification of compounds with antitumor activity in neuroendocrine tumor models.

## Conclusion

Our experiments have shown that eleven out of the eighteen studied agents had an effect on bronchial and pancreatic carcinoid cell lines with IC<sub>50</sub>-values <4 μM. This may possibly lead to better treatment options for patients with neuroendocrine tumors. We will continue by studying if treatment with various combinations of drugs has any synergistic effects in the cell lines. In addition, we aim to investigate the mechanisms of cell death for these agents.

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