The Cytotoxic Agents NSC-95397, Brefeldin A, Bortezomib and Sanguinarine Induce Apoptosis in Neuroendocrine Tumors *In Vitro*

DHANA E. LARSSON[1], MALIN WICKSTRÖM[2], SADIA HASSAN[2], KJELL ÖBERG[1] and DAN GRANBERG[1]

Departments of [1]Endocrine Oncology and [2]Medical Sciences, Division of Clinical Pharmacology, Uppsala University Hospital, Uppsala, Sweden

Abstract. *The aim of this study was to investigate the apoptosis resulting from NSC 95397, brefeldin A, bortezomib and sanguinarine in neuroendocrine tumor cell lines. Materials and Methods: A multiparametric high-content screening assay for measurement of apoptosis was used. The human pancreatic carcinoid cell line, BON-1, human typical bronchial carcinoid cell line NCI-H727 and the human atypical bronchial carcinoid cell line NCI-H720 were tested. After incubation with cytotoxic drugs, the DNA-binding dye Hoechst 33342, fluorescein-tagged probes that covalently bind active caspase-3 and chloromethyl-X-rosamine to detect mitochondrial membrane potential were added. Image acquisition and quantitative measurement of fluorescence was performed using automated image capture and analysis instrument ArrayScan. In addition, nuclear morphology was examined on microscopic slides stained with May-Grunewald-Giemsa. Results: A time- and dose-dependent activation of caspase-3 and increase in nuclear fragmentation and condensation were observed for the drugs using a multiparametric apoptosis assay. These results were confirmed with nuclear morphological examination on microscopic slides. Conclusion: NSC 95397, brefeldin A, bortezomib and sanguinarine induced caspase-3 activation with modest changes in nuclear morphology.*

Apoptosis, an important mechanism of cell death displaying nuclear fragmentation and other morphological characteristics, was originally described by Kerr *et al*. (1, 2). Apoptosis, programmed cell death, is the necessary mechanism complementary to proliferation that ensures homeostasis of all tissues. In recent years, the molecular machinery responsible for apoptosis has been elucidated, revealing a family of intracellular proteases which are responsible directly or indirectly for the morphological and biochemical changes that characterize the phenomenon of apoptosis (3, 4). One of the most important groups of proteins involved in apoptosis are the cystein aspartate-specific proteases, also called caspases, which are activated by different toxic stimuli. Three major pathways have been elucidated so far, which all result in the activation of the executor caspase enzyme caspase-3. One is the mitochondrial/cytochrome c by stimulating the intrinsic pathway, largely mediated through Bcl-2 family members, which results in activation of Apaf-1, caspase-9, and then caspase-3. The second or, the extrinsic (receptor) pathway, signals ligation of members of the tumor necrosis factor (TNF)-receptor family (*e.g*. Fas, tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) receptors) and activates caspase-8 and subsequently caspase-3. Finally, granzyme B (a cytolytic T-cell product) directly cleaves and activates several caspases, resulting in apoptosis (5-7) Regardless of the pathways, the process ends with DNA fragmentation and formation of apoptotic bodies, which are phagocyted by macrophages (8). Physiological cell death, such as in processes during fetal development, usually occurs by apoptosis. Defects in apoptotic cell death contribute to neoplastic diseases, by inhibiting normal cell turnover and promoting cell accumulation. Defects in apoptosis also facilitate tumor progression, by rendering cancer cells resistant to death mechanisms relevant to metastasis, hypoxia, growth factor-deprivation, chemotherapy and irradiation (9).

Most anticancer drugs presently used in clinical treatment have been described to induce cell death by apoptosis (2). The understanding of the mechanisms of action of drugs and the knowledge of the molecular mechanisms responsible for the regulation of apoptosis may help to establish new therapeutic agents for different tumor diseases. One *in vitro* method to identify and evaluate drugs that induce apoptosis is the study of caspase activation in cells. In this method, fluorochrome-labeled inhibitors of caspases (FLICA) that covalently bind to the active site of activated caspases are used (10). It is an

Correspondence to: Dhana Larsson, Department of Endocrine Oncology, University Hospital, S-751 85 Uppsala, Sweden. Tel: +46 186114916, Fax: +46 18553601, e-mail: dhana.larsson@medsci.uu.se

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automated information-rich image-based analysis, often called high-content screening (11, 12). With this approach, one is able to measure fluorescence intensity and localization on a cell-by-cell basis. In this work, we present evaluation of caspase-3 activity and nuclear morphology in neuroendocrine cells exposed to the cytotoxic drugs, NSC 95397, brefeldin A, bortezomib, and sanguinarine.

Even if an initial response is obtained in patients with malignant endocrine pancreatic tumors as well as bronchial carcinoids treated with chemotherapy or biotherapy, resisistance to treatment occurs sooner or later. There is thus a need for better treatments in patients with malignant neuroendocrine tumors. A screening study and combination study with anticancer drugs have been carried out on the human pancreatic carcinoid cell line BON-1, as well as the human typical bronchial carcinoid cell line NCI-H727 and the human atypical bronchial carcinoid cell line NCI-H720, with promising results (13, 14). In this study, we wanted to investigate the apoptotic effect for the above mentioned cytotoxic drugs in the three neurondocrine cell lines.

Materials and Methods

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 modified Ham's F12 medium (F12K) (Invitrogen AB, Stockholm, Sweden). The human typical bronchial carcinoid cell line NCI-H727 and the human atypical bronchial carcinoid cell line NCI-H720 were obtained from ATCC (LGC Promochem, Sweden) and maintained in RPMI-1640 medium (Invitrogen AB, Stockholm, Sweden). All three cell lines were supplemented with 10% heat-inactivated fetal calf serum (FCS), 1% glutamine and 1% penicillin/streptomycin (Sigma-Aldrich AB Stockholm, Sweden) and cultured in a humidified 5% CO_2 atmosphere at 37˚C.

Drugs preparation. Drugs were purchased from Sigma-Aldrich AB (Stockholm, Sweden) Bortezomib and NSC 95397 were dissolved in dimethyl sulphoxide (DMSO), brefeldin A was dissolved in ethanol and sanguinarine was dissolved in methanol, all to a stock concentration of 10 mM and further diluted with sterile water or phosphate-buffered saline (PBS). The FLICA probe, FAM-DEVD-FMK for measurement of caspase-3, supplied as part of the CaspaTag kit (Chemicon, Temicula, CA, USA) and chloromethyl-X-rosamine (MitoTracker Red CMXRos; Molecular Probes, Eugene, OR, USA) were dissolved in DMSO and further diluted in PBS to their final concentration. Hoechst 33342 (Sigma Aldrich AB, Stockholm, Sweden) was dissolved in water.

Morphology. Microscope slides of cells were prepared using a Cytospin 3 centrifuge (Shandon Scientific Ltd.). Slides were prepared after 24 h, 48 h and 72 h incubation for control cells exposed to PBS and treated cells exposed to NSC 95397 (6.0 μM and 30 μM), brefeldin A (0.1 μM and 0.5 μM), bortezomib (0,5 μM and 2,5 μM) or sanguinarine (1.0 μM and 5.0 μM). The slides were stained with May-Grunewald-Giemsa for morphological evalution under light microscopy, at ×40. Cells with disintegrated membrane were classified as necrotic and cells with a condensed, fragmented nucleus but intact cell membrane as apoptotic. A minimum of 500 cells in 3-4 areas per slide were assessed (data not shown).

Plate preparation and induction of apoptosis. BON-1, H727 and H720 cells were seeded, 90 μl cells in each well at a concentration of 5×10^4 cells /ml, into 96-well plates with flat optical bottom (Perkin-Elmer Inc., Welleslay, MA, USA), and left to attach 4-5 h before the addition of drugs. The number of cells seeded per well was determined by the proliferation rate and the optimal number of cells per well for apoptosis analysis. Plates were incubated at the culture conditions indicated above. NSC 95397 (5.4 μM, 27 μM and 67.5μM), brefeldin A (0.2 μM, 1.0 μM and 5.0 μM), bortezomib (1.0 μM, 5 μM and 25 μM), and sanguinarine (2 μM, 10 μM and 50 μM) were tested in duplicate at three different concentrations and each experiment was repeated three to four times.

Cell staining. The cells were incubated with the drug for 24 h, 48 h and 72 h. A multiparametric high-content screening assay previously described in detail was used (12). One hour before the end of drug exposure, the FLICA probe FAM-DEVD-FMK (carboxyfluorescein-labeled fluoromethyl ketone peptide inhibitor of caspase-3) was added at a final concentration of 20 μM to stain cells with activated caspase-3. MitoTracker Red (a fluorescent dye that stains mitochondria in living cells and whose accumulation is dependent upon mitochondrial membrane potential, MMP was added during the last 30 min of exposure at a final concentration of 100 nM to evaluate (MMP). The staining solutions were removed and the plates were washed twice with PBS followed by 20 min fixation with 3.7% formaldehyde and 10 μM Hoechst 33342. Plates were then washed twice. All plate washing was performed with a multiwash plate washer (Dynatec Laboratories, Chantilly, VA, USA). Plates were centrifuged before each aspiration to avoid loss of cells growing in suspension or cells detached due to toxic stimuli. Processed plates were kept at +4˚C for up to 48 h before analysis.

Image acquisition and cytometric analysis. In order to examine if the drugs studied were able to trigger the apoptotic machinery, processed plates were analyzed using the ArrayScan high-content screening system (Cellomics Inc., Pittsburgh, PA, USA) for automated imaging acquisition and analysis. This ArrayScan is an automated inverted fluorescence imaging microscope that identifies stained cells and reports the intensity and distribution of fluorescence in individual cells. Images were acquired to identify cells based on their nuclear Hoechst 33342 staining, fluorescence intensity and localization based on the staining from FAM-DEVD-FMK, and MitoTracker Red using suitable filters with a ×20 objective. In each well, 800 cells were analyzed. The morphology of nuclear condensation/fragmentation was characterized by the variability and intensity in the Hoechst 33342 staining. A condensed or fragmented nucleus thus results in a high value. Images and data of the fluorescence within each cell and fluorescence of the cell population within the well were stored in a Microsoft SQL database.

Results

The results are illustrated by Figures 1-4. Figure 1 and 2 illustrate the morphological changes in the human bronchial carcinoid cell line NCI-H727 and the human

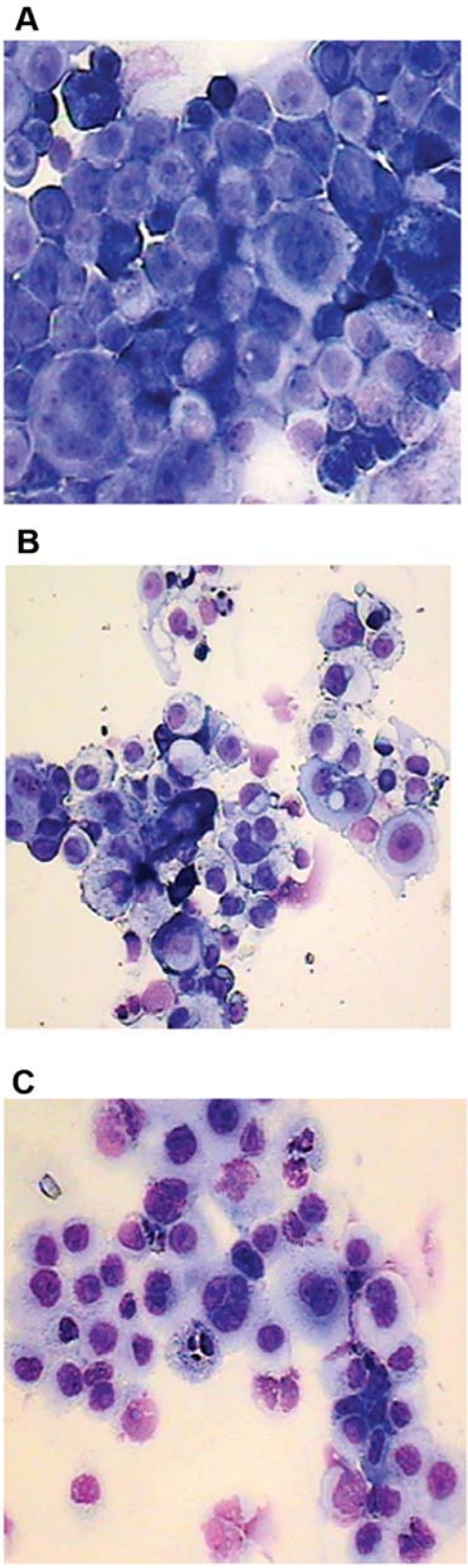

Figure 1. *Microphotographs, under light microscopy at ×40, of May-Grunewald-Giemsa-stained NCI-H727 cells exposed to (a) PBS, (b) 2.5 μM bortezomib and (c) 0.2 μM brefeldin A for 24 h and 48 h.*

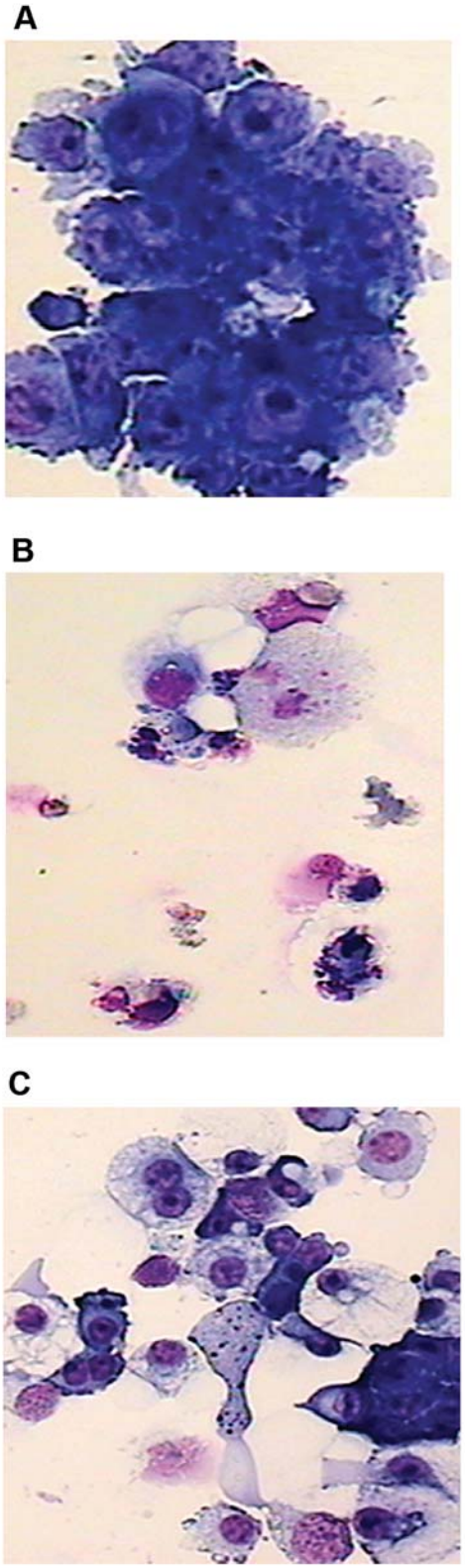

Figure 2. *Microphotographs, under light microscopy at ×40, of May-Grunewald-Giemsa stained BON-1 cells exposed to (a) PBS, (b) 2.5 μM bortezomib and (c) 0.2 μM brefeldin A for 48 h and 72 h.*

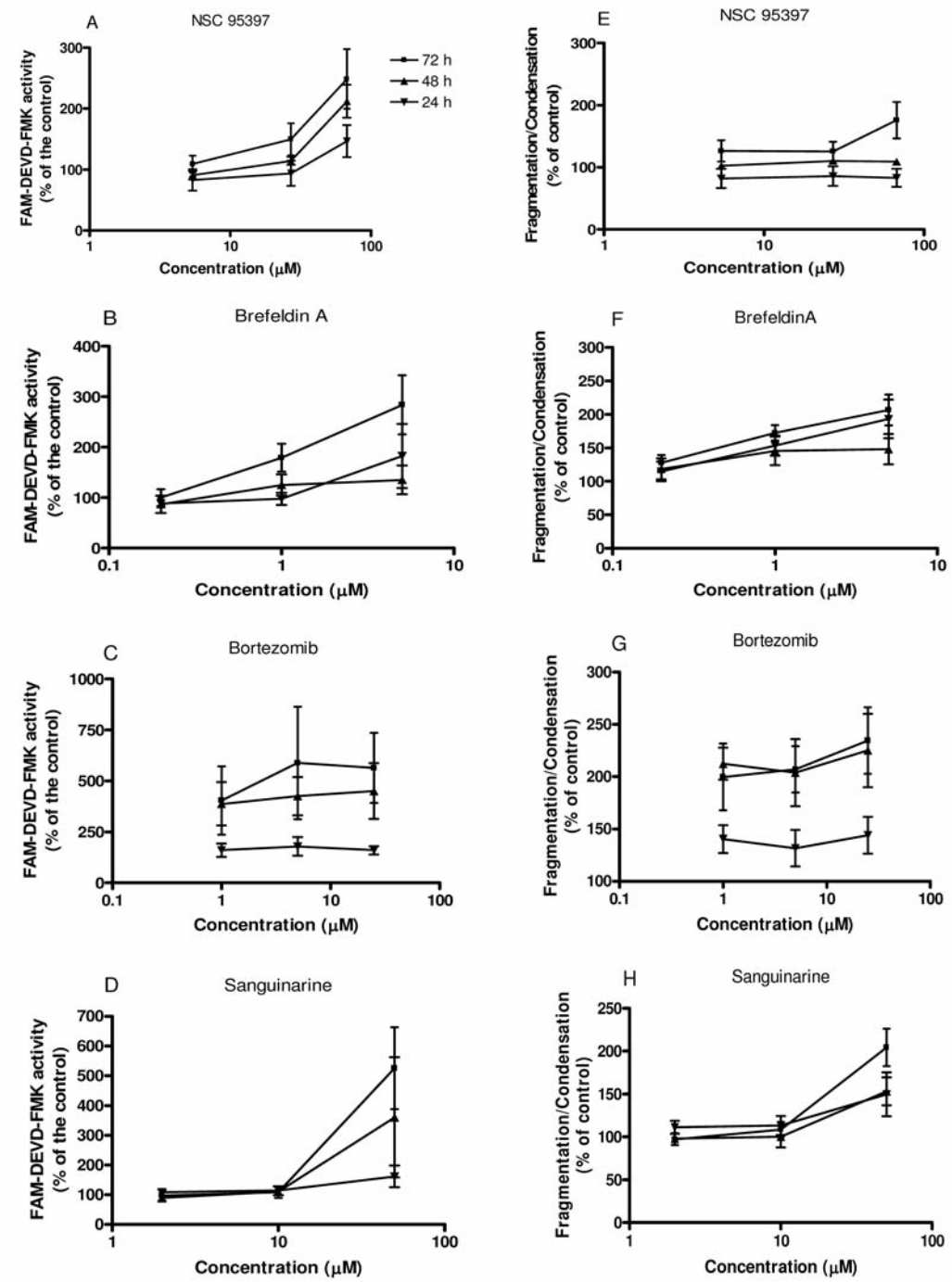

Figure 3. *Measurement of caspase-3 activation and nuclear fragmentation/condensation in NCI-H727 cells: Dose-dependent activation of caspase-3-like activity (A-D) and nuclear fragmentation/condensation (E-H) after 24, 48 and 72 h drug exposure, expressed as a percentage of the untreated control. Mean of three independent experiments ±SEM.*

pancreatic carcinoid cell line BON-1 exposed to the drugs bortezomib and brefeldin A, for 24, 48, or 72 h respectively. Cells exposed to the drugs for 72 h showed predominantly necrotic morphology with disrupted cell membranes. However, cells exposed for 48 h showed marked increase in the typical signs of apoptosis: chromatin condensation and nuclear fragmentation with less necrosis. Figures 3 and 4 illustrate the caspase-3

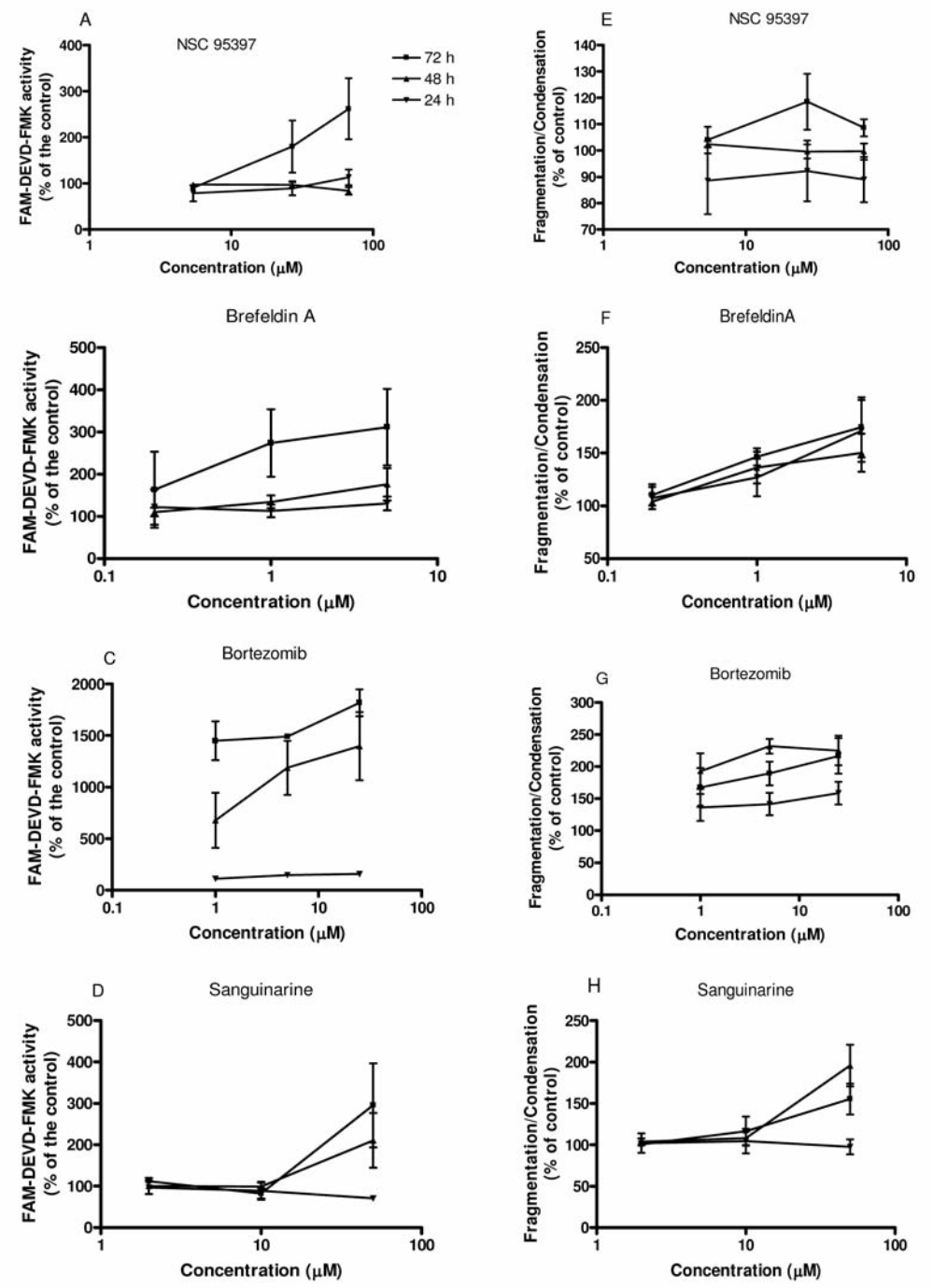

Figure 4. *Measurement of caspase-3 activation and nuclear fragmentation/condensation in BON-1 cells: Dose-dependent activation of caspase-3-like activity (A-D) and nuclear fragmentation/condensation (E-H) after 24, 48 and 72 h drug exposure, expressed as a percentage of the untreated control. Mean of three independent experiments ±SEM.*

activity and a dose-dependent increase in fragmentation/condensation in the two cell lines, NCI-H727 and BON-1, exposed to NSC 95397, brefeldin A, bortezomib and sanguinarine respectively.

All drugs tested induced a high level of caspase-3 activity and apoptotic changes in nuclear morphology. In the BON-1 cell line, caspase-3 activation and nuclear DNA fragmentation were seen 48 h after addition of NSC 95397

and bortezomib. Caspase-3 activation was also seen 48 h after addition of NSC 95397, bortezomib and sanguinarine in the NCI-H727 cell line. The same findings were also observed 72 h incubation with NSC 95397, brefeldin A, bortezomib and sanguinarine in both neuroendocrine cell lines. After 24 h, a modest decrease in MMP was observed for brefeldin A and sanguinarine at the lowest tested concentrations compared to the untreated controls (data not shown). For bortezomib and NSC 95397, no changes in MMP were observed (data not shown).

Discussion

The treatment of neuroendocrine tumors with the standard cytotoxic drugs is so far of limited effect. We previously performed a screening study on a pancreatic carcinoid (BON-1) and two bronchial carcinoid (a typical, NCI-H727 and an atypical, NCI-H720) cell lines. That study demonstrated that Bay 11-7085, bortezomib, brefeldin A, CGP-74514A, doxorubicin, emetine, idarubicin, β-lapachone, mitoxantrone, NSC 95397 and sanguinarine showed antitumor effect *in vitro* (13). We thereafter continued with a combination study, where the most active agents from the screening study were combined with four cytotoxic dugs commonly used in oncology practice. In general, NSC 95397, emetine, CGP-74514 A, brefeldin A and sanguinarine showed additive or synergistic interaction effects when combined with the standard cytotoxic drugs doxorubicin, etoposide, oxaliplatin and docetaxel in the neuroendocrine tumor cell lines *in vitro* (14).

In the present study, we have investigated the mechanisms of cell death for four of the earlier studied drugs by evaluating caspase-3 activity and nuclear morphology in two neuroendocrine tumor cell lines, BON-1 and NCI-H727. The atypical bronchial carcinoid cell line NCI-H720 was also tested, but since this cell line grows in floating aggregates, it was not suitable for the ArrayScan system and was thus not further analyzed. We found that NSC 95397, brefeldin A, bortezomib and sanguinarine induced a marked increase in caspase-3 activity as early as 48 h of incubation. The same concentrations that induced caspase-3 activation also induced changes in nuclear morphology. Only modest decreases or no change in MMP were detected using this experimental setup, which may possibly be explained by the relatively long drug exposure times, the shortest being 24 h. At longer incubation times many fragile apoptotic cells may be degraded during plate handling, leaving only cells in early or mid-phase apoptosis and with identifiable nuclei. Another explanation for the lack of decrease in MMP is that it may depend on limitations in the algorithm analyzing the parameter in the ArrayScan, a problem previously described (15). An increase in overall mitochondrial mass as measured by the ArrayScan algorithm could be due to increased mitochondrial staining, an increase in nuclear size, or both. Taken together, our observations indicate that at least brefeldin A and sanguinarine, and probably also bortezomib and NSC 95397, induce apoptosis in neuroendocrine tumor cells by activation of the extrinsic (receptor) apoptotic pathway, and at shorter drug exposure times (24 h), by activation of the intrinsic (mitochondrial) apoptotic pathway.

The focus of drug discovery in the area of oncology is to identify molecules important for apoptosis regulation in tumor cells (16). In order to develop and evaluate promising new anticancer agents, it is important to rapidly and easily identify substances with apoptosis-inducing properties. One possible approach to identify and evaluate substances that induce apoptosis may be to use a rapid and information-rich method for identifying individual cells or subpopulations of cells expressing one or more features attributable to apoptosis. High-content screening (HCS) employs fluorescent indicators to define cellular morphometry and molecular responses to compound treatment with antitumor agents.

The drug discovery process is greatly enhanced by multiparametric cellular analyses that define, often in a few multiplexed assays, the toxicity, potency, specificity and selectivity of pharmacologically active compounds (17). Image-based screening has been performed since the late 1990s, and HCS, which studies several parameters simultaneously, was first described by Giuliano and others in 1997 (11) and has since then been shown to be useful in many areas of research such as cancer (18), neurite outgrowth (19) and inflammation (20).

NSC 95397 has previously been described as a Cdc25 phosphatase inhibitor (21). Cdc25 phosphatases are involved in regulation of the cell cycle by activating Cdk/cyclin complexes (22). Bortezomib (Velcade®), a proteasome inhibitor and an indirect nuclear factor kappa B (NF-kB) inhibitor, has shown activity in early clinical trials among patients with non-Hodgkin's lymphoma and multiple myeloma (23). The ubiquitin/proteosome system in a large proteinase complex regulating the cell growth and apoptosis. Cell cycle regulators and transcription factors such as p53, cyclins and cyclin-dependent kinase (Cdk) inhibitors, and NF-kB are regulated by the ubiquitin/proteosome system. The induction of apoptosis by proteasome inhibitors leads to the accumulation of proteins such as p53, p27, proapoptotic Bad or Bax, or the stress kinase, c-Jun N-terminal kinase, (JNK1), which leads to the release of cytochrome-c and activation of the intrinsic apoptosis pathway (24-26). Sanguinarine has been shown to induce apoptotic death of human squamous carcinoma A431 cells, as well as of the androgen-responsive human prostate carcinoma cells LNCaP and androgen-unresponsive human prostate carcinoma cells DU145. Concentrations of sanguinarine above 1 μM may have toxic effects in the A431, LNCaP and DU145 cell lines (27-29). It has also been shown that induction of apoptosis by sanguinarine in human breast

cancer cells (30) and primary effusion lymphoma cells (31) are induced by activation of caspases. Treatment with sanguinarine at 3-9 h led to activation of caspase-9 and -3 (the intrinsic and extrinsic pathway respectively) with apoptosis in A549 human lung cancer cells (32). Brefeldin-A is a fungal-derived substance inhibiting protein transport. This agent is a pro-apoptotic drug that acts on the Golgi apparatus. A study on the CAC2 cell line derived from adenoid cystic carcinoma showed that brefeldin A induced cell death by apoptosis (33). Apoptosis are induced with the tested drugs by intrinsic or extrinsic pathways, or both in other cell lines. Our data are in line with such findings. In summary, our experiments have demonstrated that NSC 95397, brefeldin A, bortezomib, and sanguinarine had a considerable apoptotic effect in the studied human neuroendocrine tumor cells *in vitro*. At longer exposure times, these drugs activated the extrinsic apoptotic pathway and at shorter drug exposure times (24 h), the intrinsic apoptotic pathway, resulting in nuclear condensation and fragmentation. This makes these drugs possible candidates for further studies.

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