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Anti-tumor effects of peptide analogs targeting neuropeptide hormone receptors on mouse pheochromocytoma cells

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Abstract

Pheochromocytoma is a rare but potentially lethal chromaffin cell tumor with currently no effective treatment. Peptide hormone receptors are frequently overexpressed on endocrine tumor cells and can be specifically targeted by various anti-tumor peptide analogs. The present study carried out on mouse pheochromocytoma cells (MPC) and a more aggressive mouse tumor tissue-derived (MTT) cell line revealed that these cells are characterized by pronounced expression of the somatostatin receptor 2 (sst2), growth hormone-releasing hormone (GHRH) receptor and the luteinizing hormone-releasing hormone (LHRH) receptor. We further demonstrated significant anti-tumor effects mediated by cytotoxic somatostatin analogs, AN-162 and AN-238, by LHRH antagonist, Cetrorelix, by the cytotoxic LHRH analog, AN-152, and by recently developed GHRH antagonist, MIA-602, on MPC and for AN-152 and MIA-602 on MTT cells. Studies of novel anti-tumor compounds on these mouse cell lines serve as an important basis for mouse models of metastatic pheochromocytoma, which we are currently establishing.

Keywords

pheochromocytoma; peptide analogs; targeted tumor therapy

1. Introduction

Pheochromocytoma are potentially lethal tumors of adrenal and extra-adrenal (also called paragangliomas) chromaffin cells (Harding et al., 2005). Currently there are no reliable markers to distinguish benign from malignant tumors and there is no curative therapy (Scholz et al., 2007).

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To date, combination chemotherapy with cyclophosphamide, vincristine, and dacarbazine (CVD) and radiotherapy with ^{131}I-metaiodobenzylguanidine (MIBG) represent the two primary modes of treatment for malignant pheochromocytoma (Chrisoulidou et al., 2007). Combinations of etoposide and cisplatin, cisplatin and 5-fluorouracil, cytokine arabinoside, anthracine, and more recently, temozolomide and thalidomide also have benefit in some patients (Eisenhofer et al., 2008). However, complete remissions are rare and these therapies can only be regarded as palliative (Fassnacht et al., 2009). Thus, there is a need for identification of new targets for treatment of metastatic pheochromocytoma along with development of appropriate therapeutic agents for those targets.

Targeted therapy based on peptide analogs binding specifically to neuropeptide hormone receptors on cancer cells provides unique treatment strategy with potentially more tissue specificity and less systemic toxicity than conventional chemotherapeutic approaches (Reubi, 2003). Some support for this approach is provided by case reports (Valkema et al., 2002) and a recent publication including 28 patients treated with radio-labeled DOTATOC (Forrer et al., 2008). Since altered neuropeptide hormone receptor expression leading to hypersecretion of hormones or enhanced tumor growth is a frequent feature of adrenal tumors (Willenberg et al., 1998; Lacroix et al., 2001), targeting these aberrant receptors with novel chemotherapeutic peptide analogs may provide a logical therapeutic alternative to other approaches for pheochromocytomas and other tumors (Ziegler et al., 2009). Such targeted therapies should enable higher tumoral concentrations of antineoplastic agents compared to current systemic chemotherapeutic therapies and thereby lead to improved antitumor treatment efficacy with reduced systemic toxicity (Buchholz et al., 2006).

Over the last few decades several targeted cytotoxic hormone analogs have been synthesized by Schally et al. to yield new classes of antineoplastic agents (Schally et al., 2001). These compounds include the cytotoxic analogs of bombesin (AN-215), of somatostatin AN-238 and AN-162, of luteinizing hormone-releasing hormone (LHRH) AN-152, and others, synthesized by coupling doxorubicin or 2 pyrrolinodoxorubicin (2-pyrrolino-DOX) (AN-201) to the respective hormone analogs (Nagy et al., 1996). Recently, a new class of GHRH antagonists (MIA-313, MIA-602, MIA-604, and MIA-610) was generated by Schally and co-workers and shown to be effective anti-neoplastic agents, as for example demonstrated on ovarian cancer (Klukovits et al., 2012). Both MIA-602 and the LHRH antagonist Cetrorelix (Buchholz et al., 2009) seem to be highly promising anti-cancer compounds on endocrine tumors (Schally, 2008) and both compounds were also part of our investigation on adrenomedullary tumors.

Recently two mouse pheochromocytoma cell (MPC) lines, one more malignant (MTT) than the other have been established as originally generated from heterozygous neurofibromin-1 knock-out mice (Powers et al., 2000). Based on these cell lines various mouse models were derived, which are useful for studying metastatic pheochromocytoma and testing novel therapies (Martiniova et al., 2009; Martiniova et al., 2011; Korpershoek et al., 2012; Nolting and Grossman, 2012). Furthermore, these models are also physiologically relevant to human pheochromocytoma because of the tumoral production of norepinephrine in athymic nude mice after tail vein injection of the MPC cells (Ohta et al., 2006). Additionally, both MPC and MTT cells were recently stably transfected with luciferase and/or GFP for further tracing tumor spread *in vivo* by bioluminescence (Giubellino et al., 2012).

Aberrantly expressed neuropeptide hormone receptors are frequently found in a subgroup of adrenocortical tumors, especially in ACTH-independent macronodular adrenal hyperplasia. Based on this we explored a large pheochromocytoma microarray data base performed by our collaborating partners (Brouwers et al., 2006) and demonstrated significant differential expression of neuropeptide hormone receptors among human pheochromocytoma specimens

(Ziegler et al., 2009). Based on these microarray data we performed a comprehensive analysis of mRNA expression of various relevant receptors in human adrenal tumors and cell lines.

The present analysis extends our previous study of neuropeptide hormone receptor expression to the MPC and MTT cell lines. We also studied possible anti-tumor effects of the most promising peptide analogs targeting neuropeptide hormone receptors identified to be expressed in these cell lines.

2. Materials and methods

2.1. Cell culture

MPC and MTT cells were cultured on collagen coated flasks in RPMI 1640 including HEPES (GIBCO) with 10% horse serum (GIBCO), 5% FBS superior (BIOCHROM) and 0,1% Gentamicin (GIBCO) in a humidified 5% CO_2/95% O_2 atmosphere at 37°C. The culture medium was changed every second day. Neuropeptide antagonists and agonists were used at concentrations of 10^{-5}–10^{-8} for 24 to 72 h.

2.2. Immunohistochemical analysis of tumor cells

Immunohistochemical stainings were preformed on deparaffinized slides of tumor cells (n=3) using an automated immunostainer (Benchmark Ventana) according to the manufacturer's protocols. The primary antibody for Somatostatin Receptor Type 2 (SSTR2) was Rabbit anti mouse/anti-human polyclonal antibody (Novus Biologicals). For GHRHR we employed Rabbit anti-human (Lifespan Bio Sciences) and Rabbit anti-mouse (ABCAM) antibodies and for detecting LHRH/GnRHR we used Rabbit anti-mouse (Novus Biologicals) and Rabbit anti-human (Santa Cruz) antibodies, respectively. We used the VENTANA amplification kit as well as avidin-biotin labeling and 3, 3'- diaminobenzidine to amplify and visualize the signal. Slides were counterstained with H&E. Staining with isotype control antibodies was performed to confirm the staining specificity.

2.3. Cell viability and apoptosis assays

For evaluating cell viability we used the CellTiter-Glo Luminescent Cell Viability Assay (Promega). This assay determines the number of viable cells in culture based on quantification of the ATP present, an indicator of metabolically active cells. For analysing programmed cell death we employed the Caspase-Glo 3/7 Assay (Promega), which provides a homogeneous luminescent assay that measures caspase-3/7 activities. All assays were performed according to the manufacturer's protocols and guidelines.

2.4. Electron microscopy

For evaluating the ultrastructure of our tumor cells, they were fixed in 2.5 M glutaraldehyde in 0.1 M cacodylate buffer and refixed in 1% osmiumtetroxid solution. After dehydration in an ascending ethanol series, specimens were embedded in EPON. Ultrathin slices (60 nm) were afterwards stained with lead acetate und uranyl acetate to obtain a suitable contrast, and analyzed using an electron microscope.

2.5. Neuropeptides employed

In our current investigation we employed somatostatin octapeptide analog RC-160, targeted cytotoxic somatostatin analogs AN-162 and AN-238, LHRH antagonist Cetrorelix, cytotoxic LHRH analog AN-152 as well as GHRH antagonist MIA-602. Doxorubicin (DOX) and Dox hydrochloride were obtained from Chemex Export-Import. All analogs were synthesized in the laboratories of one of us (A.V.S.) (Zarandi et al., 1994).

2.6. Statistical analyses

In all experiments, statistical differences between experimental groups relative to appropriate controls were determined by ANOVA. Data are presented as means ± SEM. Significance of differences was tested by analysis of variance with Bonferroni's post hoc test. Differences were considered significant at values of $P < 0.05$. Cells from at least 2 different passages were used for each experimental series; n represents the number of cells or tissue culture dishes investigated.

Results

Immunohistochemical analyses

We established abundant receptor protein expression for somatostatin subtype 2 (sst2), for growth hormone releasing-hormone (GHRH) and for luteinizing hormone-releasing hormone (LHRH) on MPC and MTT cells (Figure 1).

Effect of peptide analogs on MPC

AN-162 and AN-238 targeting SSTR—Employing cytotoxic analogs of somatostatin, AN-162 and AN-238 (10^{-6} mol/l), we found strong and significant reductions in cell viability over three days of incubation of MPC with these anti-cancer compounds, while the non-targeted and more systemically acting anti-cancer drug doxorubicin was at the same concentration (10^{-6} mol/l) and study time (24–72h) not affecting MPC viability (Fig. 2A). When we applied lower concentrations of the somatostatin analogs (10^{-7}–10^{-8} mol/l) we could not document significant anti-tumor effects. In our study we could also demonstrate AN-162 (24–48h) and AN-238 (24h) to significantly increase caspase 3/7 activity with a most effective concentration of 10^{-6} mol/l (Fig. 2B). Lower concentrations of the cytotoxic somatostatin analogs (10^{-7}–10^{-8}) were not effective or not as effective as 10^{-6} mol/l, as was the case for doxorubicin.

Effects of MIA-602 as well as of Cetrorelix and AN-152 targeting GHRHR and LHRHR

Since also receptors for LHRH and GHRH were strongly expressed on our analyzed adrenal tumor cell lines and tumor tissues, we were interested to evaluate possible anti-tumor effects mediated by the cytotoxic LHRH analog AN-152, by the LHRH antagonist Cetrorelix as well as by the GHRH antagonist MIA-602 on MPC. While AN-152 (10^{-6} mol/l) highly significant reduced cell viability over three days, Cetrorelix (10^{-6} mol/l) showed highly significant anti-tumor effects only after 48h. Lower concentrations (10^{-7}–10^{-8}) of both compounds were again not effective on MPC (Fig. 2C). Studying the effects of MIA-602, we found this GHRH antagonist to slightly but significantly reduce MPC survival after 72h, at a concentration of 10^{-6} mol/l. Furthermore, mediated by activation of caspases 3 and 7, AN-152 (10^{-7}–10^{-8} mol/l) directed MPC into programmed cell death (24h-72h) (Fig. 2D).

Effect of peptide analogs on MTT cells

AN-152 and MIA-602 targeting LHRHR and GHRHR—Similar to MPC cells, the more aggressive MTT cells demonstrated pronounced expression of LHRH and GHRH receptors. AN-152 (10^{-6} mol/l) was again found to strongly and highly significant reduce MTT cell viability after 24h. Interestingly, after 2–3 days both 10^{-6} mol/l and 10^{-7} mol/l had a pronounced and significant effect on MTT cell survival, while there was no effect of AN-152 at 10^{-8} mol/l (Fig. 3A). Furthermore, at both 10^{-6} and 10^{-7} mol/l, AN-152 strongly induced programmed cell death with a most prominent effect after 24h (10^{-6} mol/l) (Fig. 3B). Finally, since also the GHRH receptor was abundantly expressed on MTT cells we were interested to studied possible anti-tumor effects mediated by the GHRH antagonist MIA-602 and could show that MIA-602 decreased MTT cell survival only at a rather high

concentration of 10^{-5} mol/l after 2–3 days (Fig. 3C). Studies on the possible effects of the targeted cytotoxic somatostatin analogs AN-162 and AN-238 on MTT cells are still ongoing.

Ultrastructural analyses

We could further confirm this pro-apoptotic mode of cell death induced by AN-152 on an ultrastructural level. Application of AN-152 (10^{-6} mol/l) revealed characteristic apoptotic changes, including apoptotic bodies, filopodia and internucleosomal DNA fragmentation, while MTT cells without AN-152 showed characteristics of normal chromaffin cells with a relatively low number of dense core vesicles (Fig. 3D).

Discussion

This study, which extends previous work in other tumor cells (Ziegler et al., 2009) to MPC and MTT cell lines relevant to neuroendocrine tumors, provides further evidence that targeted hormone receptor therapy might provide a novel approach for metastatic pheochromocytoma. Our study revealed an abundant expression of various neuropeptide hormone receptors on MPC and MTT cells as well as significant anti-tumor effects when applying highly potent and long-lasting peptide hormone analogs, specifically targeting these overexpressed receptors. Although until now, the functional role and importance of mutated and upregulated peptide receptors occasionally detected in various tumors including pheochromocytoma are not known, the present results suggest that targeting these overexpressed receptors might provide a promising therapeutic approach for patients with these tumors (Schally et al., 2011).

After receptor-ligand interaction at the cell membrane, the ligand (usually agonist) is internalized, as e.g. demonstrated for the targeted cytotoxic LHRH analog AN-152 (Emons et al., 2010). In this way, radiotracers, important for tumor imaging could easily accumulate in the cancer cell or the internalized ligand may also be able to selectively destroy the target cell, or inhibit hormone secretion as shown for somatostatin and its analogs (Schally et al., 2004). Also our present study adds further evidence for significant anti-tumor effects mediated by the cytotoxic somatostatin analogs AN-162 and AN-238 on pheochromocytoma cells *in vitro*.

Another class of potent anti-tumor compounds are analogs of LHRH as demonstrated already 30 years ago on prostate cancer (Tolis et al., 1982). Currently the LHRH analog AN-152 (AEZS-108) is provided to patients enrolled in phase II clinical trials suffering form prostate, ovarian or endometrial cancer (Engel et al., 2012). Additionally, in experimental models, analogs of LHRH have been shown to exert direct effects on human breast and bladder cancers through specific LHRH receptors, while non targeted doxorubicin was not as effective and more toxic (Schally et al., 2011; Szepeshazi et al., 2012). Also the side effects of targeted cytotoxic LHRH analogs are expected to be only minor (Emons et al., 2009). Besides AN-152 also the LHRH antagonist Cetrorelix exerted a strong anti-tumor effect on our chromaffin cell tumor model. Interestingly, Cetrorelix has recently been shown to reduce prostate size and prostatic hyperplasia via influencing inflammatory cytokines (Rick et al., 2011).

A third class of promising and highly effective anticancer compounds are GHRH antagonists. These agents show widespread anti-tumor effects and were already successfully employed over many years by Schally and co-workers in numerous experimental tumor models (Schally et al., 2008). In our study, we especially focused on MIA-602 and found significant reductions in cell viability on both MPC and MTT cells. Furthermore, just

recently, MIA-602 has been shown to significantly decrease cell viability and well known parameters of tumor spreading on three different human cancers (Bellyei et al., 2010).

In summary, our present study on mouse pheochromocytoma cells demonstrated significant anti-tumor effects of peptide analogs targeting sst2, GHRHR and LHRHR in adrenomedullary tumors. Together with our previous investigation (Ziegler et al., 2009) we provide further evidence for a successful application of targeted peptide hormone receptor therapy on adrenal tumor cells. Future *in vivo* studies on two different mouse metastatic pheochromocytoma models, based on subcutaneous and intravenous application of MPC and MTT cells, will hopefully shed more light on the possible therapeutic efficiency of various peptide analogs in experimental and pre-clinically models of metastatic pheochromocytoma.

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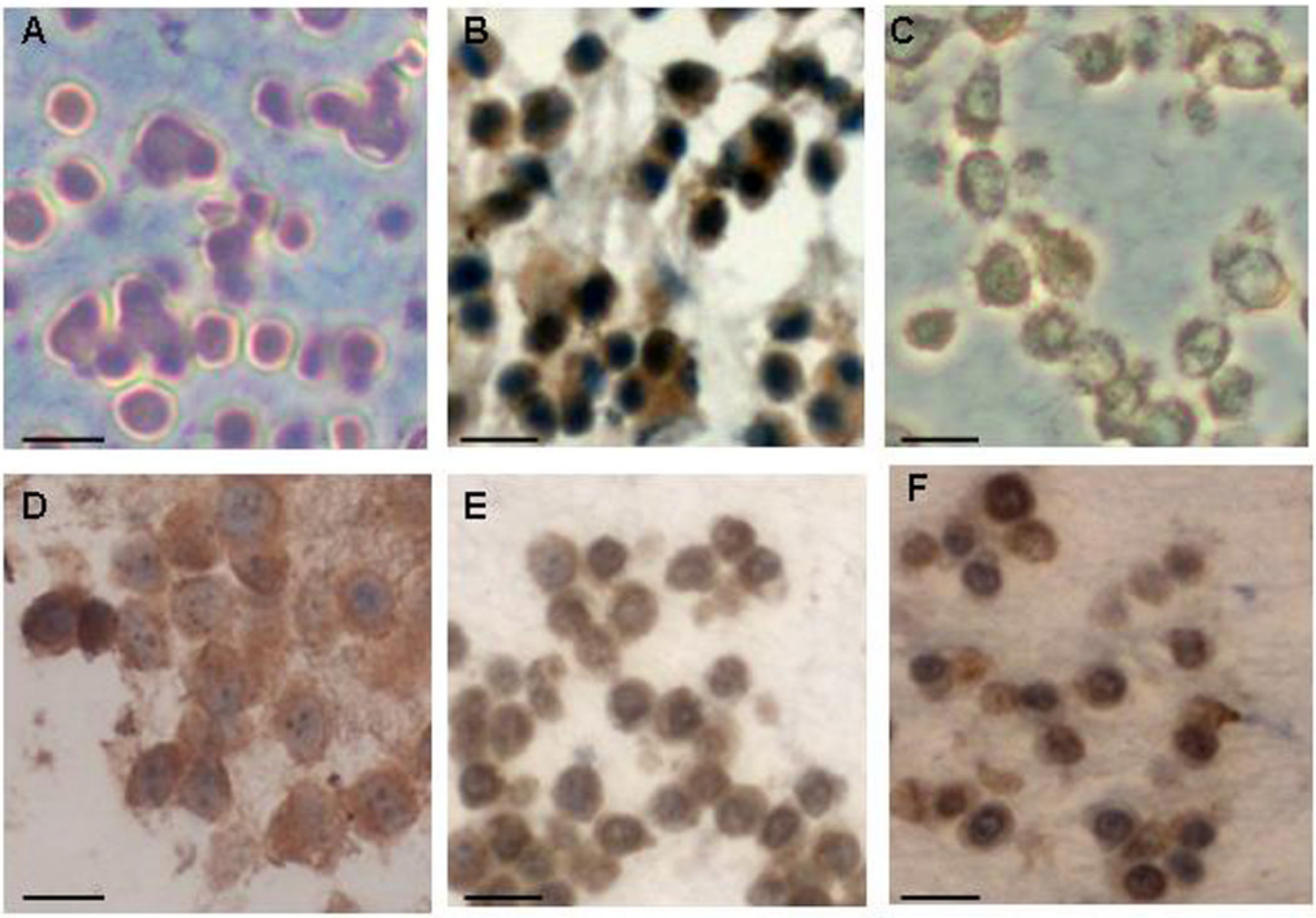

Figure 1.
Immuohistochemical analyses: protein expression of the neuropeptide hormone receptors on mouse pheochromocytoma cells (A-F). Negative control (A). MPC express sst2 (B), GHRHR (C) and LHRHR (D) and on MTT cells we found a pronounced expression of GHRHR (E) and LHRHR (F) (Scale bars: 20 μm).

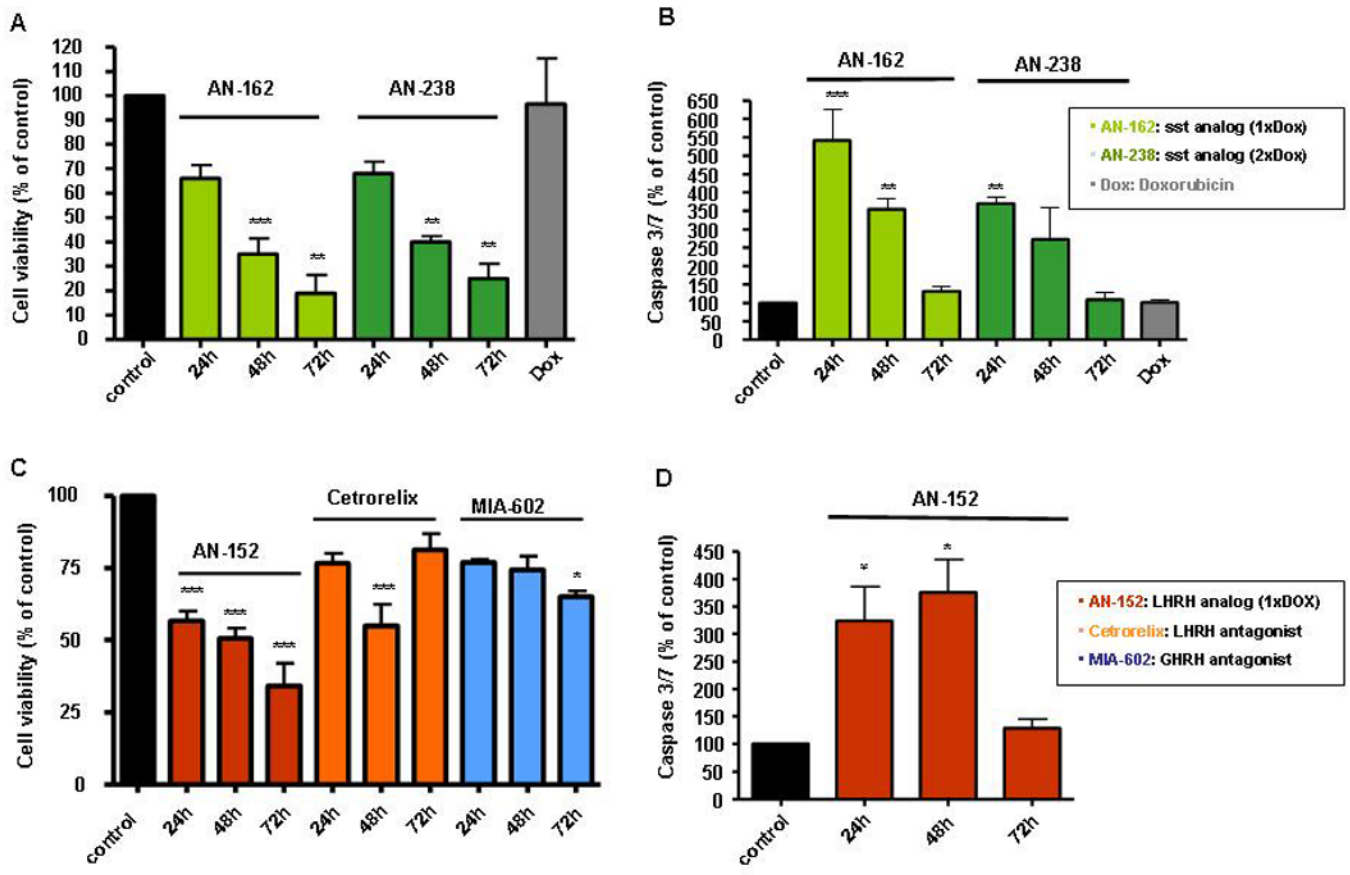

Figure 2.

Anti-tumor effects of peptide analogs targeting ss2, LHRHR and GHRHR in MPC. AN-162 and AN-238 significantly influenced MPC cell viability over 24–72h (A) and highly significant increased caspase 3/7 activity (B) over 24–72h (10^{-6} mol/l). Similarly, GHRH antagonist MIA-602, LHRH antagonist Cetrorelix and LHRH analog AN-152 reduced MPC cell viability (C). AN-152 also significantly increased programmed MPC cell death (D) (n= 3–6, *P < 0.05, **P < 0.01, ***P < 0.001 as compared to control for all assays).

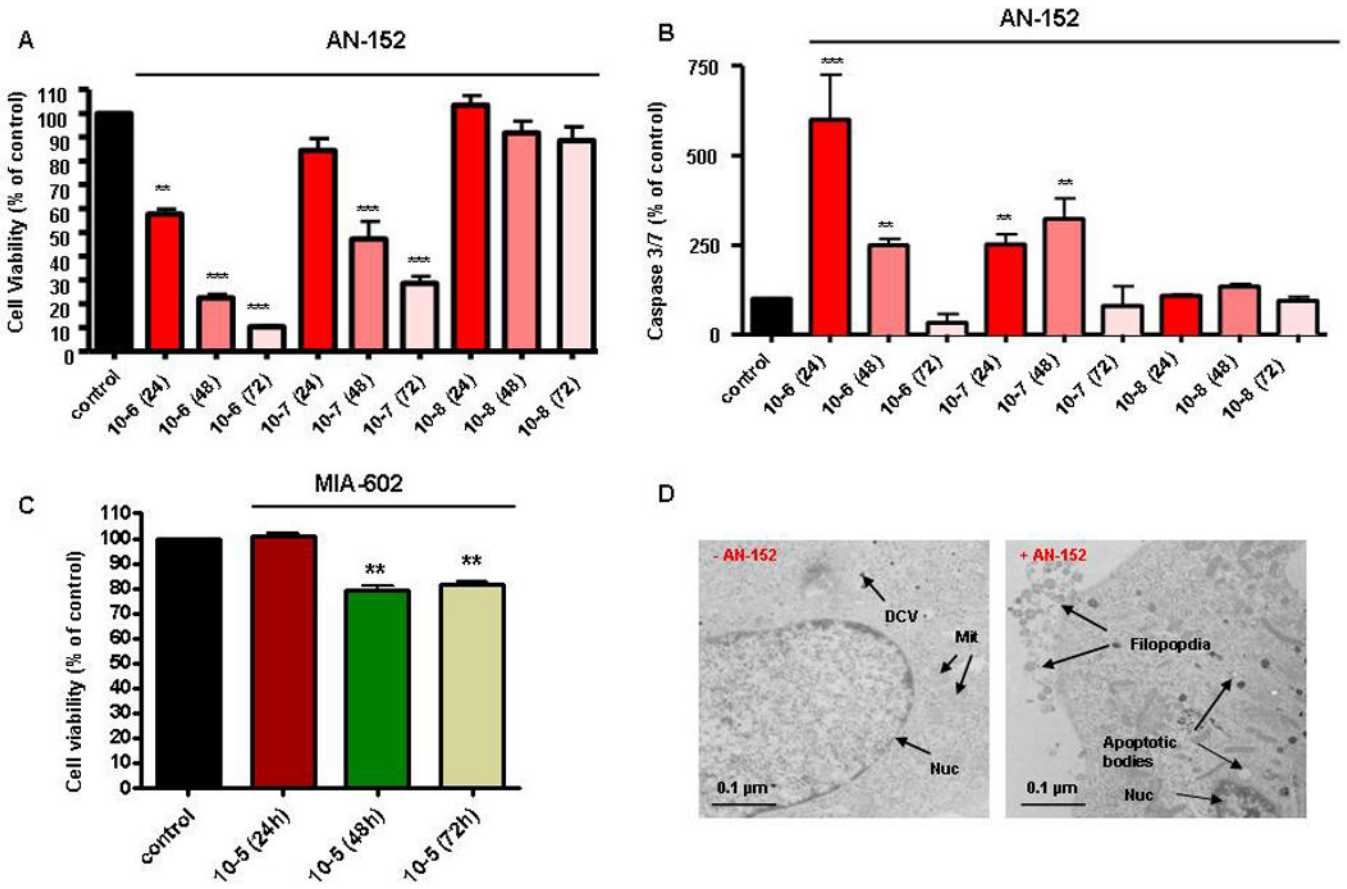

Figure 3.

Anti-tumor effects of peptide analogs targeting LHRHR and GHRHR in MTT cells. AN-152 (10^{-6}–10^{-7} mol/l) highly significant reduced MTT cell viability (A) and induced cell apoptosis (B) over 24–72h. (C) MIA-602 (10^{-5} mol/l) significantly reduced cell viability of more malignant MTT cells (48–72h). (D) Ultrastructural analyses. Confirmation of pro-apoptotic mode of cell death induced by AN-152, including shrinking of the cytoplasm away from the plasma membrane, apoptotic bodies, internucleosomal DNA fragmentation, or condensation of the cytoplasm while retaining mitochondria and endomembrane structure (Scale bar, 0.2 µm); DCV, dense-core vesicles; MIT, mitochondria; Nuc, nucleus.