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A Synthetic Peptide Derived from the Human Eosinophil-derived Neurotoxin Induces Apoptosis in Kaposi's Sarcoma Cells

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Abstract. *Several commercial preparations of human chorionic gonadotropin (hCG) have been tested as therapy for Kaposi's sarcoma (KS) in clinical trials, but with discordant outcomes. We also have found dramatic differences in the cytotoxic effects of four different commercial hCG preparations on an established KS cell line, KSIMM. A co-purified moiety (ies) present in these preparations may explain these differences. The eosinophil-derived neurotoxin ribonuclease, extended with four extra residues ((-4)EDN), has been suggested to be the putative anti-KS compound in the hCG preparations, being specifically recognized by the cells through its N terminal extension. We therefore synthesized a 16-residue peptide (MSLHV-NT12 EDN), made to resemble the active recognition sequence of (-4)EDN. MSLHV-NT12 EDN displays a dose-dependent cytotoxic effect on KSIMM (killing 50% of the cells at 9 μg/ml). The cytotoxic effect is specific for KS cells, MSLHV-NT12 EDN being harmless even at 100μg/ml for a melanoma cell line (SK-MEL-28) or for normal human fibroblasts. We also demonstrated that MSLHV-NT12 EDN induces apoptosis in KSIMM cells. In conclusion, MSLHV-NT12 EDN is a specific proapoptotic substance for KS cells, which warrants further investigation into its in vivo effects.*

Kaposi's sarcoma (KS), once seen as a rare vascular mucocutaneous tumour, has reached high incidence in recent decades, being the most common AIDS-related tumour. Even though the introduction of extremely active antiretroviral therapy had a favourable impact on KS incidence in western countries (1), the disease still represents an important health problem in other geographic areas. Moreover, the treatment of patients with AIDS-related KS is so far essentially palliative.

The prevalence of KS is higher in men than in women (2) and spontaneous regression of the disease during pregnancy has been reported. In addition, KS tumour xenographs do not establish in pregnant mice (3), suggesting the presence of an anti-KS factor related to pregnancy. hCG has been proposed as the putative anti-KS pregnancy-related factor (4). However, both *in vivo* and *in vitro*, the effectiveness of hCG preparations has been very varied, ranging from response rates over 80% to lack of effect [recently reviewed in (5)]. These discrepancies, together with the observation that highly purified or recombinant hCG is void of anti-KS activity (6, 7), led to the speculation that the putative anti-KS moiety (ies) was (were) a contaminant (s) present in different amounts in the commercial hCG preparations. Over the years, several substances have been proposed as anti-KS moieties: a degradation product of the β-hCG subunit (β-core), an hCG-associated ribonuclease, antineoplastic urinary protein (ANUP) and low molecular weight compounds termed hCG-associated factors [recently reviewed in (8)]. The hCG-associated ribonuclease, which specifically killed a KS cell line, was first described as a 18 kDa RNAse present in a crude commercial hCG preparation (9) with an N –terminal sequence similar to the human eosinophil-derived neurotoxin (hEDN) (10). A post-translantionally processed segment of the signal peptide region of hEDN, previously described in the urine of pregnant women ((-4)EDN) (11), was later shown to be

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specifically cytotoxic for a KS cell line (12). The anti-KS effect of ((-4)EDN) seems to depend on a recognition site present in the N-terminus extension (13). In the present study, using an established KS cell line (KSIMM), we showed that the anti-KS factor present in the urinary hCG extracts is different from hCG itself since the cytotoxic effect of different hCG preparations is independent of the hCG content. Moreover, we synthesized a short peptide which contained the recognition motif of the amino-terminus extended hEDN ((-4)EDN) and showed that it specifically induces apoptosis in KSIMM cells.

Materials and Methods

Materials. The KSIMM cell line was a kind gift from Dr A Albini, Instituto Nazionale per la Ricerca sul Cancro, Genova, Italy. Low-passage human diploid fibroblasts (HDF) and SK-Mel-28 (human melanoma cell line) were obtained from the Coriell Institute of Medical Research, NJ, and from the American Type Culture Collection, USA.

Profasi Serono was provided by Serono Nordic (Copenhagen, Denmark), Profasi Steris was a kind gift from Dr.Siegfried Schwarz (Univ. of Innsbruck, Austria), CG-10 was bought from Sigma (St. Louis, MO, USA) and Pregnyl from Organon (Oss, The Netherlands). Agarose NA was from Pharmacia Biotech (Uppsala, Sweden), agarose Nu Sieve GTG was from FMC BioProducts (Rockland, USA), the MTT kit and the antibody against PARP were obtained from Boehringer Mannheim Biochemicals (Mannheim, Germany) and the caspase-3 kit was purchased from BioSource. All other chemicals, unless otherwise stated, were from Sigma.

Cell culture. The cells were cultured in a humidified atmosphere with 5% CO_2 at 37˚C. The KSIMM cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal calf serum (FCS) and the SK-MEL-28 and HDF cells in Minimum Essential Medium supplemented with 10% FCS and non-essential amino acids. For experimental purposes the cells were plated at a density of 3,000-5,000 cells/cm^2 in 35-mm, 60-mm or 150-mm dishes or in 96-well culture plates. The experiments were initiated when the cells had reached sub-confluence.

Peptide construction. The following peptides MSLHVKPPQFT WAQWF (MSLHV-NT12 EDN) and PAPHFWQMLFQWHVST (internal control) were synthesized with an Applied Biosystems 433A synthesizer using Fmoc chemistries. The products were further purified to homogeneity by reverse phase-high performance liquid chromatography (RP-HPLC). The molecular masses of the RP-HPLC purified peptides were verified by matrix-assisted laser desorption/ionization time-of-flight and electrospray ionisation mass spectrometry.

Cytotoxic assay. After reaching sub-confluency, the cells were treated with the testing substances in triplicate for each concentration including the control-treated cells. MSLHV-NT12 EDN or the control peptide were first diluted in an optimal buffer dilution as described (12) and hCG preparations were diluted in PBS, prior to adding to the cells. After 48 hours of incubation with the testing substances, the number of viable cells was evaluated using the MTT viability assay, as we have previously described (14). The cell viability was calculated from the optical density (OD) and expressed as a percentage of the OD of the untreated control cultures. Each experiment was repeated three times. Data are reported as the mean ± SE.

Analysis of DNA fragmentation. After incubation for 72 hours with MSLHV-NT12 EDN (50 mg/ml), $2x10^6$ cells were collected and centrifuged at 2,000 rpm for 10 minutes. The resulting pellet was resuspended in 15 ml of water and incubated with 6 μl RNA-se A (50 mg/ml) for 20 minutes at room temperature and then mixed with loading buffer (5 μl). The samples were loaded on an agarose gel (1.7% low melting agarose (Nu Sieve GTG) and 0.1% agarose NA in Tris buffer-boric acid-EDTA). The upper part of the gel was excised above the comb and a digestion gel (0.8% agarose NA, 2% SDS and 1.25 mg/ml proteinase K) was cast. Electrophoresis was performed at 20 V for 1 hour, followed by 100 V for 3 hours. The gel was rinsed with water and then kept overnight in 100 ml buffer (10 mM Tris /1 mM EDTA, pH 8) containing 40 μl RNA-se A (50 mg/ml). Finally, the gel was rinsed with distilled water and stained with ethidium bromide and photographed in UV light.

Caspase 3 colorimetric protease assay. After a 24-hour incubation with MSLHV-NT12 EDN or the control peptide, the KSIMM cells were isolated ($5x10^6$ cells) and resuspended in 50 μl cold cell lysis buffer (Tris-buffered saline containing detergent). For each sample, 100 μg protein extract measured by Bradford's method (Biorad, Hercules, USA) was assessed for caspase-3 activity according to the manufacturer's instructions (BioSource, Nivelles, Belgium). The substrate for caspase-3, DEVD-pNA, was added to each sample and incubated at 37˚C for 2 hours. The release of free pNA was measured using a spectrometer at 405 nm.

Western blotting. After a 72-hour exposure of the KSIMM cells to MSLHV-NT12 EDN or to the control peptide, a whole cell protein extract was prepared. Total cellular proteins (125 μg), measured by Bradford's method, were subjected to SDS–polyacrylamide gel electrophoresis, transferred to a nitrocellulose membrane (Hybond, Amersham Biosciences AB, Uppsala, Sweden) and blocked for 1 hour at room temperature in 5% (w/v) skimmed milk, 0.02% (w/v) Tween 20 in PBS. Incubation with the primary antibody (anti PARP) was performed for 1 hour at room temperature. After three washes with PBS, the membranes were incubated with a biotinylated secondary antibody (Amersham Biosciences AB, Uppsala, Sweden) for 1 hour. The detection was made by enhanced chemiluminiscence according to the manufacturer's instructions (Hyperfilm-ECL, Amersham), after A 15-minute incubation with streptavidin-labelled horseradish peroxidase.

Statistical analysis. All values are presented as mean ± SE. The data for the cytotoxic assay were analysed by one-way ANOVA using Tukey as a *post hoc* test. The data for caspase-3 activation were analysed using the *t*-test.

Results

hCG urinary preparations exhibit different cytotoxic activities to KSIMM cells. From four hCG compounds tested

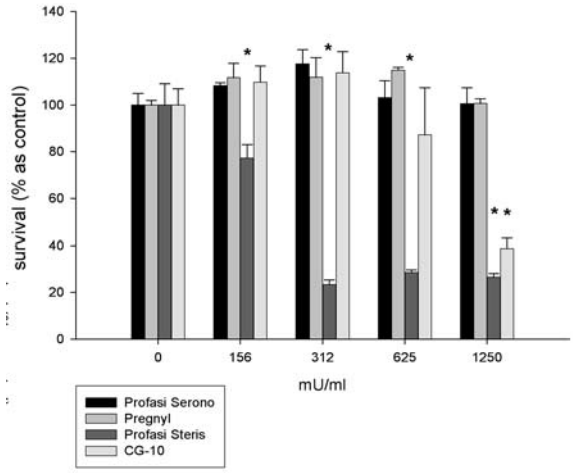

Figure 1. *Urinary hCG products have different cytotoxic effects on KSIMM.*
KSIMM cells were exposed to different hCG preparations for 48 hours and the survival rate was evaluated by MTT. The values represent means ± SEM from three different experiments (p<0.05 vs. control).*

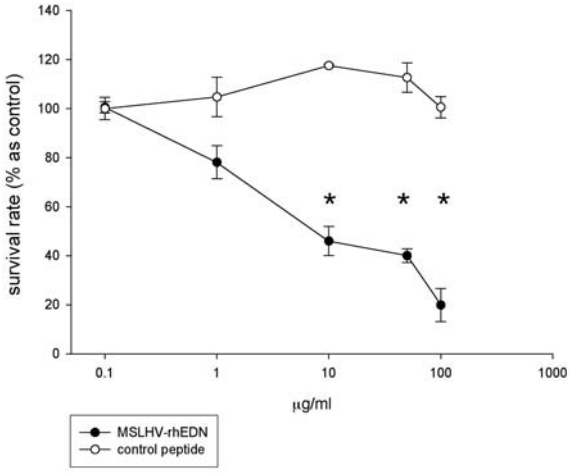

Figure 2. *MSLHV-NT12 EDN exhibits a dose-dependent cytotoxic effect on KSIMM cells.*
KSIMM cells were exposed to MSLHV-NT12 EDN and a scrambled peptide (control) for 48 hours and the survival rate was evaluated by MTT. The values represent means ± SEM from three different experiments (p<0.05 vs. control).*

(Profasi Steris, Profasi Serono, Pregnyl Organon, CG-10 Sigma), just two had a cytotoxic effect on KSIMM cells: Profasi Steris (IC_{50} at 220 mU/ml) and CG-10 (IC_{50} 1100 mU/ml). The other two hCG preparations (Profasi Serono and Pregnyl) did not affect the growth of the KSIMM cells at doses as high as 1250 mU/ml (Figure 1). The cytotoxic effects of both Profasi Steris as well as CG-10 were restricted to the KSIMM in that they did not interfere with the survival rate of human dermal fibroblasts (HDF) (data not shown).

MSLHV-NT12 EDN specifically affects the survival rate of KSIMM cells but not of SK-Mel-28 cells or normal human fibroblasts. MSLHV-NT12 EDN had a dose-dependent cytotoxic effect on the KSIMM cells with an IC_{50} of 9 µg/ml. Over 60% killing of KSIMM cells was observed at 50 µg/ml, while 100 µg/ml caused 80-90% cell death. By contrast the control peptide had no effect on the KSIMM survival rate, even at 100 µg/ml (Figure 2). The MSLHV-NT12 EDN cytotoxic effect was cell-specific in that the peptide did not affect the survival rate of a melanoma cell line (SK-Mel-28) and it was even slightly stimulatory on HDF (Figure 3).

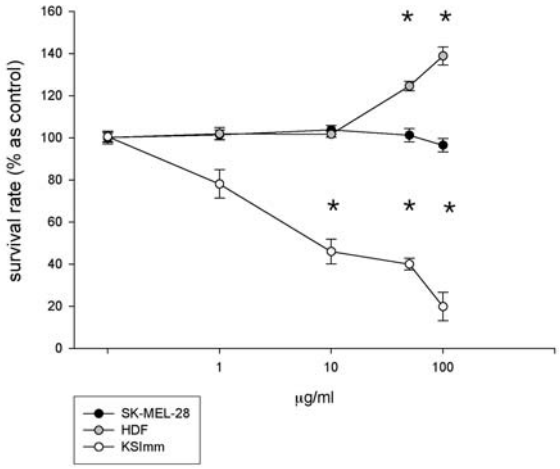

Figure 3. *MSLHV-NT12 EDN is specifically cytotoxic for KSIMM cells. KSIMM cells, SK-Mel-28 and HDF were treated with different concentrations of MSLHV-NT12 EDN for 48 hours and the survival rate was evaluated by MTT. The values represent means ± SEM from three different experiments (* p<0.05 vs. control).*

MSLHV-NT12 EDN peptide induces apoptosis in KSIMM cells. We further investigated the mechanism by which MSLHV-NT12 EDN kills KSIMM cells. As can be seen in Figure 4 A, MSLHV-NT12 EDN (50 µg/ml) induced DNA ladders on DNA agarose gel electrophoresis, formed by typical internucleosomal fragmentation, characteristic of apoptosis. In perfect agreement with this, MSLHV-NT12 EDN but not the control peptide induced proteolytic activation of caspase-3 (Figure 4B) and the apoptosis induced cleavage of cellular proteins such as PARP (Figure 4C), demonstrating that the peptide induced apoptosis in KSIMM cells.

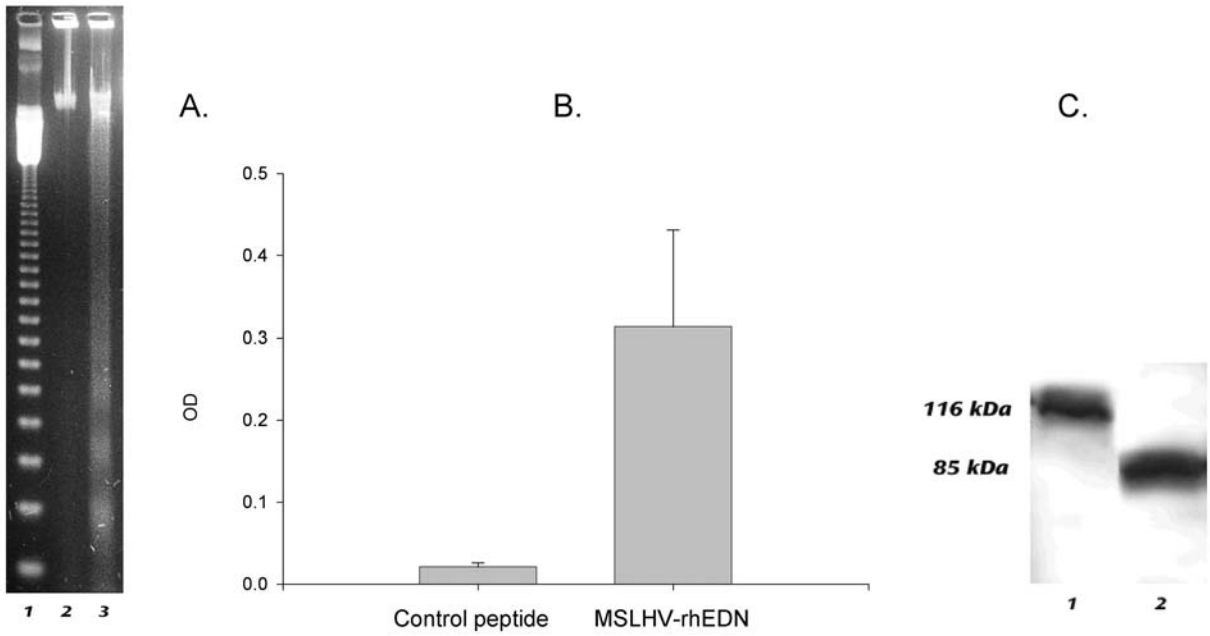

Figure 4. *MSLHV-NT12 EDN induces apoptosis in KSIMM cells.*
(A) Genomic DNA was isolated from KSIMM cells treated with 50mg/ml MSLHVNT12 EDN or control peptide for 72 hours and subsequently analysed for oligonucleosomal fragmentation on a agarose gel (A Lane 1: 100 bp-ladder DNA marker; Lane 2: control; Lane 3: 50mg/ml MSLHVNT12 EDN). (B) The cell extracts obtained from KSIMM cells treated for 24 hours with MSLHVNT12 EDN (50mg/ml) or control peptide were evaluated for caspase-3 activation by a colorimetric method. The values represent means ± SEM from three different experiments ($p<0.05$ vs. control). (C) Immunoblot analysis of PARP (p116) and its apoptosis-specific cleavage product (p85) in KSIMM cells treated for 72 hours with control (lane 1) and MSLHVNT12 EDN (50μg/ml) (lane 2).*

Discussion

After the first report of anti-KS activity of hCG *in vivo* and *in vitro* (3) several clinical trials were run with highly controversial results [recently reviewed in (5)]. It has been suggested that these discrepancies are the consequence of the presence of a moiety with anti- KS activity, which is co-purified with hCG. hCG, as a dimmer intact hormone, represents only 20-50% of the clinical grade preparations (15) thus allowing for a highly variable content of the putative anti-KS factor between companies and even between batches. In agreement with this concept, the best clinical results were obtained when the selected hCG product used was first selected based on its *in vitro* cytotoxic effect on a KS cell line (4,16).

Here we confirm that the anti-KS effect of the clinical grade urinary hCG products is very different between sources. From four hCG compounds tested only one (Profasi Steris) had a clear-cut dose-dependent cytotoxic activity on KSIMM cells. A minor cytotoxic activity was identified as well in a second compound (GC-10). The cytotoxic effect of the active hCG product was specific to the KS cells and did not affect the survival of normal human fibroblasts, which is in perfect accordance with other reports (3,7). The other two compounds (Profasi Serono and Pregnyl) tested even had a small stimulatory effect on the KSIMM proliferation rate. The same paradoxical stimulation of KS cells proliferation after treatment with some hCG products was also observed by other investigators (17), meaning that a preliminary *in vitro* test is mandatory for a clinical study on the effect of hCG on KS tumours.

One putative anti-KS substance present in hCG products is a 18 K ribonuclease, suggested to belong to the onconase family based on the N terminal sequence (9). A similar ribonuclease present in the urine of pregnant women, but not tested against tumours, had been reported before and was suggested to be the hEDN with 4 amino acids extended in the signal region ((-4EDN) (11). EDN is a member of the RNAse family of proteins, which are not cytotoxic for cultured cells but are as potent as toxins when they are injected into the cells directly (18). The N-terminal portion of several RNA-ase has been postulated to contribute to their catalytic activity, substrate specificity and cytotoxicity (19). The recombinant hEDN, extended at the amino terminus by four amino acids (serine, leucine, histidine and valine) (-4)EDN) has been reported to specifically inhibit the viability of a KS-derived cell line KS Y-1 *in vitro* (20) and it has been suggested that the extended N terminus of

the molecule is specifically recognized by tumoral cells (13). We have therefore synthesized a 16-amino-acid-peptide (MSLHV-NT12 EDN) containing the recognition motif of ((-4)hEDN) and investigated its ability to kill KSIMM cells and the mechanism of its cytotoxicity.

We found that the MSLHV-NT12 EDN had a strong cytotoxic effect on KSIMM cells. A concentration of 50 μg/ml induced over 60% killing of KSIMM cells within 48 hours. The cytotoxic effect of this short peptide was comparable, although at a higher concentration, to the effects of the full (-4)hEDN protein reported for KSY1 cells (20). However the MSLHV-NT12 EDN concentration used is still in the range of concentrations compatible with its potential *in vivo* use. As was initially reported for the full protein ((-4)EDN), the short peptide synthesized by us (MSLHV-NT12 EDN) has a cell-specific effect, being virtually harmless for the melanoma cell line or human fibroblast cells.

Biochemical changes induced by MSLHV-NT12 EDN in KSIMM cells displayed the hallmarks of apoptosis and this was associated with caspase-3 activation and PARP degradation. However, the exact mechanism by which MSLHV-NT12 EDN is able to induce cell-specific apoptosis is still unknown. After the completion of the (-4)EDN crystallographic structure, a very elegant explanation of its mechanism of action has been provided (13). The enzymatic activity of (-4)EDN was lower than that of EDN, indicating that anti-KS activity is not related to the ability of the molecule to degrade RNA, but the N-terminal extension represents a recognition motif for a specific binding site at the cellular membrane level. That may explain the cytotoxic selectivity of this modified EDN on KS-derived cells. After (-4)EDN binds at a specific site on the cell membrane, the molecule is internalised and it is allowed to reach its intracellular target and to induce cell death. These data suggest that the cell recognition of EDN ribonuclease is largely dependent upon the amino acid sequence in the extended N-terminal portion of the molecule. This is in perfect accordance with our data which show that even a small peptide, which preserves just the first 16 amino acids of the N-terminus extended EDN, has a specific proapoptotic effect for KS cells. Moreover we found that the cytotoxic effect of MSLHV-NT12 EDN is abolished when the cells are co-incubated with suramin, a polysulfonated naphthylurea, known to block the cell surface binding of various growth factors (21) (data not shown).

In conclusion, our data indicate that the anti-KS moiety, present in urinary extracted hCG products, is different from the hormone itself. We could also demonstrate that a small peptide (MSLHV-NT12 EDN), which contains the recognition motif of ((-4)EDN), specifically induces apoptosis in KSIMM cells. Using synthetic small peptides instead of full proteins clearly has advantages with regards to the ease of manipulation, the purity of the products and cost-effectiveness. Therefore, due to its anti-KS capabilities *in vitro*, the MSLHV-NT12 EDN peptide seems to be promising for clinical trials in KS therapy.

Acknowledgements

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References

1 Jacobson LP, Yamashita TE, Detels R, Margolick JB, Chmiel JS, Kingsley LA, Melnick S and Munoz A: Impact of potent antiretroviral therapy on the incidence of Kaposi's sarcoma and non-Hodgkin's lymphomas among HIV-1-infected individuals. Multicenter AIDS Cohort Study. J Acquir Immune Defic Syndr *21 Suppl 1*: S34-S41, 1999.

2 Antman K and Chang Y: Kaposi's sarcoma. N Engl J Med *342*: 1027-1038, 2000.

3 Lunardi-Iskandar Y, Bryant JL, Zeman RA, Lam VH, Samaniego F, Besnier JM, Hermans P, Thierry AR, Gill P and Gallo RC: Tumorigenesis and metastasis of neoplastic Kaposi's sarcoma cell line in immunodeficient mice blocked by a human pregnancy hormone. Nature *375*: 64-68, 1995.

4 Gill PS, Lunardi-Ishkandar Y, Louie S, Tulpule A, Zheng T, Espina BM, Besnier JM, Hermans P, Levine AM, Bryant JL and Gallo RC: The effects of preparations of human chorionic gonadotropin on AIDS-related Kaposi's sarcoma. N Engl J Med *335*: 1261-1269, 1996.

5 Simonart T, Van Vooren JP and Meuris S: Treatment of Kaposi's sarcoma with human chorionic gonadotropin. Dermatology *204*: 330-333, 2002.

6 Lang ME, Lottersberger C, Roth B, Bock G, Recheis H, Sgonc R, Sturzl M, Albini A, Tschachler E, Zangerle R, Donini S, Feichtinger H and Schwarz S: Induction of apoptosis in Kaposi's sarcoma spindle cell cultures by the subunits of human chorionic gonadotropin. AIDS *11*: 1333-1340, 1997.

7 Samaniego F, Bryant JL, Liu N, Karp JE, Sabichi AL, Thierry A, Lunardi-Iskandar Y and Gallo RC: Induction of programmed cell death in Kaposi's sarcoma cells by preparations of human chorionic gonadotropin. J Natl Cancer Inst *91*: 135-143, 1999.

8 Bisacchi D, Noonan DM, Carlone S, Albini A and Pfeffer U: Kaposi's sarcoma and human chorionic gonadotropin: mechanisms, moieties and mysteries. Biol Chem *383*: 1315-1320, 2002.

9 Griffiths SJ, Adams DJ and Talbot SJ: Ribonuclease inhibits Kaposi's sarcoma. Nature *390*: 568, 1997.

10 Griffiths SJ, Bramley TA, Menzies GS and Adams DJ: Co-purification of a ribonuclease and human chorionic gonadotrophin beta-core protein from human urine and displacement of 125I-human luteinizing hormone from *Candida albicans* binding sites by ribonucleases. Mol Cell Endocrinol *134*: 69-76, 1997.

11 Sakakibara R, Hashida K, Tominaga N, Sakai K, Ishiguro M, Imamura S, Ohmatsu F and Sato E: A putative mouse oocyte maturation inhibitory protein from urine of pregnant women: N-terminal sequence homology with human nonsecretory ribonuclease. Chem Pharm Bull (Tokyo) *39*: 146-149, 1991.

12 Newton DL, Nicholls PJ, Rybak SM and Youle RJ: Expression and characterization of recombinant human eosinophil-derived neurotoxin and eosinophil-derived neurotoxin-anti-transferrin receptor sFv. J Biol Chem *269*: 26739-26745, 1994.
13 Chang C, Newton DL, Rybak SM and Wlodawer A: Crystallographic and functional studies of a modified form of eosinophil-derived neurotoxin (EDN) with novel biological activities. J Mol Biol *317*: 119-130, 2002.
14 Catrina SB, Catrina AI, Sirzen F, Griffiths W, Bergman T, Biberfeld P, Coculescu M and Mutt V: A cytotoxic, apoptotic, low-molecular weight factor from pineal gland. Life Sci *65*: 1047-1057, 1999.
15 Manjunath P and Sairam MR: Biochemical, biological, and immunological properties of chemically deglycosylated human choriogonadotropin. J Biol Chem *257*: 7109-7115, 1982.
16 Hermans P, Clumeck N, Picard O, Van Vooren JP, Duriez P, Zucman D, Bryant JL, Gill P, Lunardi-Iskandar Y and Gallo RC: AIDS-related Kaposi's sarcoma patients with visceral manifestations. Response to human chorionic gonadotropin preparations. J Hum Virol *1*: 82-89, 1998.
17 Simonart T, Hermans P, Van Vooren JP and Meuris S: Paradoxical pro-Kaposi's sarcoma activity of preparations of human chorionic gonadotropin. Blood *94*: 376-377, 1999.
18 Saxena SK, Rybak SM, Winkler G, Meade HM, McGray P, Youle RJ and Ackerman EJ: Comparison of RNases and toxins upon injection into *Xenopus* oocytes. J Biol Chem *266*: 21208-21214, 1991.
19 Newton DL, Boque L, Wlodawer A, Huang CY and Rybak SM: Single amino acid substitutions at the N-terminus of a recombinant cytotoxic ribonuclease markedly influence biochemical and biological properties. Biochemistry *37*: 5173-5183, 1998.
20 Newton DL and Rybak SM: Unique recombinant human ribonuclease and inhibition of Kaposi's sarcoma cell growth. J Natl Cancer Inst *90*: 1787-1791, 1998.
21 Coffey RJ Jr, Leof EB, Shipley GD and Moses HL: Suramin inhibition of growth factor receptor binding and mitogenicity in AKR-2B cells. J Cell Physiol *132*: 143-148, 1987.

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