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Changes in elemental concentrations in LNCaP cells are associated with a protective effect of neuropeptides on etoposide-induced apoptosis

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Abstract

One of the mechanisms that has been put forward for the development of the androgen-resistant status is neuroendocrine differentiation. Neuroendocrine cells secrete neuropeptides that may represent one of the possible molecular bases by which hormone-dependent prostate cancer cells could escape treatment. LNCaP prostate cancer cells were treated with either etoposide or neuropeptides. Morphological changes related to apoptosis and cell viability were assessed. Changes in intracellular ion content were quantitatively analyzed by electron probe X-ray microanalysis. Etoposide treatment consistently induces a decrease in K and an increase in Na, which are inhibited by bombesin or calcitonin. The Na/K ratio increased markedly after exposure to etoposide, and both bombesin and calcitonin blocked this increase. Etoposide also caused changes in the intracellular P and S concentrations that to a large extent could be blocked by neuropeptides. These results support the hypothesis that neuropeptides confer anti-apoptotic capabilities onto non-neuroendocrine cells in close proximity to neuroendocrine cells.

Keywords: Apoptosis; Prostate carcinoma; Electron probe X-ray microanalysis; Intracellular ions; Neuropeptides; Bombesin; Calcitonin

1. Introduction

Prostatic adenocarcinoma reveals extensive and multilocal neuroendocrine features in ~10% of cases by using the neuroendocrine marker, chromogranin A. These tumors are generally aggressive and often resistant to hormonal therapy (Fixemer et al., 2002). Ultrastructural studies, biochemical analyses, and histochemical staining provide evidence for functionally diverse subtypes of NE cells within the prostate that secrete a wide range of peptides known to stimulate cell growth in an autocrine and paracrine fashion (Clegg et al., 2003).

Neuroendocrine secretory products and their interactions with epithelial prostate cells are currently being investigated to gain a better understanding of their significance in the pathogenesis of the prostate gland disorders, their prognosis and therapy (Wilson et al., 2001). Calcitonin (CT), one such neuropeptide, has been associated with the growth of prostate cancer (Aprikian et al., 1998; Chien et al., 2001). Bombesin acts as a survival and migratory factor for androgen-independent prostate cancers; it may also influence growth, invasiveness, metastatic processes and angiogenesis in prostatic carcinoma (Hansson and Abrahamsson, 2001) and can induce LNCaP growth in the absence of androgens (Lee et al., 2001).

We previously demonstrated that the neuropeptides, bombesin and calcitonin, inhibit etoposide-induced apoptosis, both in androgen-dependent (LNCaP) and in androgen-independent (PC-3 and Du 145) prostate cancer cell lines (Salido et al., 2000). Furthermore, we have shown that apoptosis is associated with changes in elemental content of the cells, and bombesin and calcitonin inhibit apoptosis-associated some of these elemental changes (Salido et al., 2002). These findings strengthen the link between apoptosis and changes in the intracellular elemental content.

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Apoptosis is accompanied by major changes in ion compartmentalization and transmembrane potentials, with transient mitochondrial swelling and subsequently a loss of plasma membrane potential related to the loss of cytosolic K^+ (Salido et al., 2002). Electron probe X-ray microanalysis also gives information about the distribution of elements at the cell and tissue level. This technique combines the chemical analysis with the high-resolution localization at the level of the electron microscope, thus correlating chemical with ultrastructural details.

The approach taken in the present study mimics the presence of neuroendocrine cells in prostatic carcinoma. We have investigated whether bombesin and calcitonin inhibit etoposide-induced changes in intracellular elemental concentrations in LNCaP cells in a similar manner in androgen-independent cell lines. Neuroendocrine cells, thus, become an indicator of poor prognosis in patients with prostate carcinoma that is independent of hormonal status of epithelial cell tumors.

2. Materials and methods

2.1. Study design

To determine whether neuropeptides inhibit the etoposide-induced ionic changes in intracellular ion concentrations in the androgen-dependent prostate cancer cells, we used the androgen-dependent cell line LNCaP (Salido et al., 2002). Cells were grown in RPMI 1640 medium containing 5% fetal bovine serum (FBS) for 3 days and then changed to medium containing 5% steroid-free serum (SFS) for 2 days. The cultures were treated with either 150 μM etoposide or bombesin (or calcitonin), and either alone or in combination with etoposide. Cells were examined by light and fluorescence optical microscopy and scanning electron microscopy to assess morphological changes of apoptosis. Changes in intracellular ion content were analyzed by electron probe X-ray microanalysis at 100 kV in the STEM mode of a Hitachi H7100 electron microscope, with an Oxford Instruments ISIS energy dispersive spectrometer system.

2.2. Cell culture

LNCaP prostate cancer cells provided by ATCC (Teddington, Middlesex, UK) were originally obtained from a lymph node metastasis at passage 18. Cells were grown in RPMI 1640 (ICN Biomedicals, Aurora, OH) supplemented with 10% FBS (Boehringer, Heidelberg, Germany), 4% penicillin–streptomycin (Biochrom, Berlin, Germany) and 0.4% gentamycin (Gibco, Paisley, Scotland) under standard conditions in a water-saturated atmosphere of 5% CO_2 in air until the experiment was started. Experiments involved unsynchronized exponentially growing cells.

2.3. Etoposide-induced apoptosis. Inhibition of etoposide-induced apoptosis

We have used an etoposide-induction protocols previously validated by Salido et al. (2002). Briefly, $2–5 \times 10^5$ cells/well were seeded in microplates. After 3 days, the medium was switched to 5% SFS (Biogenesis, Bournemouth, England) supplemented with RPMI 1640, and 48 h later 150 μM etoposide (Sigma, Steinheim, Germany) was introduced for 48 h. To evaluate the inhibition of etoposide-induced apoptosis, cells were exposed to combined treatments with etoposide (as described above) and bombesin (Sigma; 1 nM) or calcitonin (Sigma; 500 pg/ml). A control group cultured in the standard medium was established in each experiment. Positive controls were treated with bombesin (1 nM) or calcitonin (500 pg/ml), as described above.

2.4. Apoptosis quantification

2.4.1. Light and fluorescent microscopy

Cells were monitored during the experiment with a Nikon Diaphot phase contrast microscope to assess morphological changes. Once the experiment was finished, cultured cells were collected by trypsinization and cytospun on to glass slides. At least four slides were obtained for each group. Two of them were fixed in 10% formalin, stained with H&E and examined by light microscopy. Two more slides were air-dried, fixed in methanol at −20° for 20 min, and the cell nuclei stained with DAPI at room temperature and in darkness for 20 min. The cultures were mounted with the anti-fading medium containing *o*-phenylendiamine (Sigma) in glycerol (Merck, Darmstadt, Germany) and kept in the dark at −20 °C until examination by fluorescence microscopy in the range 300–400 nm.

At least 200 cells were counted per experiment, and the percentage of apoptotic cells was defined as (number of apoptotic cells/total cell number) × 100. Morphological changes, such as nuclear roundness and condensation, presence of apoptotic bodies, cell surface alterations, blebbing, detachment and rounding up of treated cells, were subjectively assessed.

2.4.2. Scanning electron microscopy

Cells cultured on glass coverslips were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, postfixed in 1% OsO4 in the same buffer, dehydrated in a graded acetone series and critical-point dried. They were coated with gold and examined in an LEO (Cambridge, UK) 1530 field emission scanning electron microscope at 1 kV.

2.4.3. Growth kinetics and cell viability

Growth kinetics and cell viability were determined by the XTT viability assay and the trypan blue exclusion test. The XTT assay (Roche, Palo Alto, CA) was carried out according to the manufacturer's instructions.

2.5. X-ray microanalysis in the scanning-transmission electron microscope

For analysis in the scanning-transmission electron microscope (STEM) the cells were cultured on titanium grids (Agar Scientific, Stansted, UK) covered with a Formvar film (Merck). Cells were cultured as described above. After the exposure period, the grids were rinsed briefly in cold distilled water (4 °C), frozen in liquid nitrogen–cooled liquid propane (−180 °C), freeze-dried in vacuum overnight at −130 °C and slowly brought to room temperature under vacuum. After coating with a conductive carbon layer, X-ray microanalysis was performed at 100 kV in the STEM mode of a Hitachi (Tokyo, Japan) H7100 electron microscope with an Oxford Instruments ISIS energy dispersive spectrometer system (Oxford Instruments, Oxford, UK). Quantitative analysis was carried out based on the peak-to-continuum ratio after correction for extraneous background, and by comparing the spectra from the cells with those of a standard, which consisted of known concentrations of mineral salts in a 20% gelatin and 5% glycerol matrix, frozen, cryosectioned and freeze-dried to resemble the specimen in its physical and chemical properties. Spectra were acquired for 100 s and only one spectrum was obtained for each cell.

2.6. Statistical analysis

Initial statistical comparison of element concentrations was made using analysis of variance (ANOVA), where significant differences were established. Homogeneity of means was tested using Bonferroni's test. Correlation coefficients and linear regression equations were also calculated to assess the strength of the relationship between elements.

3. Results

After the androgen-withdrawal protocol, 6.6% of apoptosis was seen in untreated cells. Etoposide treatment of LNCaP cells resulted in a dose- and time-dependent cytotoxicity, accompanied by induction of apoptosis. The combined treatment with etoposide and neuropeptides decreased the percentage of apoptotic cells from 42% in etoposide-treated cultures to 14.5% in bombesin plus etoposide group, and to 25% in etoposide plus calcitonin treated cells. No significant apoptosis was observed prior to the androgen withdrawal protocol (Fig. 1).

Apoptotic cells are rounded, and detached from neighboring cells. In the etoposide-treated group most cells are in advanced stages of apoptosis, with membrane alterations, such as blebs. The presence of neuropeptides blocks these apoptotic changes, no blebs are observed, and cells remain larger and more attached to the substrate than in the etoposide-treated group (Fig. 2). These changes are clearly associated with elemental changes as determined by X-ray microanalysis, as described below.

3.1. Growth kinetics and cell viability

Etoposide treatment resulted in a decreased cell viability (48%) with respect to untreated cells. In the presence of neuropeptides, an increase in cell viability was noticed in etoposide-treated cells. In those cells treated with bombesin or calcitonin alone, cell viability improved with respect to untreated cells and also to cells exposed to etoposide and in the case of combined treatment with etoposide and neuropeptides (Fig. 3).

3.2. X-ray microanalysis in the scanning-transmission electron microscope

In the LNCaP cell line, etoposide treatment consistently induced a decrease in K and an increase in Na. These changes were inhibited by bombesin or calcitonin (Fig. 4). The Na/K ratio in LNCaP cells increased

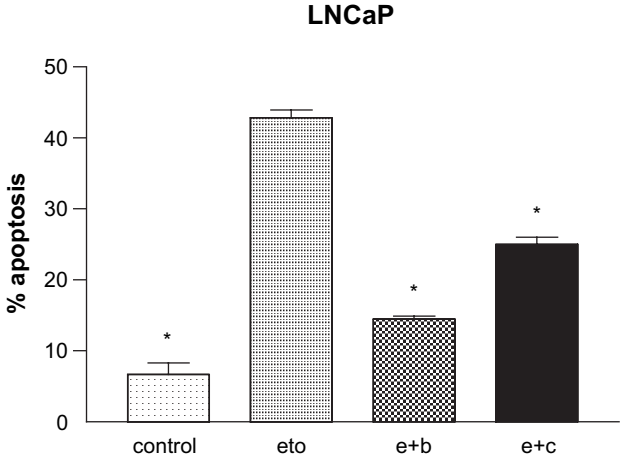

Fig. 1. Percentage of apoptotic cells. Etoposide treatment after the androgen-withdrawal protocol results in a significant induction of apoptosis in LNCaP androgen-dependent cells. The presence of neuropeptides bombesin and calcitonin causes a significant decrease in the percentage of apoptotic cells when compared to etoposide-treated cells. Data, shown as means and standard deviation (SD), representative of at least 10 experiments, are given after examination by three independent observers. $*P < 0.001$; when compared with etoposide-treated cells.

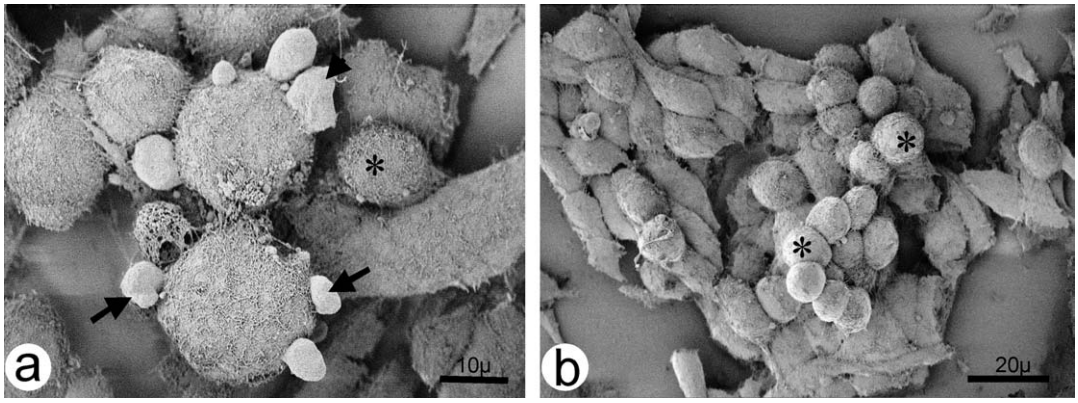

Fig. 2. Scanning electron micrographs of LNCaP prostate carcinoma cells: (a) etoposide-treated cells and (b) etoposide + bombesin treated cells. Apoptotic cells (*) are rounded, and detached from neighboring cells. In the etoposide-treated group most cells are in advanced stages of apoptosis as shown in the micrograph, with membrane alterations, such as blebs (↑). The presence of neuropeptides blocks apoptotic changes. Most cells do not pass to the latest stages of apoptosis, no blebs are observed, and cells remain larger and more attached to the substrate than in the etoposide-treated group.

markedly after exposure to etoposide, and both bombesin ($P < 0.001$) and calcitonin ($P < 0.001$) blocked this increase (Fig. 5).

Etoposide treatment caused an increase in the concentration of P, S and Cl (Fig. 4), resulting in a decrease in the ratio of P to S and an increase in the ratio of Cl to K (Fig. 6). The addition of the neuropeptides resulted in inhibition of the increase in Cl/K ratio and also in an increase of P/S ratio (Fig. 6). Bombesin, but not calcitonin blocked changes in chloride concentrations and both neuropeptides inhibited the changes observed in the rest of elements analyzed. Calcitonin was more effective in blocking changes in K, S, and P, whereas bombesin was more effective in blocking changes in Mg, Na and Cl.

4. Discussion

Apoptosis plays a major role in prostate cancer development (Matsushima et al., 1999). Here we provide evidence that neuropeptides bombesin and calcitonin block apoptosis-related ion changes in etoposide-induced apoptosis in LNCaP cells.

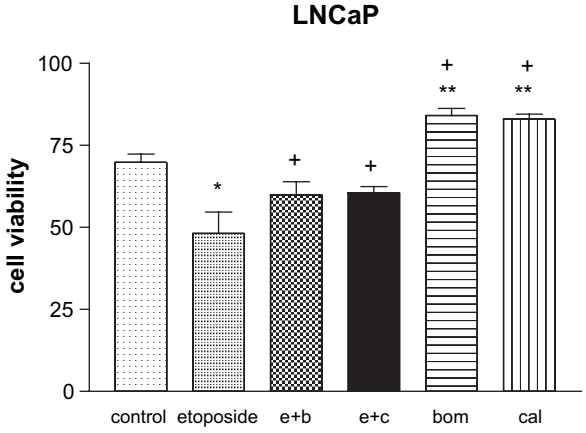

Fig. 3. Cell viability assessed after different treatments. Treatment with etoposide alone decreased cell viability of LNCaP cells, after the androgen withdrawal protocol. The addition of neuropeptides to etoposide-treated cells resulted in an improved cell viability when compared to etoposide-treated cells with values slightly lower than in control cells. When cells were treated with bombesin or calcitonin alone cell viability was significantly ($P < 0.001$) higher than in the rest of groups. Data, shown as means and SD, representative of at least 10 experiments, are given after examination by three independent observers. *$P < 0.01$ when compared with control cells, **$P < 0.001$ when compared with etoposide-treated cells; +$P < 0.001$ when compared with neuropeptides treated cells.

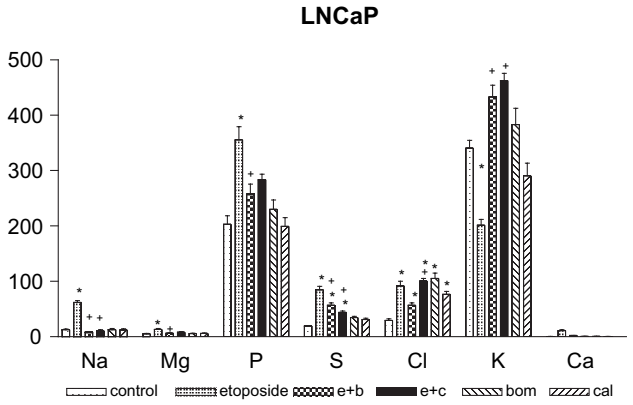

Fig. 4. Elemental concentrations in LNCaP cells analyzed in the STEM. Means and standard error of the mean for control cells, cells treated for 48 h with etoposide, cells treated for 48 h with etoposide in the presence of bombesin (e+b), cells treated for 48 h with bombesin alone (bom), cells treated for 48 h with etoposide in the presence of calcitonin (e+c), and cells treated for 48 h with calcitonin alone (cal) are given. The data are expressed in mmol/kg dry weight. Statistically significant differences from control cells are denoted by an asterisk (*) ($P < 0.001$), and statistically significant differences between etoposide-treated cells and cells treated with etoposide + bombesin or calcitonin are indicated by (+) ($P < 0.001$). Data based on 25–45 measurements per group, two separate experiments.

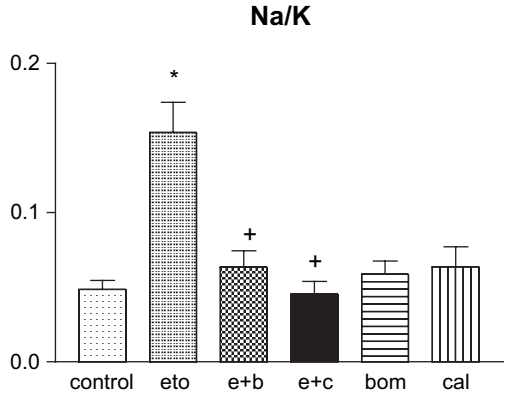

Fig. 5. Elemental Na/K ratio in LNCaP cells. For explanation of the symbols see the legend of Fig. 4. Statistically significant differences between control and etoposide-treated cells are denoted by an asterisk (*) ($P < 0.001$), and statistically significant differences between etoposide-treated cells and cells treated with etoposide + bombesin or calcitonin are indicated by (+) ($P < 0.001$). Data based on 30–60 measurements per group, three separate experiments.

There appears to be a direct relationship between the density of NE cells and enhancement of prostate cancer characteristics, such as increased Gleason grade, lose of androgen sensitivity, and autocrine/paracrine activity. It has been suggested that NE cells provide paracrine stimuli for the propagation of local carcinoma cells and that NE differentiation is associated with the progression of prostate cancer toward an androgen-independent state (Cox et al., 1999; Hansson and Abrahamsson, 2001; Ito et al., 2001; Goodin and Rutherford, 2002; Segawa et al., 2001).

The approach taken in the present study mimics the presence of neuroendocrine cells in prostatic carcinoma; it makes possible to correlate the morphological changes and elemental patterns that appear in neuropeptide-induced resistance to apoptosis. Etoposide induces a decrease in the cellular K concentration, and an increase in the cellular Na concentration. Calculation of the Na/K ratio, a sensitive indicator of cell injury, shows that this ratio increases after etoposide treatment. We have described similar ionic changes after induction of apoptosis in androgen-independent prostate cell lines (Salido et al., 2002) and also in other systems by other groups (Fernández-Segura et al., 1999). These changes may be due to activation of K^+ channels, or to inhibition of the Na^+/K^+-ATPase, presumably due to lack of ATP. Most likely the changes are due to both factors. Activation of K^+ channels is one of the common features of apoptosis and prevention of etoposide-induced apoptosis in thymocytes by blocking K channels has been reported earlier (Dallaporta et al., 1998; Maeno et al., 2000). The addition of bombesin or calcitonin to etoposide-treated cells reduces the percentages of apoptotic cells, with an increase in K and a decrease in Na, and subsequent decrease of the Na to K ratio, as an objective expression of the increase in the viability of the neoplastic cells in the presence of neuropeptides.

The data also show an increase in the cellular Cl concentration, which can also be expressed as an increased Cl/K ratio. This may appear to contradict the notion that chloride channel activation occurs at the onset of apoptosis (Maeno et al., 2000). However, if the increase in the cellular Na/K ratio is to be interpreted as an effect of energy deficiency, the cell would be unable to maintain its low intracellular chloride concentration, and chloride ions would flow into the cell along the electrochemical gradient.

The data further indicate that apoptosis is associated with an increase in both P and S, but more in S, which results in a decreased P/S ratio. The P signal mainly represents nucleotides and phosphorylated proteins, whereas the S signal mainly represents protein-bound sulfur. This correlates to the morphological changes associated with apoptosis, The fact that these changes are very marked, and can be inhibited to a large extent by neuropeptides, indicates that they are important in the process of apoptosis.

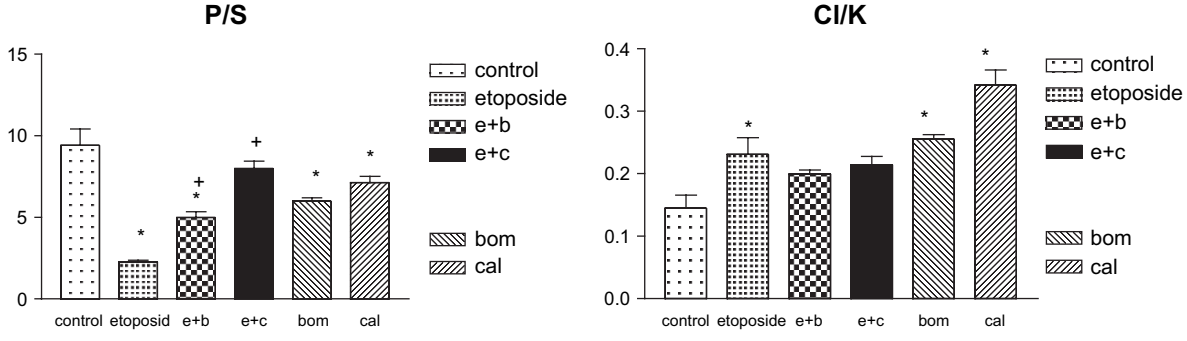

Fig. 6. Elemental ratios in LNCaP cells analyzed in the STEM (a) P/S ratio, (b) Cl/K ratio. For explanation of the symbols see the legend of Fig. 4. Statistically significant differences with control cells are denoted by an asterisk (*), and statistically significant differences between etoposide-treated cells and cells treated with etoposide + bombesin or calcitonin are indicated by (+) ($P < 0.01$). Data based on 25–45 measurements per group, two separate experiments. The increase in P/S ratio is blocked by calcitonin ($P < 0.001$) and bombesin ($P < 0.01$).

This protective effect on etoposide-induced apoptosis in LNCaP cells appears to be quite similar to that we have described earlier for androgen-independent cells (Salido et al., 2000, 2002). This confirms that neuropeptides confer anti-apoptotic capabilities on non-neuroendocrine cells in close proximity to neuroendocrine cells, and that this occurs both in androgen-dependent and androgen-independent cells. It can therefore be speculated that certain neuroendocrine peptides can increase the survival and further growth of neighboring cells (Ismail et al., 2002) and thereby contribute to the aggressive clinical course of prostate tumors containing neuroendocrine elements (Hoosein, 1998). According to our data, it therefore appears that neuroendocrine cells can be good indicators of poor prognosis in patients with prostate carcinoma, independently of hormonal status of the epithelial cell tumors.

In conclusion, our evidence shows that the presence of neuroendocrine cells and their secretory products confers anti-apoptotic capabilities on non-neuroendocrine cells in close proximity to neuroendocrine cells, both in androgen-sensitive and androgen-insensitive cells. New therapeutic protocols and trials need to be developed to test drugs based on neuroendocrine hormones, and hopefully, this will lead to the development of entirely new therapeutic modalities.

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