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# Induction of Apoptosis in Human Breast Cancer and Trophoblast Tumor Cells by Galectin-1

IRMI WIEST[1], CHRISTIAN SELIGER[2], HERMANN WALZEL[3], KLAUS FRIESE[1] and UDO JESCHKE[1]

[1]*Ludwig Maximilians University of Munich, First Department of Obstetrics & Gynaecology, Maistrasse 11, D-80337 Munich;*
[2]*Ludwig Maximilians University of Munich, Institute of Pathology, Thalkirchner Str. 36, D-80337 Munich;*
[3]*University of Rostock, Institute of Medical Biochemistry & Molecular Biology, Schillingallee 70, D-18057 Rostock, Germany*

**Abstract.** *Galectin-1 (gal-1), a member of the mammalian β-galactoside-binding proteins, preferentially recognizes Galβ1-4GlcNAc (LacNAc) sequences of oligosaccharides associated with several cell surface glycoconjugates. As demonstrated histochemically, gal-1 recognizes appropriate glycoepitopes on human breast cancer cells (MCF7) and on human chorionic carcinoma cells (BeWo). Gal-1 is expressed in many malignant and normal tissues. A high level of expression is found in lymphatic organs, which feature high rates of apoptosis. Furthermore, it is known that galectin-1 can initiate T cell apoptosis. In this study, we examined the apoptotic potential of gal-1 in vitro on MCF7 and BeWo cells. After growing both cell lines on chamber-slides for three days, apoptosis was induced by incubation with 30 μg gal-1 per ml serum-free medium for 6, 9 and 20 hours. To avoid false increased rates of apoptosis by deletion of FCS, all approaches were done with and without FCS. Apoptotic cells were detected by in situ nick translation. The rate of apoptosis was determined by counting 1500 cells per chamberslide. The normal rate of apoptosis ranged between 0.1% and 0.3%. The incubation of both cell lines with 30 μg/ml gal-1 in serum-free medium for 6 and 9 hours marginally raised the number of apoptotic cells. An increase of apoptosis was only shown by additional stimuli like hyperthermia, removal of $CO_2$ and FCS for 20 hours. Impressing findings were manifested in an older passage of BeWo cells, in which only omission of FCS caused apoptotic rates for up to 25% after 6 hours. The presence of mycoplasma in this BeWo passage was shown by PCR. Our results demonstrated, that galectin-1 shows apoptotic potential in both the epithelial tumour cell lines examined only with additional stress stimuli.*

*Correspondence to:* PD Dr. rer. nat. habil. Udo Jeschke, Ludwig Maximilians University of Munich, First Department of Obstetrics and Gynaecology, (Director: Prof. Dr. med. habil. K. Friese), Maistrasse 11, 80337 Munich, Germany. Tel: 0049-89-5160-4266, Fax: 0049-89-5160-4916, e-mail: udo.jeschke@med.uni-muenchen.de

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The galectin family members are defined by a conserved amino acid sequence motif in the carbohydrate recognition domain (CRD) and an affinity for β-galactosides. Galectin-1 (gal-1), the earliest described member of the family, is a homodimeric protein with a carbohydrate recognition domain of 134 amino acids (1). Expression of gal-1 has been specially identified in lymphoid organs such as thymus, lymph nodes, in activated macrophages and T cells and in immune-privileged sites such as placenta and cornea, suggesting an important role in generating and maintaining immune tolerance (2). Although LacNAc is the basic ligand recognized by gal-1, the proto-type galectin binds with increased avidity to multiple Galβ1-4GlcNAc sequences presented on branched N-linked or on repeating LacNAc residues on N- and O-linked glycans. Gal-1, having a single CRD, forms a non-covalently associated homodimer to become functionally bivalent under physiological conditions. The bivalent nature of gal-1 facilitates glycan-mediated cell surface receptor cross-linking, believed to be essential in inducing signaling events (3, 4). Extracellularly, by recognition of glycan ligands, gal-1 exerts distinct biological effects in various tissues and on cells, including cell adhesion (5, 6), metastasis (7), cell growth regulation (8, 9), immunosuppression (10) and apoptosis (3).

Binding experiments of breast cancer tumor cells to laminin and plasma or placental fibronectin showed that binding was generally reduced by treatment of cells with gal-1 (11). Cell binding of gal-1 proved to be very effective for blocking cell association to fibronectin after its preincubation with cell suspensions. Reduced expression of gal-1 seems to be associated with a positive lymph node status in the breast cancer group. These results can be interpreted to reflect cell-

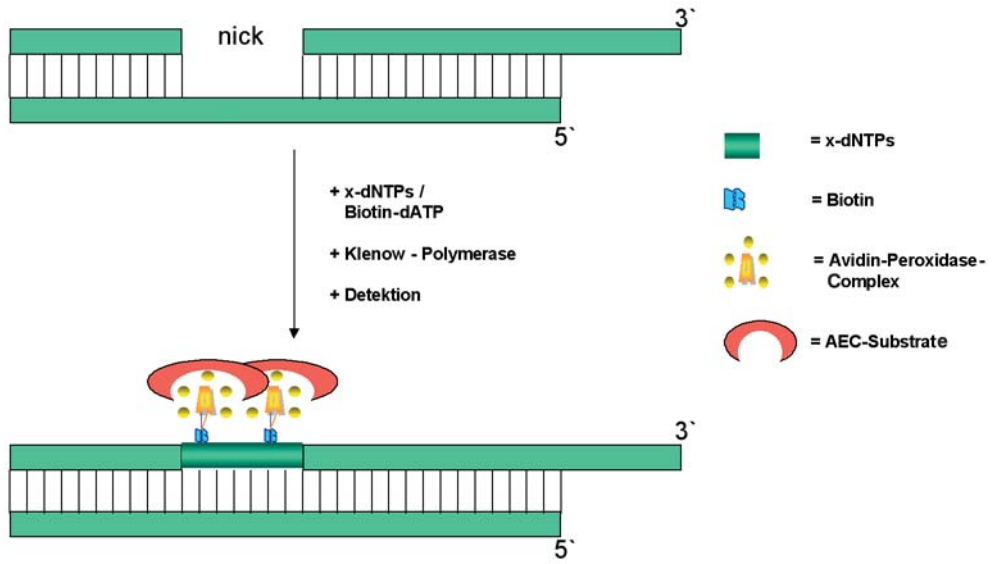


Figure 1. *Principle of detection of DNA fragmentation by in situ nick-translation technique (ISNT).*

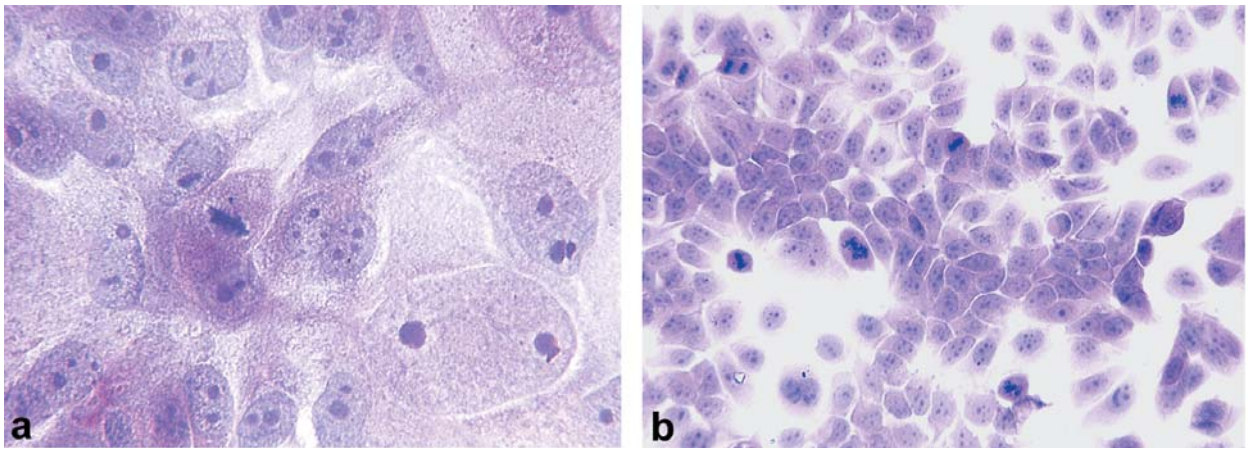


Figure 2. *a. HE staining of BeWo cells (25x). b. HE staining of MCF7 cells (10x).*

type-dependent requirements of galectin ligand presentation during the metastatic cascade (11).

Gal-1 is also expressed in the placenta. The placenta plays a key role in the maintenance of local tolerance and allows the mother to accept the embryo until completion of pregnancy. The complex process of tolerance accompanying the survival of the fetus is controlled at the embryo-maternal interface by factors deriving from decidualized endometrium and from the trophoblast itself. Trophoblasts develop various strategies to evade the damaging attack by the maternal immune response, including expression of non-classical MHC class I antigens and of complement regulatory proteins (12). Choriocarcinoma cell lines were evaluated as an experimental model of trophoblast-derived immunoregulation (13). The cell line BeWo was established by Pattillo *et al.* (14). In contrast to Jeg3, cultures of BeWo choriocarcinoma cells contain two co-existing phenotypes: a cytotrophoblast-like and a syncytiotrophoblast-like phenotype (15). We found in these cells a moderate staining for mucin 1 (MUC 1) but a strong expression of the Thomsen-Friedenreich (TF) tumor antigen (16). This pattern probably represents the syncytiotrophoblast-like phenotype because the syncytiotrophoblast *in vivo* also strongly expresses TF and MUC 1 (17). In this article we describe that the binding of placental gal-1 on breast cancer and trophoblast tumor cells induces apoptosis in MCF7 and BeWo cells *in vitro* when combined with additional stress such as removal of serum, hyperthermia, low $CO_2$ and mycoplasma.

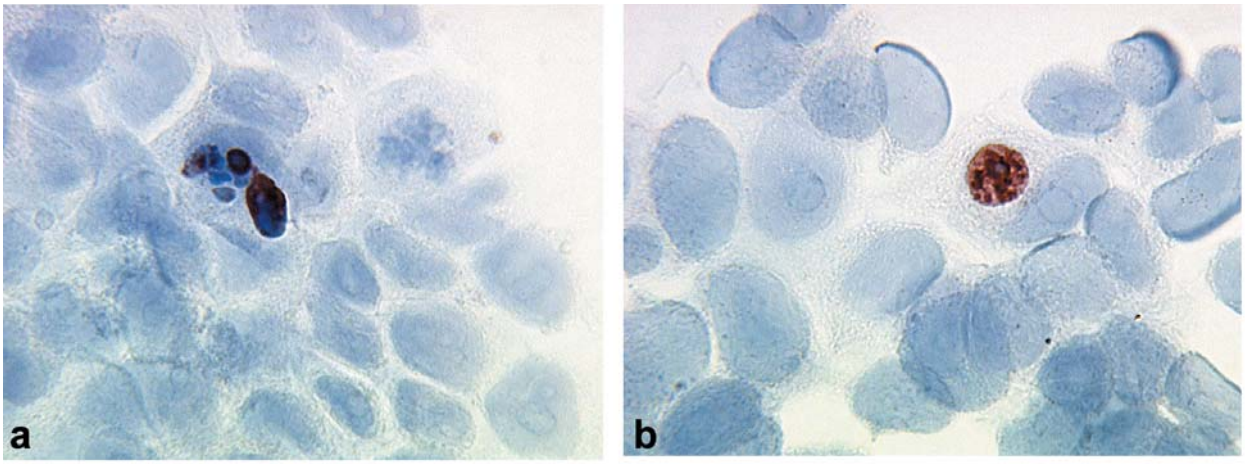


Figure 3. *a. Detection of few apoptotic cells after cultivation of BeWo cells in serum-free medium for up to 9 hours (25x). b. Detection of few apoptotic cells after cultivation of MCF7 cells in serum-free medium for up to 9 hours (40x).*

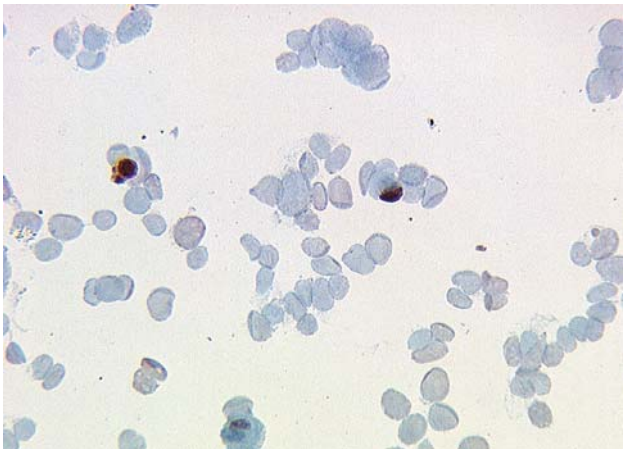

Figure 4. *Induction of apoptosis by galectin-1 on MCF7 cells with additional hyperthermia (10x).*

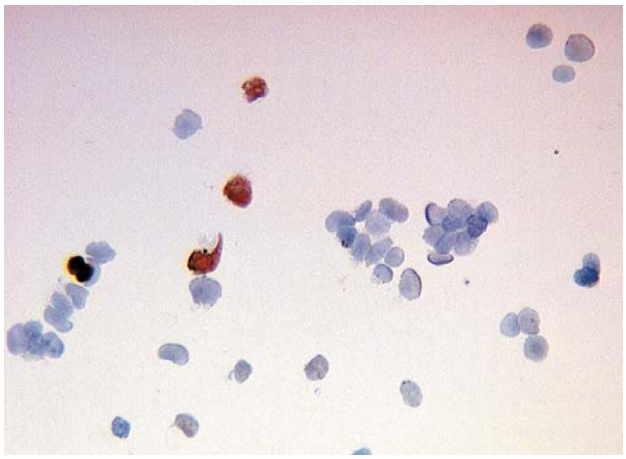

Figure 5. *Induction of apoptosis by galectin-1 on MCF7 cells with additional omission of $CO_2$ (10x).*

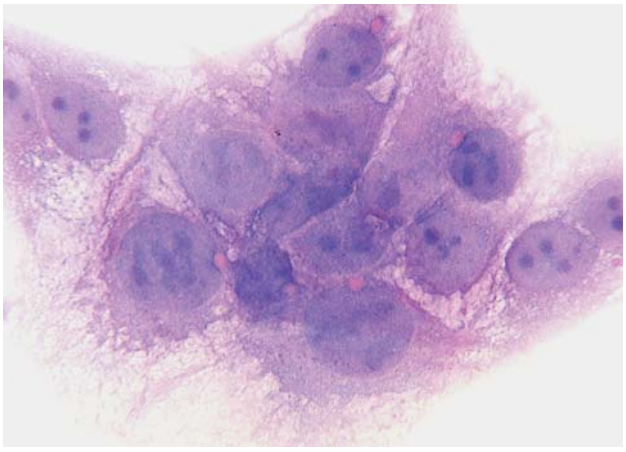

Figure 6. *HE staining of BeWo cells +mycoplasma (25x).*

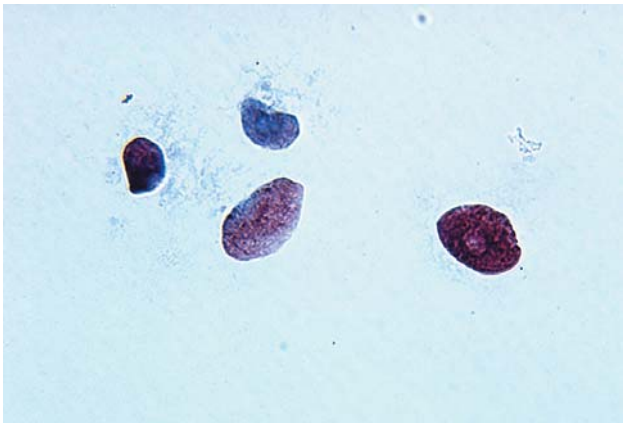

Figure 7. *Detection of large numbers of apoptotic cells after cultivation of BeWo cells +mycoplasma in serum-free medium for up to 9 hours (25x).*

## Materials and Methods

*Purification of Galectin-1.* Gal-1 was obtained from term placental tissue by lactose extraction with EDTA-MePBS (20 mM sodium phosphate, pH 7.2, 150 mM NaCl, 4 mM 2-mercaptoethanol, 2 mM EDTA) and purified by sequential affinity chromatography on asialofetuin Sepharose 4B (18) followed by lactosyl agarose. Then the protein was purified to homogeneity by anion exchange chromatography on a Resource Q column (19).

*Cells.* The human breast cancer cell line MCF7 and the human trophoblast tumor cell line BeWo were obtained from the European Collection of Cell Culture (ECACC, England) and used in different passage numbers.

*Preparation of cells.* The BeWo and MCF7 cell lines were seeded at optimal concentrations on chamber-slides (Falcon, USA,) and grown to 70% confluency in DMEM (Biochrom, Germany) supplemented with 10% FCS (PAA, Germany) and 1% MEM-Vitamines (Biochrom). Slides were washed carefully with PBS (Biochrom) and fixed in 4% PFA (Merck, Germany) for 20 minutes at room temperature. The absence of mycoplasma was proved by PCR (AppliChem, Germany).

*Controls.* The normal rate of apoptosis was determined by growing MCF7 and BeWo cells under optimal conditions on chamber-slides. To avoid false increased rates of apoptosis by obligatory depletion of FCS during gal-1 incubation, both cell lines were grown for 3 days as usual and then slides were incubated for 2, 3, 4, 6, 9 and 20 hours after removing FCS. After 2 days, both cell lines were exposed to temperatures up to 38.5˚C for 48 hours and, in another approach, after 3 days of regular culturing, $CO_2$ was removed for 1.5 hours to screen the apoptotic potential of the additional stimuli with and without serum.

*Inducing apoptosis by galectin-1.* MCF7 and BeWo cells were grown on chamber-slides for 3 days. Then slides were carefully washed with PBS and incubated with 30 μg/ml gal-1 in serum-free medium for 6, 9 and 20 hours. In addition to 6- and 9- hour gal-1 incubation, both cell lines were stressed by other stimuli, namely by hyperthermia up to 38.5˚C for 48 hours and by total removal of $CO_2$ for 1.5 hours.

*In situ nick-translation (ISNT).* Staining of DNA fragmentation and apoptotic bodies was performed on chamber-slides using the *in situ* nick-translation technique (ISNT). The slides were incubated with Proteinase K (20 μg/ml, Qiagen, Germany) for 15 minutes at room temperature. After being rinsed with distilled water, endogenous peroxidase was quenched in 0.3% hydrogen peroxide for 10 minutes. The slides were rinsed in distilled water and then equilibrated in nick buffer (Tris, $MgCl_2$, β-mercaptoethanol, BSA 20 mg/ml, distilled water) for 10 minutes at room temperature. The ISNT was carried out by incubating the slides with dNTPs and biotinylated 7-dATP (Gibco, USA) diluted in nick buffer for 65 minutes at 37˚C. The chamber-slides were rinsed in terminating buffer (0.3 mol/l sodium chloride and 0.03 mol/l sodium citrate) for 15 minutes at room temperature. After being washed in PBS, the slides were incubated with extravidin-peroxidase (Sigma, Germany) for 30 minutes at room temperature. Staining was performed using AEC-substrate (DAKO, Denmark). The slides were subsequently counterstained with haemalaun, washed and mounted. The specificity of ISNT reactivity was confirmed by human epidermis and lymph node. Negative controls were performed by incubation in nick buffer without dNTPs and biotinylated 7-dATP (Figure 1).

*Mycoplasma test/PCR.* The absence of mycoplasma was proved using the PCR Mycoplasma Test Kit (AppliChem, Germany), which allows detection of various mycoplasma species. After amplification of a conserved and mycoplasma-specific 16S rRNA gene region using two specific primers, detection was done by agarose gel electrophoresis.

## Results

*Controls.* The normal rate of apoptosis in both cell lines ranged between 0.1% and 0.25%, with BeWo cells innately having a higher metabolic rate. The cells showed regular growth and a good range of mitosis (Figure 2a, 2b). There was no increase of apoptosis by omission of FCS for 2, 3, 4, 6 and 9 hours (Figure 3a, 3b). The absence of serum for 20 hours greatly affected the cells and caused a progression of apoptosis up to 50%. Hyperthermia insignificantly affected both cell lines. Hyperthermia in association with absence of FCS increased the rate of apoptosis in MCF7 cells by up to 0.55% and in BeWo cells by up to 0.7%. Total removal of $CO_2$ for 1.5 hours interfered in the apoptosis of both cell lines with rates from 0.6 to 1.0%, but raised apoptosis up to 1.6% only in BeWo cells in the absence of FCS.

*Inducing apoptosis by galectin-1.* The incubation of both cell lines with 30 μg/ml gal-1 in serum-free medium for 6 and 9 hours influenced only BeWo cells by enhancing apoptosis to a maximum of 0.45%. Not until 9 hours of gal-1 incubation did the MCF7-cells react with rates up to 0.45%. The additional exposure to temperatures up to 38.5˚C for 48 hours increased apoptosis in MCF7 cells (Figure 4). Meanwhile BeWo cells reacted with rates up to 3.6%. Removal of $CO_2$ for 6- and 9- hour incubation with gal-1 raised the rate of apoptosis up to 10% in both cell lines (Figure 5). Incubation with gal-1 and serum-free medium for 24 hours killed 95% of all remaining cells.

*Apoptosis in mycoplasma-infected BeWo cells.* Impressive findings were manifested in one older passage of BeWo cells, which showed minimal elevated rates of apoptosis during ideal culture conditions (Figure 6). Omission of FCS alone caused a significant increase of apoptosis depending on the time of incubation and showed rates up to 25% after 6 hours (Figure 7). The presence of mycoplasma in this cell line was shown by PCR.

## Discussion

The present data demonstrate that binding of gal-1 to breast cancer cells MCF7 and trophoblast tumor cells BeWo and additional stress induced apoptosis in these cells. The normal apoptotic rates of both cell lines were 0.1 to 0.3%, respectively. Omission of serum, temporary removal of $CO_2$ and hyperthermia (38.5˚C) for up to 9 hours did not result in remarkably higher apoptotic rates in either cell line tested. Only after 20 hours was a rise of apoptotic cells up to 50% observed.

Addition of gal-1, on the other hand, induced apoptosis up to 0.45% in BeWo cells after 6 hours and in MCF7 cells after 9 hours. Addition of gal-1 in combination with hyperthermia induced apoptosis up to 3.5% in BeWo cells, whereas MCF7 cells showed apoptosis only in 1.6% of the cells.

Omission of $CO_2$ for 1.5 hours in combination with gal-1 induced apoptosis in both cell lines in 10% of all cells even after 6 hours. Similar results were obtained after 9 hours of incubation. Cultivation of BeWo and MCF7 cells with gal-1 and serum-free medium led to apoptotic rates up to 95% after 20 hours of incubation.

A striking result was obtained with the mycoplasma-contaminated BeWo cells, which showed drastically increased apoptotic rates in 25% of all cells after cultivation in serum-free medium for only 6 hours. Mycoplasma is obstructive of the metabolic pathways of cultured cells. The stress of missing serum might release their endonucleases, which are able to induce apoptosis (20).

Gal-1 is initially synthesized in the trophectoderm of the expanded blastocyst immediately *prior* to implantation, suggesting a role in the attachment of the embryo to the uterine epithelium (21). Investigations of Walzel *et al.* (22) and Vicovac *et al.* (23) showed that gal-1 is strongly expressed by the syncytiotrophoblast in term and first trimester placenta, whereas the villous cytotrophoblast was negative. The invading cytotrophoblast was weakly stained and BeWo cells expressed gal-1 particulary strongly (23). Pregnancy is often compared with a successful transplant, where semiallogenic trophoblast cells escape recognition and destruction by the mother's immune system. The cell line BeWo is derived from a trophoblast tumor and contains two coexisting phenotypes: a cytotrophoblast-like and a syncytiotrophoblast-like phenotype (24). Expression and binding of gal-1 could play a role in protection of these cells against the immune system. In an experimental model of arthritis, gal-1 was found to possess anti-inflammatory activity, probably in relation to its ability to promote Th1 cell apoptosis, switching the immune response toward a Th2 phenotype (25). Recently, it has been shown that administration of gal-1 inhibited the acute inflammatory response in a mouse model (26). Gal-1, a member of a family of conserved β-galactoside–binding proteins, has been shown to induce *in vitro* apoptosis of activated T cells and immature thymocytes (25). Gal-1 could play a similar role in breast cancer (11). Binding of gal-1 to these cells and induction of apoptosis in activated T cells could also play a role in protection of these cells against the immune system. Our investigations showed that cancer cells are protected against induction of apoptosis with gal-1.

In summary, gal-1 induces apoptosis in breast cancer and trophoblast tumor cells *in vitro* only in combination with additional stress such as long-term removal of serum, hyperthermia and temporary omission of $CO_2$.

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