Animal Science Papers and Reports vol. 40 (2022) no. 1, 5-22
Institute of Genetics and Animal Biotechnology
of the Polish Academy of Sciences, Jastrzębiec, Poland

Comparative effects of Bcl-2 inhibition in canine and human breast cancer in vitro models - a review*

Lajos Raduly[1, 2], Diana Gulei[3], Anca Jurj[1], Cristian Moldovan[3], Emilia Balint[5], Ioan Marcus[2], Orsolya Sarpataki[2], Schuyler S Korban[6], Ioana Berindan-Neagoe[1]**

[1] Research Center for Functional Genomics, Biomedicine and Translational Medicine, Iuliu Hatieganu University of Medicine and Pharmacy, 23 Marinescu Street, 4th floor, 400337, Cluj-Napoca, Romania

[2] University of Agricultural Sciences and Veterinary Medicine, Faculty of Veterinary Medicine, 3-5 Calea Manastur Street, 400374, Cluj-Napoca, Romania

[3] MEDFUTURE - Research Center for Advanced Medicine, Iuliu-Hatieganu University of Medicine and Pharmacy, 23 Marinescu Street, 400337, Cluj-Napoca, Romania

[5] University of Agronomic Sciences and Veterinary Medicine of Bucharest, Faculty of Veterinary Medicine, 59 Bd. Marasti Street, 011464, Bucharest, Romania

[6] Department of Natural and Environmental Sciences, University of Illinois at Urbana-Champaign, Urbana, IL 61801, USA

(Accepted December 16, 2021)

*This work was supported by Project PNCDI III 2015-2020 entitled "Increasing the performance of scientific research and technology transfer in translational medicine through the formation of a new generation of young researchers" – ECHITAS, Research grant No. 128/2014; PN-II-PT-PCCA-2013-4-2166 "New strategies for improving life quality and survival in cancer patients: molecular and clinical studies of the tumor genome in deuterium-depleted water treatment augmentation - GenCanD", project PN-III-P1-1.2-PCCDI-2017-0782, entitled "Advanced innovative approaches for predictive regenerative medicine"- REGMED., as well as Raduly Lajos-Zsolt won a scholarship from the European Social Found, Human Capital Operational Programme 2014-2021, project no. POCU/380/6/13/125171-ACAMED.
**Corresponding authors: ioananeagoe29@gmail.com, ioana.neagoe@umfcluj.ro

Breast cancer is among the leading malignant pathologies in terms of incidence and mortality rates; therefore, rapid development of effective drug treatments, particularly for triple negative breast cancer (TNBC), is of critical importance. One promising approach to speed up the process of drug discovery capitalizes on repositioning of specific therapeutic molecules used for malignancies that have similar altered molecular profiles. In this study, ABT-199, a small molecule that elicits selective inhibition of Bcl-2 and effective against chronic lymphocytic leukemia, is repositioned for treating TNBC using both human and canine breast cancer cell lines. It is observed that ABT-199 elicits similar inhibitory effects, including apoptosis, cell cycle, and migration, on both human and canine breast cancer cells.

KEYWORDS: **breast cancer / canine models / comparative oncology / mammalian cancer model**

Introduction

Based on Globocan 2020 data, breast cancer is the second leading type of malignancy worldwide, and the fifth in terms of all cancers [Bray *et al*. 2018, Sung *et al*. 2021]. For the female population in particular, incidence of breast cancer is one of the most common malignancies worldwide, and it is among the deadliest pathologies. Thus, there is a significant demand for improved diagnostics, as well as for effective therapeutic management of breast cancer [Braicu *et al.* 2013, Chiorean *et al.* 2013, Cojocneanu Petric *et al.* 2015, Lotter *et al*. 2021, Huminiecki *et al.* 2017, 2020, Tewari *et al.* 2017a, Huminiecki and Horbańczuk 2018, Wang *et al.* 2018].

The use of reliable animal models in preclinical studies that closely mimic the status of oncological human subjects, as well as their responses and side effects in clinical studies is key to rapidly translate and advance findings from research trials to clinical practice [Rowell *et al*. 2011, Pinho *et al.* 2012, Graim *et al.* 2020, Korkmaz and Ustun 2021]. Currently, imunocompromised mice serve as standard models for conducting *in vivo* research studies due to their amenable phenotypes to accept engraftment of human cancer cells or tissue sections; thus, allowing for tracking and follow-up on development of these cells/tissues during treatment. However, there are multiple factors that do not overlap with clinical scenarios, particularly of non-spontaneous cancer development, lack of immune interplay, and non-synonymous hormonal balance [Park *et al*. 2016]. Thus, several studies have proposed the use of the canine as a closely-related model for studying human breast cancer due to resemblance of several factors, including those that overlap with clinical scenarios as mentioned above, between canine and human mammary malignancies [Khanna *et al.* 2006, Gardner *et al.* 2016, Graim *et al*. 2020].

The process of normal breast tissue development in both humans and canines is regulated by various apoptotic pathways. Moreover, one of the main processes in mammary tissue homeostasis is that of apoptosis induced by mitochondrial activity and regulated by the Bcl-2 super-family of proteins [Delbridge and Strasser 2015, Alhoshani *et al.* 2020]. Interactions between pro-and anti-apoptotic members of the Bcl-2 family maintain mitochondrial integrity and regulate breast cell apoptosis

[Shimizu *et al.* 2004, Youle and Strasser 2008, Alhoshani *et al.* 2020]. A deregulation of this interaction contributes to breast cancer development, as well as in acquisition of a therapeutic-resistant phenotype of breast cancer cells [Shimizu *et al.* 2004, Patel *et al.* 2009, Shen *et al*. 2021]. Studies have demonstrated that the anti-apoptotic protein Bcl-2 is overexpressed in cancers, particularly in hematological malignancies, such as lymphoma and leukemia [Delbridge and Strasser 2015, Haselager *et al*. 2020]. Based on these findings, specific Bcl-2 inhibitors, a class of small molecules, have been developed, and used in cancer therapy. For breast cancer, only a limited number of clinical studies have been conducted using these Bcl-2 small molecule inhibitors [Williams and Cook 2015]. Among these small molecules, ABT-737 and ABT-263 are both associated with in vitro and in vivo inhibitory effects in breast cancer, as well as in other types of cancer [Young *et al.* 2016, Panayotopoulou *et al.* 2017, Wolf 2017, Wu *et al*. 2017]. Furthermore, ABT-199 is another small molecule that elicits a more selective inhibition of Bcl-2, and it has a good effect on chronic lymphocytic leukemia [Patel *et al*. 2017, Seymour *et al.* 2017, Pollyea *et al*. 2021]. However, there are only a few studies that have investigated apoptotic effects of ABT-199 on breast cancer [Deng and Letai 2013, Merino *et al*. 2016, Alhoshani *et al.* 2020]. It is important to point out that expression of Bcl-2 in cancer has different prognosis, as this also depends on the status of hormone receptors. In general, Bcl-2 has an anti-apoptotic role; however, it has been demonstrated that in breast cancer subtypes positive for hormone receptors, the Bcl-2 protein is associated with a good prognosis [Martinez-Arribas et al. 2007, Dawson *et al*. 2010, Honma *et al*. 2015, Eom *et al*. 2016, Young *et al*. 2021].

In this study, effects of the small molecule ABT-199 on human and canine breast cancers are investigated. In particular, a triple negative breast cancer (TNBC) human breast cancer model is used as it has an aggressive phenotype, has limited therapeutic options, and it is correlated with increased expression of Bcl-2 which is further associated with poor prognosis [Honma *et al*. 2015]. Therefore, in vitro inhibitory effects and comparative responses of ABT-119 in human and canine breast cancer cells have been determined, and are assessed.

Material and methods

Cell lines and in vitro culture

Cell culture in vitro studies refers to the removal of cells from a tissue and their subsequent growth in a specific artificial environment.

The following three cell lines, HS578T, PC114, and CMT-U27, were used in this study. The HS578T human (Homo sapiens) breast cancer cell line was obtained from ATCC, while P114 and CMT-U27 dog (Canis lupus familiaris) breast carcinoma cell lines were kindly provided by Prof. Gerard Rutteman (The Netherlands) and Prof. Eva Hellmen (Sweden), respectively.

The human cell line HS578T was cultured in a Dulbecco's Modified Eagle Medium (DMEM) containing 4.5 g/l glucose, and supplemented with 10% Fetal Bovine Serum

(FBS), 1% L-glutamine, 1% non-essential amino acids (NEAA), and 143µl insulin. The two canine cell lines were cultured in either an RPMI medium supplemented with 10% FBS and 1% L-glutamine for P114 or a DMEM low-glucose medium supplemented with 10% FBS and 1% L-glutamine for CMT-U27. All culture media and supplements were purchased from Sigma-Aldrich, while the Bcl-2 small molecule inhibitor ABT-199 was purchased from Selleckchem [Ciocan-Cartita *et al.* 2020].

The MTT cell viability assay

The MTT assay is used to evaluate cellular metabolic activity as an indicator of cell viability, proliferation and cytotoxicity, on the reduction of a yellow tetrazolium salt to purple formazan crystals by metabolically active cells. The assay was conducted by incubating 1×10^5 cells/well, of each of HS578T, P114, and CMT-U27 cell lines, in 96-well culture plates maintained in 5% CO_2 atmosphere incubators for a period of 24 h at 37ºC. Following incubation, each of the cell culture lines were treated with different doses (from 1µM to 500 µM) of ABT-199. After 48 h, the medium was discarded, and a 150 µl MTT solution was added to each of the wells. Following a 2 h incubation period at 37ºC, the MTT solution was discarded, and the formazan crystal was solubilized with 100µl DMSO. To assay for cell viability, the absorbance was measured at 570/690 nm in a microplate reader (Synergy H1 Hybrid Reader Biotek) [Braicu *et al.* 2019].

Assessment of cellular morphology using dark-field microscopy

After 48 h of treatment with ABT-199, cells of each of the lines from each of the treatments were fixed with 4% PFA (paraformaldehyde), and incubated for 15 min at room temperature. Following fixation, cells were washed three times with 1X PBS, and then placed, along with a mounting medium, onto microscope slides. Cellular morphological changes in each of the treated cell lines were evaluated via dark-field microscopy using an Olympus BX43 microscope [Mehta *et al.* 2021].

Evaluation of cellular apoptosis using fluorescence flow cytometry.

In the early stages of apoptosis, changes occur at the cell surface at the cell mambrane level. One of these biological processis is the translocation of phosphatidylserine from the inner side of the plasma membrane to the outer layer. Annexin-V presents a high affinity for phosphatidylserine exposed on the outer layer of apoptotic cells. Propidium iodide is utilized as a DNA stain to distinguish necrotic cells from Annexin-V-labeled cell population.

Each of the three cell lines were incubated in 6-well culture plates for 24 h at 37 °C temperature, and then treated with 100µM ABT-199. After 48 h, cells were collected, re-suspended in 100 µl of Annexin V-FITC staining solution, and incubated in the dark for 15 min. Subsequently, cells were centrifuged, and the supernatant was discarded. For each of the pellets, 100-200µl of a propidium iodide (PI) staining solution was added. Stained cells were then analyzed using a fluorescence-activated cell sorting (FACS Canto II) flow cytometer (BD-Becton, Dickinson), and data were

analyzed with a FACS Diva version 6.0 software [Ciocan-Cartita *et al.* 2020].

Cell cycle assays using fluorescence flow cytometry

Basic cell cycle analysis by flow cytometry identify cells in different stages of division according to the DNA content. Each of the three cell lines were incubated in 6-well culture plates for 48 h at 37°C temperature, and then treated with 100μM ABT-199. After 48 h, cells were washed with cold phosphate buffered saline (PBS; 1X), and fixed in 70% ethanol at 4°C. Following fixation, cells were mixed with RNase A, stained with propidium iodide (PI) for 30 min in the dark, and then washed twice with PBS 1X. Cell cycle assays were performed using a FACS flow cytometer, and data were analyzed with FACS Diva version 6.0 software [Alhoshani *et al*. 2020].

DNA contents and mitochondrial transmembrane activities of apoptotic cells by fluorescence microscopy

For analysis of nuclear DNA contents and mitochondrial transmembrane activities of apoptotic cells, the Multi-Parameter Apoptosis Kit (Cayman) was used, and cell staining was conducted according to the manufacturer's protocol. Cells from each of the three cells lines treated with 100μM ABT-199 where stained with either Hoechst 33342 and Tertamethylrhodamineethyl ester (TMRE). Stained cells were analyzed at appropriate UV-wavelengths for Hoechst 33342 staining (at 350/460 nm) for nuclear DNA content, and for TMRE staining (at 560/595 nm) for measuring mitochondrial transmembrane activity potential using an Olympus IX71 inverted microscope.

Autophagy assays by fluorescence microscopy

Autophagy represents a biological process that involves the degradation and digestion of intracellular components by the lysosome enabling cells to efficiently mobilize and recycle cellular constituents, but also prevents the accumulation of damaged organelles, misfolded proteins, and invading microorganisms. Evaluation of autophagy was performed using an Autophagy/Cytotoxicity Dual Staining Kit (Abcam), according to the recommended protocol. For the each of the three cell lines a Monodansylcadaverine (MDC) staining was used for autophagy vacuole staining, while and PI staining was used for cellular death. After 24 h of treatment, cells were visualized under an Olympus IX71 inverted microscope [Budisan *et al.* 2019].

Scratch assays (wound healing assay)

The wound healing process starts as cells polarize toward the wound, initiate protrusion, migrate, and close the wound area. These processes represents the behaviour of individual cells as well as the entire cell population.

Each of the cell lines were separately treated with ABT-199 for 48 h in 6-well plates. Following treatment, cell proliferation was blocked by pretreatment with mitomycin C (40 μg/ml) for 30 min. Using a 20-μl pipette tip, a single scratch was then made in each well, and cells were maintained in culture medium with 10% serum. Distances between the two edges of the gap were scaled at 6, 24, 30, 48, and 72 h [Jiao *et al*. 2021].

Results

Gene Order Conservation of the Bcl-2 between human and canine genomes

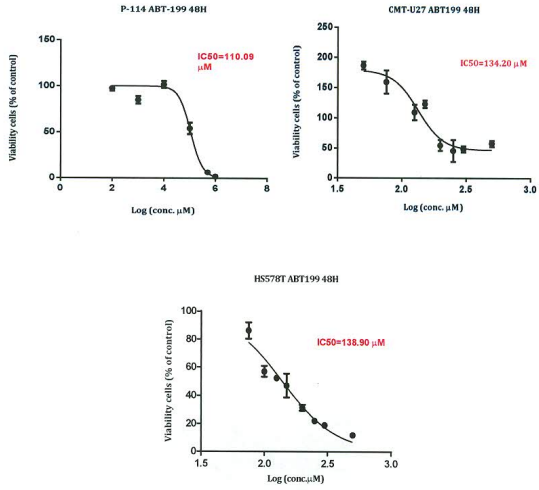
To assess the conservation status of the Bcl-2 between the human and canine genomes, orthologues of the human Bcl-2 have been identified within the Ensemble genome browser (Fig. 1A). Among a total of 185 orthologues, a Canis lupus familiaris Bcl-2 is found to have a Gene Order Conservation (GOC) score of 100% and a Whole Genome Alignment (WGA) coverage (values range between 0 and 100) of 100 with that of the human Bcl-2, and at a high level of confidence (Fig. 1B). A GeneTree ENSGT00950000182651 for the human Bcl-2, generated with the same browser, is found to contain 1442 genes, 1305 speciation nodes, 65 duplications, 71 ambiguous

Fig. 1. Sequence comparisons of the Bcl-2 gene family between human and canine genomes. (A) Locations of Bcl-2 along chromosomes 1 and 18 of canine and human genome sequences, respectively. (B) Gene Order Conservation (GOC) scores and Whole Genome Alignment (WGA) coverage values (0 to 100) between human and canine Bcl-2. (C) The GeneTree ENSGT00950000182651 for the human Bcl-2 and its dog orthologues are presented in highlighted mode.

and 0 gene split events, and this is found to be consistent with findings in the Bcl-2 orthologues in dog, as shown in a highlighted mode (Fig. 1C).

ABT-199 inhibits cell viability in both human and canine in vitro breast cancer models

When the three breast cancer cell lines of P114, CMT-U27, and HS578T are treated with different concentrations of ABT-199 for 48 h, it is observed that there is a dosage-dependent inhibition of viability in all three cell lines. Moreover, it is observed that the half-maximum inhibitory concentration, IC50, for all three cell lines is similar, at an approximate level of 100 µM ABT-199 (Fig. 2). Thus, for subsequent experiments, a concentration of 100µM of the small molecule inhibitor ABT 199 is used to compare responses of these cell lines to this treatment.



Small molecule	Cell line	Breast cancer type	IC50 (µM)
ABT-199	P-114	Canine mammary carcinoma	110.09
ABT-199	CMT-U27	Canine mammary carcinoma	134.20
ABT-199	HS578T	Human mammary carcinoma	138.90

Fig. 2. MTT cell viability assay and IC50 values following treatment of P114, CMT-U27, and HS578T breast cancer cell lines with increasing doses of ABT-199 for a period of 48 h. ABT-199 inhibits cell viability in all three breast cancer cell lines with an IC50 of 110.09 µM for P-114 cell line, 134.20 µM for CMT-U27 cell line, and 138.90 for HS578T cell line.

Flow cytometry apoptosis assays following treatment of each of the three cell lines with either 100 µM ABT-199 or untreated (control) for 48 h have provided an assessment of the effects of this small molecule in inducing cell death. It is found that the P114 canine cell line treated with ABT-199 shows 98.60% necrotic cells compared to 3.80% necrotic cells in untreated control cells (Fig. 3A). Moreover, the CMT-U27 canine cell line treated with ABT-199 yields 61.30% necrotic cells, while untreated (control) cells yield 2.88% necrotic cells (Fig. 3B). Whereas, the HS578T human cell

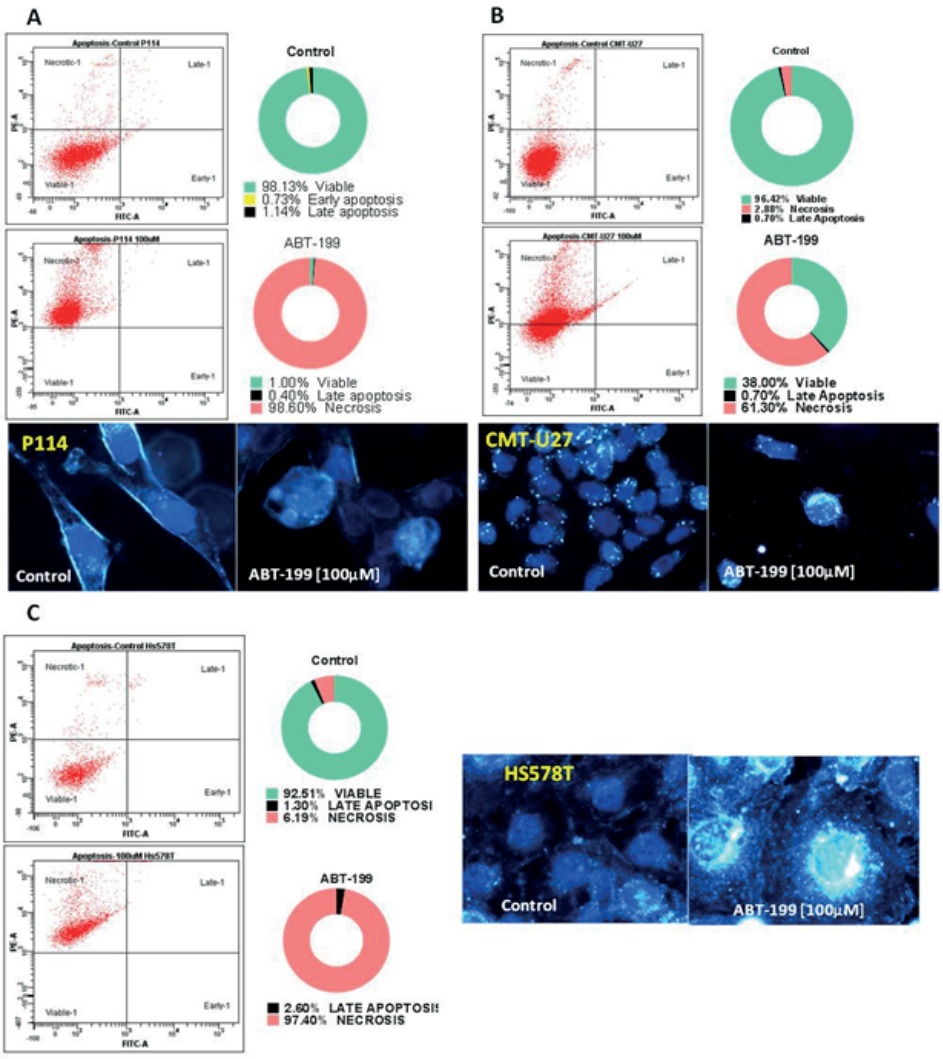


Fig. 3. Evaluation of cell death in three breast cancer cell lines following treatments with 100 µM ABT-199 for 48 h using flow cytometry and dark-field microscopy. (A) The P114 canine cell line shows 98.13% viable cells, 0.73% early apoptosis, and 1.14% late apoptosis in untreated (control) cells, while it exhibits 1.00% viable cells, 0.40% late apoptosis cells, and 98.60% necrosis cells in treated cells. (B) The CMT-U27 canine cell line shows 96.42% viable cells, 2.88% necrotic cells, and 0.70% late apoptosis cells in untreated (control) cells, while it exhibits 38.00% viable cells, 0.70% late apoptosis cells, and 61.30% necrosis cells in treated cells . (C) The HS578T human cell line shows 92.51% viable cells, 1.30% late apoptosis cells, and 6.19% necrosis cells in untreated (control) cells group; whereas, this cell line shows 0.00% viable cells, 2.60% late apoptosis cells, and 97.40% necrosis cells in treated cells.

line treated with ABT-199 yields 97.4% necrotic cells compared to 6.2% in untreated (control) cells (Fig. 3C). Overall, flow cytometry findings reveal that both the P114 canine cell line and the HS578T human cell line are highly sensitive to treatment with the small molecule ABT-199, thereby shifting their phenotypic status towards a necrotic phenotype. Moreover, these findings indicate that the principal mechanism for cell death by ABT-199 is via induction of necrosis, and then followed by apoptosis.

Dark-field microscopy observations further confirm that the cell death mechanism induced by ABT-199 involves changes in cell morphology, consisting of membrane break down, nuclear changes, and apoptotic body formation (Fig. 3). Moreover, in concordance with flow cytometry analysis, P114 and HS578T cell lines exhibit the highest morphological changes in cell viability in response to ABT-199 treatment (Fig. 3).

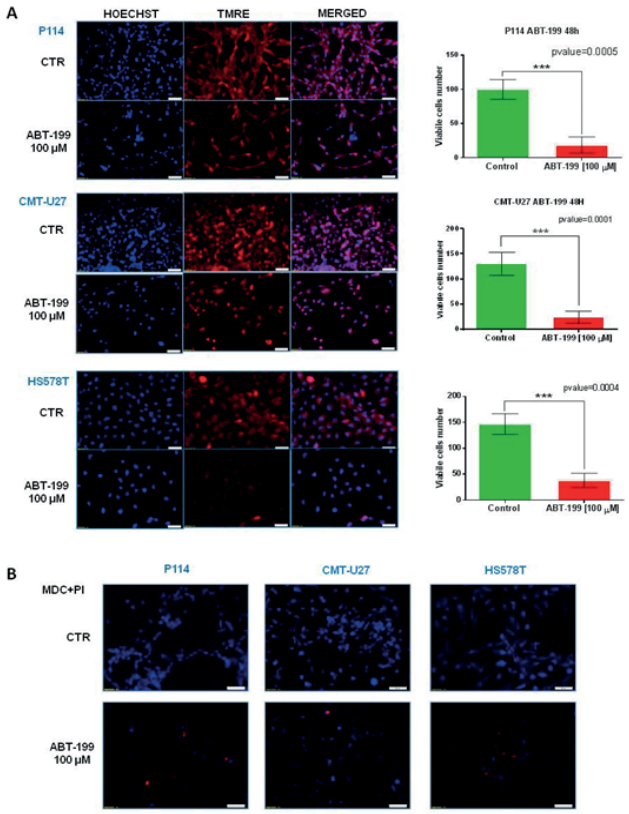


Fig. 4. Mitochondrial membrane potential activity and nuclear morphology evaluation of P114, CMT-U27, and HS578T breast cancer cell lines. (A) Observations of mitochondrial potential activity inhibition and nuclear morphology changes using TMRE and Hoechst staining/Olympus IX71 inverted fluorescence microscopy following treatment of each of the cell lines with 100 µM of ABT-199 for 48 h; image magnification 20X. (B) Autophagy evaluation by MDC and PI staining after 48 h of treatment of each of the cell lines with 100 µM of ABT-199.

Interestingly, fluorescence microscopy assays have revealed that ABT-199 treatments of each of the three cell lines for 48 h result in decreased mitochondrial membrane potential activities and morphological changes, including nuclear DNA condensation, nuclear shrinkage, and fragmentation in all three cell lines (Fig. 4A). Furthermore, autophagy evaluations of these treated cell lines, have detected decreased numbers of autophagic vacuoles along with increased numbers of apoptotic cells (Fig. 4B). Interestingly, control cells stained with MDC demonstrate basal autophagy while stained treated cells demonstrate decreased autophagy; moreover, increased PI staining of necrotic cells is readily observed in ABT-199-treated cells compared to that of control cells (Fig. 4B).

ABT-199 impairs cell migration in both human and canine in vitro breast cancer models

For cell lines CMT-U27 and HS578T treated with 100 µM ABT-199, a significant reduction in migratory capacity is observed (Fig. 5A). This is clearly demonstrated in the abilities of control cells to completely close the gap in the scratch assay, while this is not observed in ABT-199-treated cells, wherein the gap remains high visible after 48 h of treatment (Fig. 5A). Following quantification of the wounded area, it

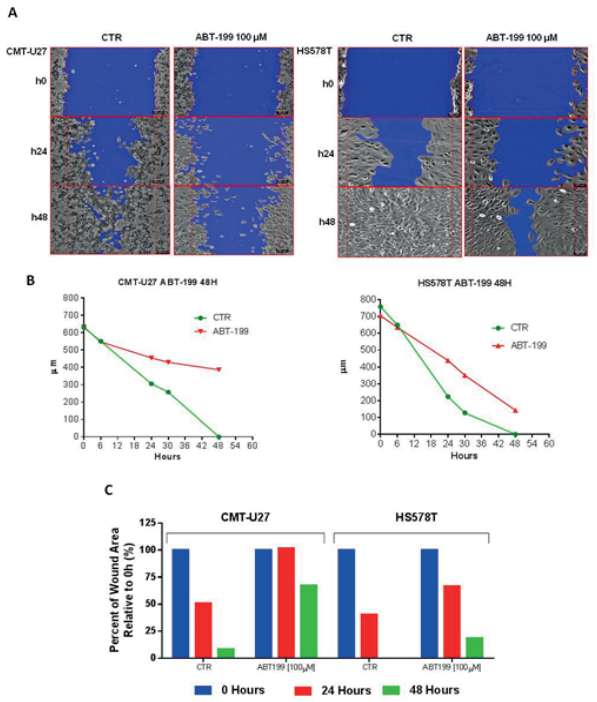


Fig. 5. Scratch assays and quantification of cell migration of the two cell lines, CMT-U257 and HS578T, following treatment with 100 µM ABT-199 for 48 h. (A) Migratory capacity; And (B, C) Quantification of wound area in control cells versus ABT-199 treated cells. Pictures were taken at 0 h, 24 h, and 48 h with an Olympus IX71 microscope (objective 10X).

is observed that ABT-199 is most efficient in canine cell line CMT-U27, followed closely by the human cell line HS578T cell line, and then those of treated cells of both cell lines, CMT-U257 and HST578T (Fig. 5B).

The cell cycle arrest in the G1 phase is induced by ABT-199

For evaluation of cell growth inhibition induced by ABT-199, cell cycle assays of cells either treated or untreated (control), was conducted using flow cytometry.

It is observed that all three cell lines, P114, CMT-U27, and HS578T, are associated with a drop of cells in the G2 phase in ABT-199 treated cells compared to untreated (control) cells.

For ABT-199 treated cells of the canine line P114, their S phase distribution slowly increased between 4.7 and 8.2%; whereas, their G2 phase distribution decreased from 16.7 to 11.2%, and their G1 phase distribution increased from 73.9 to 79.5% (Fig. 6). For the canine CMT-U27 cells treated with ABT-199, their S phase distribution dropped from 9.6 to 5.6%, while their G2 phase distribution dropped from 29.4 to 15.2%, but their G1 phase distribution increased from 56.7 to 72.7%

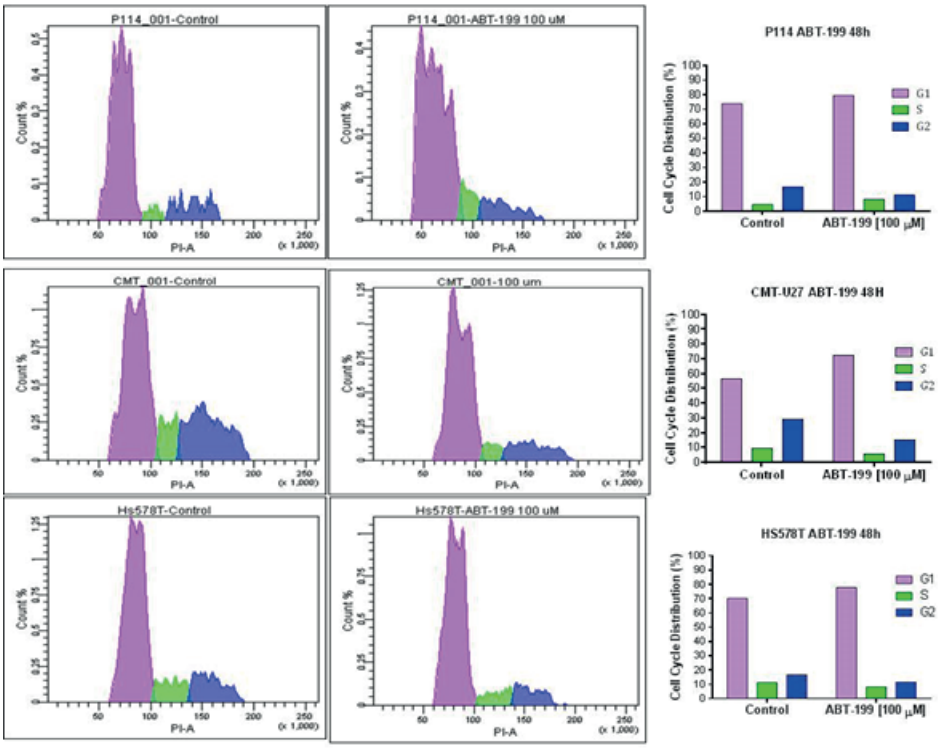


Fig. 6. Flow cytometry for cell cycle analysis following treatment with 100 µM ABT-199 for 48 h. It is observed that ABT-199 predominantly induces cell cycle arrest at the G1 phase in all three cell lines, P114, CMT-U27, and HS578T, when compared to untreated cells (control).

(Fig. 6). Moreover, for ABT-100 treated cells of the human HS578T line, the S phase distribution decreased from 11.1 to 8.1%; whereas, distribution of cells in the G2 phase dropped from16.6 to 11.5%, and distribution of cells in the G1 phase increased from 70.3 to 77.8% (Fig. 6).

Discussion

Currently, research efforts are underway to pursue novel therapeutic strategies for treating breast cancer, and these include approaches focused on nanopharmacology [Tomuleasa *et al*. 2014, Jurj *et al*. 2017, Liu *et al*. 2021], gene therapy, both for either replacement or inhibition [Braicu *et al.* 2014], dietary-based management [Braicu *et al*. 2017], immunotherapy [Bayraktar *et al.* 2019], and metastasis focused approaches [Park and Choi 2016]. Yet, one of the most promising strategies could on drug repositioning, wherein specific inhibitors of one disease are repurposed for treating another disease that has similar molecular mechanism(s). Thus, the use of an already approved drug for one disease could significantly reduce both the time frame and cost for its alternative use as a new therapeutic for another disease [Xue *et al.* 2018, Sukumar e*t al.* 2021].

Among various pathologies, breast cancer ranks first in terms of both incidence and mortality, with numerous reported cases of patient resistance to various therapies or reports of disease recurrence [Klimov *et al*. 2017, Ogata *et al*. 2021]. In particular, TNBC is deemed as one of the most aggressive subtypes within the breast carcinoma histological panel, and it is also known as one of the most challenging in terms of therapeutic options and patient response [Brewster *et al.* 2014, Ogata *et al*. 2021, Sukumar *et al.* 2021]. Despite numerous clinical and molecular differences among the different breast cancer subtypes, one of the striking features of these subtypes pertains to the role of the therapeutic Bcl-2 anti-apoptotic protein target in treating these cancer subtypes. It is well known that the efficacy of the Bcl-2 target is related to the status of hormone receptors, wherein, this therapeutic anti-apoptotic protein has a positive prognosis in cancer subtypes positive for such hormone receptors, and a negative prognosis for cancer subtypes lacking such hormone receptors [Martinez-Arribas *et al*. 2007, Dawson *et al.* 2010, Honma *et al.* 2015, Eom *et al*. 2016, Alhoshani *et al*. 2020]. Therefore, efforts to identify new therapeutic for the TNBC subtype can capitalize on the Bcl-2 anti-apoptotic protein and expression of corresponding hormone receptors.

In this study, in vitro responses of three cell lines, including two canines and one human, of the TNBC subtype to the action of ABT-199, a next generation Bcl-2 inhibitor, have been evaluated. In comparison to various small molecule inhibitors of Bcl-2, the ABT-199 small molecule has high levels of specificity for the Bcl-2; therefore, it is selected in this study as a likely repurposed therapeutic treatment for TNBC. Moreover, two canine breast cancer cell lines are used in this study to assess comparative responses of these canine cell lines to that of a human breast cancer line to an ABT-199 therapeutic treatment. This comparative analysis will evaluate the

utility of these canine cell lines as reliable model systems for investigating mammary cancers.

Following treatments of the three breast cancer cell lines with different concentrations of ABT-199, it is observed that ABT-199 elicits apoptotic effects on both human and canine breast cancer cell lines, thus further confirming previous findings of the influence of ABT-199 on breast cancer cells [Alhoshani *et al.* 2020, Campbell *et al*. 2021]. Furthermore, it is observed that ABT-199 at 100 μM reduces autophagy (based on similar percentages of apoptotic cells), cell migration (based on similar mitochondrial potential activity inhibition and nuclear morphology changes), and induces cell cycle arrest (based on similar patterns of distribution of cells in the S, G2, and G1 phases) following flow cytometric analyses and dark-microscopy observations. Interestingly, these collective findings are found to be similar in both canine and human breast cancer cell lines. Considering that the Bcl-2 gene family is highly conserved, based on GOC, WGA, and GeneTree phylogeny, in human (located on chromosome 18) and canine (located on chromosome 1) genomes, it is anticipated that the functionality of the Bcl-2 gene family is likely to be similar. Thus, it is to be expected that similar inhibitory responses are observed on all three ABT-199 treated cancer cell lines [Young *et al.* 2021]. Furthermore, these findings suggest that the two canine cells lines used in this study are useful as reliable in vitro model systems for investigating human breast cancer.

Overall, these findings indicate that the activity of the small molecule ABT-199 elicits an inhibitory effect of breast cancer cells via inhibition of the Bcl-2 anti-apoptotic protein along with induction of tumor suppressor mechanism(s). However, further studies should be conducted to further investigate such an induction of a tumor suppressor mechanism. Nevertheless, the ABT-199 can be useful for repurposing for treatment of TNBC. Furthermore, these canine cell lines would serve as good in vivo models for investigating human breast cancers, particularly following more detailed characterization of canine histological subtypes for this pathology.

Conclusions

Developing new therapeutic strategies for breast cancer, particularly for the aggressive TNBC subtype, remains a major challenge. Moreover, identifying reliable experimental models for pursuing new therapeutic strategies for TNBC is also of major interest. In this study, it is found that repurposing of a small molecule ABT-199, which acts as an inhibitor of the Bcl-2 anti-apoptotic protein, elicits appropriate selective inhibitory responses of breast cancer cells of both canine and human cell lines. Therefore, the ABT-199 drug is likely to be an effective therapeutic drug for treatment of breast cancer. Moreover, the use of canine breast cancer cell lines can serve as appropriate models for evaluating such drug treatment strategies, and should be further evaluated in serving as in vivo models for developing reliable preclinical testing and therapeutic treatment of TNBC.

Author contributions: Conceptualization, Lajos-Zsolt Raduly and Ioana Berindan-Neagoe; methodology, Lajos-Zsolt Raduly, Ancuta Jurj, Cristian Moldovan, Emilia Balint, and Orsolya Sarpataki; data curation and supervision, Ioana Berindan-Neagoe, Ioan Marcus for writing-original draft preparation; and writing-review and editing, Lajos-Zsolt Raduly, Diana Gulei, Schuyler Korban, and Ioana Berindan-Neagoe.

Conflicts of interest: Declare conflicts of interest or state "The authors declare no conflict of interest." Authors must identify and declare any personal circumstances or interest that may be perceived as inappropriately influencing the representation or interpretation of reported research results. Any role of the funders in the design of the study; in the collection, analyses or interpretation of data; in the writing of the manuscript, or in the decision to publish the results must be declared in this section. If there is no role, please state "The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results".

REFERENCES

1. ALHOSHANI A., ALATAWI F.O., AL-ANAZI F.E., ATTAFI I.M., ZEIDAN A., AGOUNI A., EL GAMAL H.M., SHAMOON L.S., KHALAF S., KORASHY H.M., 2020 - "BCL-2 Inhibitor Venetoclax Induces Autophagy-Associated Cell Death, Cell Cycle Arrest, and Apoptosis in Human Breast Cancer Cells." ***OncoTargets and Therapy*** 13, 13357-13370.
2. BAYRAKTAR S., BATOO S., OKUNO S., GLUCK S., 2019 - "Immunotherapy in breast cancer." ***Journal of Carcinogenesis*** 18, 2.
3. BRAICU C., CATANA C., CALIN G.A., BERINDAN-NEAGOE I., 2014 - "NCRNA combined therapy as future treatment option for cancer." ***Current Pharmaceutical Design*** 20(42), 6565-6574.
4. BRAICU C., GULEI D., RADULY L., HARANGUS A., RUSU A., BERINDAN-NEAGOE I., 2019 - "Altered expression of miR-181 affects cell fate and targets drug resistance-related mechanisms." ***Molecular Aspects of Medicine*** 70, 90-105.
5. BRAICU C., MEHTEROV N., VLADIMIROV B., SARAFIAN V., NABAVI S.M., ATANASOV A.G., BERINDAN-NEAGOE I., 2017 - "Nutrigenomics in cancer: Revisiting the effects of natural compounds." ***Seminars in Cancer Biology*** 46, 84-106.
6. BRAICU C., PILECZKI V., IRIMIE A., BERINDAN-NEAGOE I., 2013 - "p53siRNA therapy reduces cell proliferation, migration and induces apoptosis in triple negative breast cancer cells." ***Molecular and Cellular Biochemistry*** 381(1-2), 61-68.
7. BRAY F., FERLAY J., SOERJOMATARAM I., SIEGEL R.L., TORRE L.A., JEMAL A., 2018 - "Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries." *CA:* ***A Cancer Journal for Clinicians*** 68(6), 394-424.
8. BREWSTER A.M., CHAVEZ-MACGREGOR M., BROWN P., 2014 - "Epidemiology, biology, and treatment of triple-negative breast cancer in women of African ancestry." ***The Lancet. Oncology*** 15(13), e625-634.
9. BUDISAN L., GULEI D., JURJ A., BRAICU C., ZANOAGA O., COJOCNEANU R., POP L., RADULY L., BARBAT A., MOLDOVAN A., MOLDOVAN C., TIGU A.B., IONESCU C., ATANASOV A.G., IRIMIE A., BERINDAN-NEAGOE I., 2019 - "Inhibitory Effect of CAPE and Kaempferol in Colon Cancer Cell Lines-Possible Implications in New Therapeutic Strategies." ***International Journal of Molecular Sciences*** 20(5).

10. CAMPBELL K.J., MASON S.M., WINDER M.L., WILLEMSEN R.B.E., CLOIX C., LAWSON H., ROONEY N., DHAYADE S., SIMS A.H., BLYTH K., TAIT S.W.G., 2021 - "Breast cancer dependence on MCL-1 is due to its canonical anti-apoptotic function." ***Cell Death and Differentiation*** 28(9), 2589-2600.
11. CHIOREAN R., BRAICU C., BERINDAN-NEAGOE I., 2013 - "Another review on triple negative breast cancer. Are we on the right way towards the exit from the labyrinth?" ***Breast*** 22(6), 1026-1033.
12. CIOCAN-CARTITA C.A., JURJ A., RADULY L., COJOCNEANU R., MOLDOVAN A., PILECZKI V., POP L.A., BUDISAN L., BRAICU C., KORBAN S.S., BERINDAN-NEAGOE I., 2020. - "New perspectives in triple-negative breast cancer therapy based on treatments with TGFbeta1 siRNA and doxorubicin." ***Molecular and Cellular Biochemistry*** 475(1-2), 285-299.
13. CIOCAN-CARTITA C. A., JURJ A.,ZANOAGA O., COJOCNEANU R., POP L.A., MOLDOVAN A., MOLDOVAN C., ZIMTA A.A., RADULY L., POP-BICA C., BUSE M., BUDISAN L., VIRAG P., IRIMIE A., GOMES DIAS S.M., BERINDAN-NEAGOE I., BRAICU C., 2020 - "Correction to: New insights in gene expression alteration as effect of doxorubicin drug resistance in triple negative breast cancer cells." ***Journal of Experimental & Clinical Cancer Research*** *CR* 39(1), 287.
14. COJOCNEANU PETRIC R., BRAICU C., RADULY L., ZANOAGA O., DRAGOS N., MONROIG P., DUMITRASCU D., BERINDAN-NEAGOE I., 2015 - "Phytochemicals modulate carcinogenic signaling pathways in breast and hormone-related cancers." ***OncoTargets and Therapy*** 8, 2053-2066.
15. DAWSON S.J., MAKRETSOV N., BLOWS F.M., DRIVER K.E., PROVENZANO E., LE QUESNE J., BAGLIETTO L., SEVERI G., GILES G.G., MCLEAN C.A., CALLAGY G., GREEN A.R., ELLIS I., GELMON K., TURASHVILI G., LEUNG S., APARICIO S., HUNTSMAN D., CALDAS C., PHAROAH P., 2010 - "BCL2 in breast cancer: a favourable prognostic marker across molecular subtypes and independent of adjuvant therapy received." ***Britishh Journal of Cancer*** 103(5), 668-675.
16. DELBRIDGE A.R. STRASSER A., 2015 - "The BCL-2 protein family, BH3-mimetics and cancer therapy." ***Cell Death and Differentiation*** 22(7), 1071-1080.
17. DENG J., LETAI A., 2013 - "Priming BCL-2 to kill: the combination therapy of tamoxifen and ABT-199 in ER+ breast cancer." ***Breast Cancer Research*** *BCR* 15(5), 317.
18. EOM Y.H., KIM H.S., LEE A., SONG B.J., CHAE B.J., 2016 - "BCL2 as a Subtype-Specific Prognostic Marker for Breast Cancer." ***Journal of Breast Cancer*** 19(3), 252-260.
19. GARDNER H.L., FENGER J.M., LONDON C.A., 2016 - "Dogs as a Model for Cancer." ***Annual Review of Animal Biosciences*** 4, 199-222.
20. GRAIM K., GORENSHTEYN D., ROBINSON D.G., CARRIERO N.J., CAHILL J.A., CHAKRABARTI R., GOLDSCHMIDT M.H., DURHAM A.C., FUNK J., STOREY J.D., KRISTENSEN V.N., THEESFELD C.L., SORENMO K.U., TROYANSKAYA O.G., 2020 - "Modeling molecular development of breast cancer in canine mammary tumors." ***Genome Research*** doi: 10.1101/gr.256388.119.
21. HASELAGER M.V., KIELBASSA K., TER BURG J., BAX D.J.C., FERNANDES S.M., BORST J., TAM C., FORCONI F., CHIODIN G., BROWN J.R., DUBOIS J., KATER A.P., ELDERING E., 2020 - "Changes in Bcl-2 members after ibrutinib or venetoclax uncover functional hierarchy in determining resistance to venetoclax in CLL." ***Blood*** 136(25), 2918-2926.
22. HONMA N., HORII R., ITO Y., SAJI S., YOUNES M., IWASE T., AKIYAMA F., 2015 - "Differences in clinical importance of Bcl-2 in breast cancer according to hormone receptors status or adjuvant endocrine therapy." ***BMC Cancer*** 15, 698.
23. HUMINIECKI L., ATANASOV A.G., HORBAŃCZUK J., 2020 – Etiology of atherosclerosis informs choice of animal models and tissues for initial functional genomic studies of resveratrol. ***Pharmacological Research*** 156,104598.

24. HUMINIECKI L., HORBAŃCZUK J., 2018 – The functional genomic studies of resveratrol in respect to its anti-cancer effects. ***Biotechnology Advances*** Doi: 10.1016/J.Biotechadv.2018.02.011.
25. HUMINIECKI L., HORBAŃCZUK J., ATANASOV A.G., 2017 – The functional genomic studies of curcumin. ***Seminar Cancer in Biology*** Doi.Org/10.1016/J.Semcancer.2017.04.002.
26. JIAO D., ZHANG J., CHEN P., GUO X., QIAO J., ZHU J., WANG L., LU Z., LIU Z., 2021 - "HN1L promotes migration and invasion of breast cancer by up-regulating the expression of HMGB1." ***Journal of Cellular and Molecular Medicine*** 25(1), 397-410.
27. JURJ A., BRAICU C., POP L.A., TOMULEASA C., GHERMAN C.D., BERINDAN-NEAGOE I., 2017 - "The new era of nanotechnology, an alternative to change cancer treatment." ***Drug Design, Development and Therapy*** 11, 2871-2890.
28. KHANNA C., LINDBLAD-TOH K., VAIL D., LONDON C., BERGMAN P., BARBER L., BREEN M., KITCHELL B., MCNEIL E., MODIANO J.F., NIEMI S., COMSTOCK K.E., OSTRANDER E., WESTMORELAND S., WITHROW S., 2006 - "The dog as a cancer model." ***Nature Biotechnology*** 24(9), 1065-1066.
29. KLIMOV S., RIDA P.C., ALESKANDARANY M.A., GREEN A.R., ELLIS I.O., JANSSEN E.A., RAKHA E.A., ANEJA R., 2017 - "Novel immunohistochemistry-based signatures to predict metastatic site of triple-negative breast cancers." ***British Journal of Cancer*** 117(6), 826-834.
30. KORKMAZ U., USTUN F., 2021 - "Experimental Breast Cancer Models: Preclinical Imaging Perspective." ***Current Radiopharmaceuticals*** 14(1), 5-14.
31. LIU T., SONG S., WANG X., HAO J., 2021 - "Small-molecule inhibitors of breast cancer-related targets: Potential therapeutic agents for breast cancer." ***European Journal of Medicinal Chemistry*** 210, 112954.
32. LOTTER W., DIAB A.R., HASLAM B., KIM J.G., GRISOT G., WU E., WU K., ONIEVA J.O., BOYER Y., BOXERMAN J.L., WANG M., BANDLER M., VIJAYARAGHAVAN G.R., GREGORY SORENSEN A., 2021 - "Robust breast cancer detection in mammography and digital breast tomosynthesis using an annotation-efficient deep learning approach." ***Nature Medicine*** 27(2), 244-249.
33. MARTINEZ-ARRIBAS F., ALVAREZ T., DEL VAL G., MARTIN-GARABATO E., NUNEZ-VILLAR M.J., LUCAS R., SANCHEZ J., TEJERINA A., SCHNEIDER J., 2007 - "Bcl-2 expression in breast cancer: a comparative study at the mRNA and protein level." ***Anticancer Research*** 27(1A), 219-222.
34. MEHTA N., SAHU S.P., SHAIK S., DEVIREDDY R., GARTIA M.R., 2021 - "Dark-field hyperspectral imaging for label free detection of nano-bio-materials." ***Wiley Interdisciplinary Reviews. Nanomedicine and Nanobiotechnology*** 13(1), e1661.
35. MERINO D., LOK S.W., VISVADER J.E., LINDEMAN G.J., 2016 - "Targeting BCL-2 to enhance vulnerability to therapy in estrogen receptor-positive breast cancer." ***Oncogene*** 35(15), 1877-1887.
36. OGATA R., KISHINO E., SAITOH W., KOIKE Y., KUREBAYASHI J., 2021 - "Resistance to cyclin-dependent kinase (CDK) 4/6 inhibitors confers cross-resistance to other CDK inhibitors but not to chemotherapeutic agents in breast cancer cells." ***Breast Cancer*** 28(1), 206-215.
37. PANAYOTOPOULOU E.G., MULLER A.K., BORRIES M., BUSCH H., HU G., LEV S., 2017 - "Targeting of apoptotic pathways by SMAC or BH3 mimetics distinctly sensitizes paclitaxel-resistant triple negative breast cancer cells." ***Oncotarget*** 8(28), 45088-45104.
38. PARK G. T., CHOI K.C., 2016 - "Advanced new strategies for metastatic cancer treatment by therapeutic stem cells and oncolytic virotherapy." ***Oncotarget*** 7(36), 58684-58695.
39. PARK J.S., WITHERS S.S., MODIANO J.F., KENT M.S., CHEN M., LUNA J.I., CULP W.T.N., SPARGER E.E., REBHUN R.B., MONJAZEB A.M., MURPHY W.J., CANTER R.J., 2016 - "Canine cancer immunotherapy studies: linking mouse and human." ***Journal for Immunotherapy of Cancer*** 4, 97.

40. PATEL M.P., MASOOD A., PATEL P.S., CHANAN-KHAN A.A., 2009 - "Targeting the Bcl-2." ***Current Opinion in Oncology*** 21(6), 516-523.
41. PATEL V.M., BALAKRISHNAN K., DOUGLAS M., TIBBITTS T., XU E.Y., KUTOK J.L., AYERS M., SARKAR A., GUERRIERI R., WIERDA W.G., O'BRIEN S., JAIN N., STERN H.M., GANDHI V., 2017 - "Duvelisib treatment is associated with altered expression of apoptotic regulators that helps in sensitization of chronic lymphocytic leukemia cells to venetoclax (ABT-199)." ***Leukemia*** 31(9), 1872-1881.
42. PINHO S.S., CARVALHO S., CABRAL J., REIS C.A., GARTNER F., 2012 - "Canine tumors: a spontaneous animal model of human carcinogenesis." ***Translational Research: The Journal of Laboratory and Clinical Medicine*** 159(3), 165-172.
43. POLLYEA D.A., PRATZ K., LETAI A., JONAS B.A., WEI A.H., PULLARKAT V., KONOPLEVA M., THIRMAN M.J., ARELLANO M., BECKER P.S., CHYLA B., HONG W.J., JIANG Q., POTLURI J., DINARDO C.D., 2021 - "Venetoclax with azacitidine or decitabine in patients with newly diagnosed acute myeloid leukemia: Long term follow-up from a phase 1b study." ***American Journal of Hematology*** 96(2), 208-217.
44. ROWELL J.L., MCCARTHY D.O., ALVAREZ C.E., 2011 - "Dog models of naturally occurring cancer." ***Trends in Molecular Medicine*** 17(7), 380-388.
45. SEYMOUR J.F., MA S., BRANDER D.M., CHOI M.Y., BARRIENTOS J., DAVIDS M.S., ANDERSON M.A., BEAVEN A.W., ROSEN S.T., TAM C.S., PRINE B., AGARWAL S.K., MUNASINGHE W., ZHU M., LASH L.L., DESAI M., CERRI E., VERDUGO M., KIM S.Y., HUMERICKHOUSE R.A., GORDON G.B., KIPPS T.J., ROBERTS A.W., 2017 - "Venetoclax plus rituximab in relapsed or refractory chronic lymphocytic leukaemia: a phase 1b study." ***The Lancet Oncology*** 18(2), 230-240.
46. SHEN S., XU X., LIN S., ZHANG Y., LIU H., ZHANG C., MO R., 2021 - "A nanotherapeutic strategy to overcome chemotherapeutic resistance of cancer stem-like cells." ***Nature Nanotechnology*** 16(1), 104-113.
47. SHIMIZU S., KANASEKI T., MIZUSHIMA N., MIZUTA T., ARAKAWA-KOBAYASHI S., THOMPSON C.B., TSUJIMOTO Y., 2004 - "Role of Bcl-2 family proteins in a non-apoptotic programmed cell death dependent on autophagy genes." ***Nature Cell Biology*** 6(12), 1221-1228.
48. SUKUMAR J., GAST K., QUIROGA D., LUSTBERG M., WILLIAMS N., 2021 - "Triple-negative breast cancer: promising prognostic biomarkers currently in development." ***Expert Review of Anticancer Therapy*** 21(2), 135-148.
49. SUNG H., FERLAY J., SIEGEL R.L., LAVERSANNE M., SOERJOMATARAM I., JEMAL A., BRAY F., 2021 - "Global cancer statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries." ***CA: A Cancer Journal for Clinicians***.
50. TEWARI D., MOCAN A., PARVANOV E.D., SAH A,N., NABAVI S.N., HUMINIECKI L., MA Z.F., LEE Y.Y., HORBAŃCZUK J.O., ATANASOV A.G., 2017a – Etnopharmacological approaches for theraphy of jaundice – Part I. ***Frontiers in Pharmacology*** Doi.Org/10.3389/ Fphar.2017.00518.
51. TOMULEASA C., BRAICU C., IRIMIE A., CRACIUN L., BERINDAN-NEAGOE I., 2014 - "Nanopharmacology in translational hematology and oncology." ***International Journal of Nanomedicine*** 9, 3465-3479.
52. WANG D., ÖZEN C., ABU-REIDAH I.M., CHGURUPATI S., PATRA J.K., HORBAŃCZUK J.O., JÓŹWIK A., TZVETKOV N.T., UHRIN P., ATANASOV A.G., 2018 – Vasculoprotective effects of pomegranate (Punica Granatum L.). ***Frontiers in Pharmacology*** 9, 544 Doi: 10.3389/ Fphar.2018.00544.
53. WILLIAMS M.M., COOK R.S., 2015 - "Bcl-2 family proteins in breast development and cancer: could Mcl-1 targeting overcome therapeutic resistance?" ***Oncotarget*** 6(6), 3519-3530.

54. WOLF P., 2017 - "BH3 Mimetics for the Treatment of Prostate Cancer." ***Frontiers in Pharmacology*** 8, 557.
55. WU X., WANG L., QIU Y., ZHANG B., HU Z., JIN R., 2017 - "Cooperation of IRAK1/4 inhibitor and ABT-737 in nanoparticles for synergistic therapy of T cell acute lymphoblastic leukemia." ***International Journal of Nanomedicine*** 12, 8025-8034.
56. XUE H., LI J., XIE H., WANG Y., 2018 - "Review of Drug Repositioning Approaches and Resources." ***International Journal of Biological Sciences*** 14(10), 1232-1244.
57. YOULE R.J., STRASSER A., 2008 - "The BCL-2 protein family: opposing activities that mediate cell death." ***Nature Reviews. Molecular Cell Biology*** 9(1), 47-59.
58. YOUNG A., BU W., JIANG W., KU A., KAPALI J., DHAMNE S., QIN L., HILSENBECK S.G., Y. DU Y.N., LI Y., 2021 - "Targeting the Pro-survival Protein BCL-2 to Prevent Breast Cancer." ***Cancer Prevention Research.***
59. YOUNG A.I., LAW A.M., CASTILLO L., CHONG S., CULLEN H.D., KOEHLER M., HERZOG S., BRUMMER T., LEE E.F., FAIRLIE W.D., LUCAS M.C., HERRMANN D., ALLAM A., TIMPSON P., WATKINS D.N., MILLAR E.K., O'TOOLE S.A., GALLEGO-ORTEGA D., ORMANDY C.J. OAKES S.R., 2016 - "MCL-1 inhibition provides a new way to suppress breast cancer metastasis and increase sensitivity to dasatinib." ***Breast Cancer Research : BCR*** 18(1), 125.