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"In Preclinical Models, Bk Channel Subunits Influence Responses to Endocrine Therapy and Encourage the Development of Breast Cancer."

Hari Prasad Sonwani[1], Meenakshi Bharkatia[2], Surada. Prakash Rao[3], Ashish Majumdar[4], Madhuri Baghel[1]*

[1]apollo College Of Pharmacy, Anjora Durg 491001(C.G),India

[2]b.N College Of Pharmacy, B.N University Udaipur(R.J), India

[3]columbia Institute Of Pharmacy, Raipur(C.G),India

[4]columbia College Of Pharmacy, Raipur(C.G),India

Corresponding author: Dr. Madhuri Baghel

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KEYWORDS

breast cancer, estrogen receptor, leucine-rich repeat 49 containing 26, mouse mammary tumor virus polyoma middle T antigen, selective Estrogen Receptor Modulator 51, tamoxifen, Voltage- and Ca2+-activated K+ channels of big conductance.

ABSTRACT:

Context and Objective: Breast tumour cells with malignant characteristics are encouraged by pore-forming α subunits of the voltage- and Ca2+-activated K+ channel with large conductance (BKα). Leucine-rich repeat containing 26 (LRRC26) protein, also known as BKγ1, is one example of an auxiliary component that may be needed to enable BK current activation at a depolarized resting membrane potential, which is commonly observed in non-excitable tumor cells.

Experimental Methodology The anti-tumor effects of BKα loss were studied in primary MMTV-PyMT cell cultures, a syngeneic transplantation model of breast cancer produced from these cells, and breast tumor-bearing MMTV-PyMT transgenic BKα knockout (KO) mice. In the context of endocrine therapy, the therapeutic value of BK channels was evaluated using human breast cancer cell lines that expressed high (MDA-MB-453) or low (MCF-7) levels of BKα and BKγ1 and in MDA-MB-157 that is BKα-negative Important Findings In preclinical models, BKα enhanced the onset and overall survival of breast cancer. On the other hand, the in vitro proliferation of human and murine breast cancer cells was inhibited by BKγ knockdown or absence of BKα. Tamoxifen and its main active metabolites, at low doses, increased BKα/γ1-positive breast cancer cell proliferation without affecting the estrogen receptor-controlled genomic signaling. Lastly, tamoxifen lengthened the BKα KO recipient mice's relative survival time, but not that of the wild-type tumor cell recipient mice

Conclusion and Significance Functional BK channels are necessary for the initiation, development, and tamoxifen sensitivity of breast cancer. This supports further research into the role of BK channels in carcinogenesis and how anti-estrogen medication affects clinical outcomes

Abbreviations: BK, voltage- and Ca2+-activated K+ channel of big conductance; E2, 17β-estradiol; ER, estrogen receptor; KD, knock-down; KO, knock-out; LRRC26, leucine-rich repeat containing 26; MMTV-PyMT, mouse mammary tumor virus polyoma middle T antigen; OS, overall survival; RFS, recurrence-free survival; SERM, selective estrogen receptor modulator; TCGA, The Cancer Genome Atlas; TFS, tumor-free survival; TNBC, triple-negative breast cancer.

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Introduction:

In both excitable and non-excitable cells, the pore-forming α subunit of the KCa1.1 channel, a voltage- and Ca2+-activated K+ channel with large conductance (BKα), regulates a wide range of physiological processes (Latorre et al., 2017). This involves the control of vascular tone, auditory tuning of hair cells, the creation of circadian rhythms, the processing of nociceptive signals in inflammatory tissue, the coordination of motor skills, and significant functions in regulating the size and number of fat cells, the secretion of hormones, the homeostasis of K+ and glucose, and the processing of nociceptive signals (Dufer et al., 2011; Illison et al., 2016; Lu et al., 2014; Rieg et al., 2007; Ruettiger et al., 2004; Sausbier et al., 2004, 2005; Zhou et al., 2012). The BKα subunits form a tetramer mechanistically that is cooperatively regulated by both membrane voltage and Ca2+ (Li & Yan, 2016) to induce a rapid outflow of K+ from cells. A range of intracellular and extracellular components that contribute to the BK channel complex, as well as alternatively spliced mRNA transcripts of the Kcnma1 gene that codes for it, exert a functional regulation. The co-assembly with non-pore-forming regulatory BKβ and BKγ subunits affects the pharmacological sensitivities of the channel, its sensitivity to Ca2+ and voltage, and the rate and speed of BKα channel activation or deactivation (Gonzalez-Perez & Lingle, 2019). According to Yan and Aldrich (2012), the four newly identified BKγ subunit family members exist as single-span transmembrane (TM) proteins with an LRRC (long leucine-rich repeat containing) domain, which might be essential for the modulation and connection of channels (Li, Guan, Yen, Zhang, & Yan, 2016). Prostate adenocarcinoma and breast cancer cell lines (Gessner et al., 2005; Liu et al., 2012; Yan & Aldrich, 2010) as well as other epithelial cell types, such as the lactating epithelium of the mammary gland (Yang et al., 2017), have been shown to express auxiliary subunits like BKγ1 (also known as LRRC26). It is noteworthy that BKα channel currents can be activated and then shift by -145 mV to the near-physiological membrane potential of non-excitable cells, including tumor cells, through interaction with BKγ1. (Gonzalez-Perez, Xia, & Lingle, 2014; Yan & Aldrich,

2012). Aberrant expression or function of BKα can cause perturbed K+ permeability, which can lead to alterations in regulatory volume and promote the growth and survival of cancerous cells changed the phenotypes of migration and adhesion of tumor cells (Huang & Jan, 2014; Pardo & Stuhmer, 2014). It has been demonstrated that BKα affects the behaviors of tumor cells in several hormone-dependent malignancies, such as prostate, cervical, and breast cancer. Additionally, as glioblastoma and melanoma are typically hormone-insensitive, it has been suggested as a

Figure 1 Ion transport proteins. Ion transport proteins are classified into three groups: channels, exchangers, and pumps. These three types of proteins maintain the homeostasis of the intracellular ions either through facilitated or active transport, and changes in the ionic intracellular concentrations can initiate cellular processes. In blue, a chlorine channel is represented; in red, a non-selective cation channel; in purple, the Na+/Ca2+ exchanger; and in green, the plasma membrane Ca2+ ATPase. EC, extracellular; Cl−, chloride, Na+, sodium; Ca2+, calcium; ATP, adenosine triphosphate

therapeutic target in both conditions (Bloch et al., 2007; Edalat et al., 2016; Han et al., 2007; Mazar et al., 2010; Ramirez et al., 2018). For instance, enrichment of a BK splice variant unique to gliomas, which forms a channel with increased Ca2+ sensitivity, encourages a pro-migratory and pro-invasive phenotype in tumor cells. This BK variation boosted the radio resistance of stem cell-like subpopulations of glioblastoma (Edalat et al., 2016; Liu, Chang, Reinhart, Sontheimer, & Chang, 2002; Ransom, Liu and Sontheimer (2002); Rosa and colleagues (2017); Steinle and colleagues (2011)). BK has been linked to high tumor grade and stage, proliferation, and malignancy in relation to breast cancer (Khaitan et al., 2009; Oeggerli et al., 2012). BK is also present in human tumour tissue and in breast cancer cell lines (Gambade et al., 2016; Haren et al., 2010; Khatun et al., 2016; Mound, Rodat-Despoix, Bougarn, Ouadid-Ahidouch, &Matifat, 2013). When Penitrem A, an indole diterpene alkaloid made by Penicillium species, was used to bind BK channels in vitro, it caused various breast cancer cell lines to go into G1 arrest by upregulating the cyclin-dependent kinase inhibitor p27. Penitrem A works best when used with medications that target human epidermal growth factor receptors (HER), such as lapatinib and gefitinib shown

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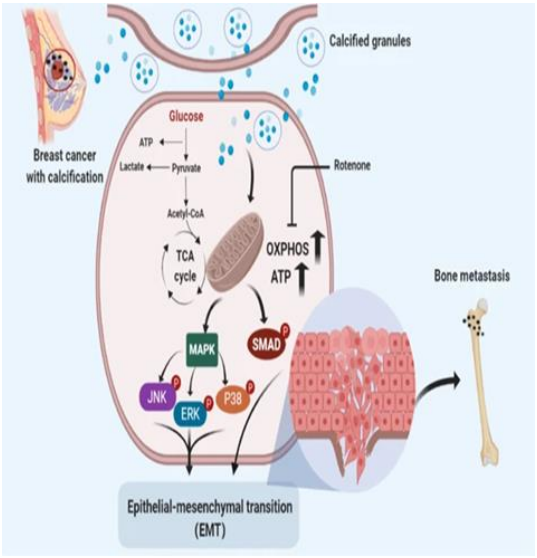
a cooperative anti-proliferative effect, suggesting a possible role for BK channels as anti-cancer targets (Goda, Siddique, Mohyeldin, Ayoub, & El Sayed, 2018). Iberiotoxin (IbTx), a specific BK channel blocker, inhibited BK currents and the proliferation-promoting effects of prolonged increases in intracellular Ca2+ concentration, even though the basal proliferation rate of various breast cancer cell lines was insensitive to IbTx (Roger, Potier, Vandier, Le Guennec, & Besson, 2004). On the other hand, triple-negative breast cancer (TNBC) advances more quickly due to epigenetic BKγ1 depletion (Miyagawa et al., 2018). All of this



indicates that estrogen receptor (ER), progesterone receptor, and HER2/neu signaling may be connected to any carcinogenic function of the BK channel complex in breast cancer. This idea is consistent with research from models of birds and dogs as well as The selective estrogen receptor modulator (SERM) tamoxifen, a frequently used anti-oestrogen for the treatment of ER-positive breast cancer (Dick, Rossow, Smirnov, Horowitz, & Sanders, 2001), can stimulate BK activity in human MCF-7 breast cancer cells (Coiret, Borowiec, Mariot, Ouadid-Ahidouch, &Matifat, 2007; Duncan, 2005). Therefore, BK and its auxiliary β and γ subunits could serve as extra targets for anti-hormonal therapy (Dick et al., 2001; Duncan, 2005). This is especially true because 17β-estradiol (E2) can up-regulate BK in human and murine neuroblastoma cell lines through an ER-dependent mechanism (Li & Qiu, 2015). In line with a non-genomic activation of BK channels by E2 and its derivatives, which has been demonstrated in non-cancerous tissues (Hristov, Parajuli, Provence, Rovner, & Petkov, 2017; Maher, Hunter, & Bow, 2016), estrogens may also directly impact ion channel functioning Provence, Hristov, Parajuli, & Petkov, 2015; Mabley, Lippiat, & Allen, 2013). It has been suggested that BK channels play a similar role in androgen responsiveness (Khatun et al., 2018), but it is still unclear whether the BK multi-protein complex has any clinical significance for the development of hormone-sensitive breast cancer or the effectiveness of anti-hormonal therapy in treating it. In this study, we examined the relationship between the ER pathway and the oncogenic effects of BK in mouse models of polyoma middle T antigen (PyMT) or HER2/cNeu oncogene-driven BKα-proficientand BKα-deficient mice, as well as in human breast cancer cells. Our goal was to ascertain the function of the BK channel complex in tumor cell behavior, particularly in response to growth factors, sex hormones, and anti-estrogens like tamoxifen.

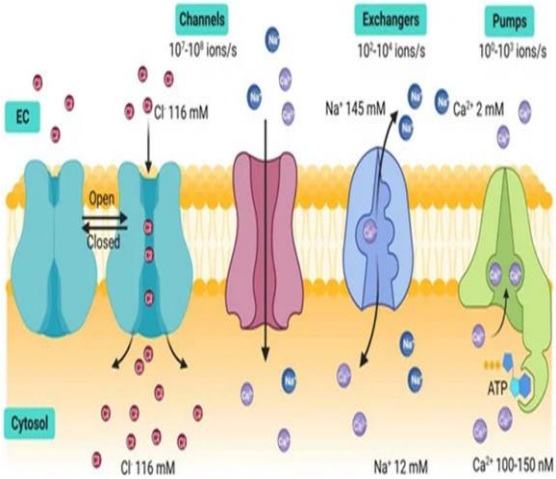


Figure 1 Ion transport proteins. Ion transport proteins are classified into three groups: channels, exchangers, and pumps. These three types of proteins maintain the homeostasis of the intracellular ions either through facilitated or active transport, and changes in the ionic intracellular concentrations can initiate cellular processes. In blue, a chlorine channel is represented; in red, a non-selective cation channel; in purple, the Na+/Ca2+ exchanger; and in green, the plasma membrane Ca2+ ATPase. EC, extracellular; Cl−, chloride, Na+, sodium; Ca2+, calcium; ATP, adenosine triphosphate

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METHODS

Spontaneous breast cancer mouse models induced by PyMT and HER2/cNeu as oncogenes

All animal care and experimental procedures were approved by the local Ethics Committee for Animal Research (Regierungspraesidium Tubingen). Animal studies are reported in compliance with the ARRIVE guidelines (Kilkenny et al., 2010). The induction and progression of spontaneous breast tumors were examined in female mice transgenic for the mouse mammary tumour virus polyoma middle T antigen (MMTV-PyMT; The Jackson Laboratory, Stock No: 002374), wild-type (WT), and BK knockout (KO) on an FVB/N background (backcrossed from FVB/N × C57BL/6 × Sv129 hybrids for >8 generations) (Guy, Cardiff, & Muller, 1992; Sausbier et al., 2004). Alternatively, tumor-prone mice expressing human EGF receptor 2 (HER2/cNeu) on a hybrid FVB/N × C57BL/6 × Sv129 background (Guy, Cardiff, & Muller, 1992). In a particular pathogen-free setting, spontaneous tumor induction was evaluated in groups of three or more litter-matched transgenic BK WT and BK KO female mice kept in conventional Makrolon type III cages with wood-chip bedding. Animals were checked for tumor development at least twice a week. regular 12-hour light/dark cycle, controlled humidity and temperature, and unlimited access to food and drink The first instance of a positive tumor palpation was used to establish tumor-free survival (TFS), and a maximal tumor diameter of 12 or 15 mm was used to estimate overall survival (OS). CO2 asphyxia was used to kill the animals once they reached the experimental endpoint. According to the protocol outlined by Steudel et al. (2017), tumors were prepared for histopathology, mRNA expression analysis, and the creation of primary cell cultures.

Allotransplant MMTV-PyMT mouse breast cancer model

Twelve-week-old BK-WT or BK KO FVB/N patients had their fourth right mammary gland syngeneic ally transplanted with primary MMTV-PyMT WT or BK KO cells (FVB/N). For allotransplant tests, only female FVB/N mice (Charles River, Sulzfeld, Germany) were utilized because this breed of mice is known to enhance the emergence of tumors (Davie et al., 2007). The experimental mice were placed on a heating pad, given an intraperitoneal injection of 100 µg g-1 ketamine (Inresa, Freiburg, Germany) and 10 µg g-1 xylazine (Bernburg, Bernburg, Germany), and had their mammary glands meticulously shaved and sanitized. A caudocranial incision of roughly 10 mm was made between the ventral midline and the mammary gland to gain entry into the mammary fat pad, which is home to 106 MMTV-PyMT WT or BK KO cells in 50 µl Prior to the wound being closed, PBS (Thermo Fisher Scientific, Waltham, USA) were injected. Metamizole was given in two dosages (200 µg g-1) to relieve post-operative pain. As previously mentioned, TFS and OS were assessed (Steudel et al., 2017).

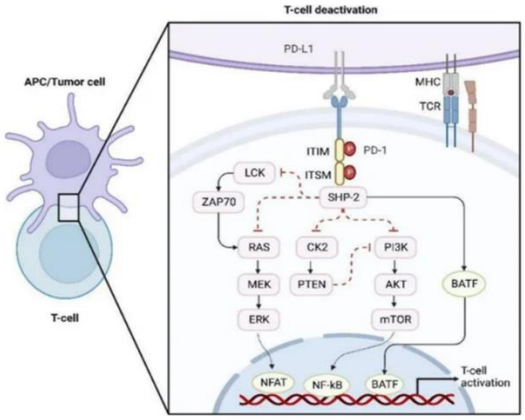

Figure 2 PD-1/PD-L1-mediated inhibition of T cell activation. The binding of PD-1 to PD-L1 recruits SHP-2, thereby weakening the TCR signaling pathway mediated by LCK and inhibiting the RAS-MEK-ERK and PI3K-Akt-mTOR pathways. In addition, PD-1 activation induces the expression of BTAF, which inhibits the expression of effectors for T cell activation. Collectively, the activation of T cells can be inhibited by PD-1/PD-L1-mediated inhibiting of these signaling pathways. Abbreviations: PD-1, programmed cell death protein 1; PD-L1, programmed cell death 1 ligand 1; TCR, T cell receptor; MHC, major histocompatibility complex; APC, antigen-presenting cell; ITIM, immunoreceptor tyrosine inhibitory motif; ITSM, immunoreceptor tyrosine-based switch motif; P, phosphorylation; LCK, lymphocyte-specific protein-tyrosine kinase; ZAP70, zeta chain of T cell receptor associated protein kinase 70; SHP-2, src homology-2 domain-containing protein tyrosine phosphatase; BATF, basic leucine zipper transcriptional factor ATF-like; RAS, rat sarcoma; MEK, mitogen-activated extracellular signal-regulated kinase; ERK, extracellular regulated protein kinase; NFAT, nuclear factor of

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activated T cell; CK2, casein kinase II; PTEN, phosphatase and tensin homolog; PI3K, phosphoinositide 3-kinase; AKT, protein kinase B; mTOR, mammalian target of rapamycin; NF-κB, nuclear factor kappa-B.

Ovariectomy

After syngeneic inoculation of MMTV-PyMT WT or BK KO cells into FVB/N recipients, the impact of tamoxifen on tumor progression in vivo was evaluated (Charles River, Sulzfeld, Germany). Tumor-bearing mice were ovariectomized when the tumor reached a diameter of 5 mm. This was done to mimic a post-menopausal state and rule out the influence of estrous cycle-dependent estrogen oscillations. In summary, mice were anesthetized with 100 µg g-1 ketamine and 10 µg g-1 xylazine. Then, the central back was cleaned, shaved, and a 10 mm skin incision was performed along the dorsal midline. Another little incision that provided access to the abdominal cavity was made every 5 mm laterally of the midline. After identifying the corresponding ovary, it was gently lifted and encircled by varying amounts of of fat was extracted and ligated from the distal uterine horn. Once more, the skin was closed and cleaned. For pain treatment, carprofen (5 mg kg-1) was given prior to surgery, right after following surgery, and for 48 hours following surgery.

Human breast cancer cell lines and primary MMTV-PyMT cell culture

IMEM, L-15, and DMEM were used to cultivate human MDA-MB-157 and MDA-MB-453 cells, mouse MMTV-PyMT WT and BK KO cells, and MCF-7 cells, respectively. For human or murine cells, 5% or 10% FCS was added to the media along with 1% penicillin-streptomycin (both from Thermo Fisher Scientific, Waltham, USA). FCS was switched out for FCS stripped of charcoal for anti-estrogen/hormone stimulation (CCS). While MDA-MB-157 and MDA-MB-453 cells were maintained in an incubator at 37°C and water-saturated room air, mouse cells and MCF-7 cells were cultured at 37°C and 5% CO2. Trypsin (Thermo Fisher Scientific, Waltham, USA) diluted 1:10 in PBS was used for 3–5 minutes in the incubator to achieve cell dissociation and detachment. Following 400 x centrifugation at g for Cells were resuspended and counted after three to five minutes in order to seed a specific number of cells for further development or experimentation.

Treatment with tamoxifen

Seven days after ovariectomy, tamoxifen or placebo was administered as subcutaneous pellets. Under ketamine (100 µg·g−1) and xylazine (10 µg·g−1) anesthesia, the skin at the right lateral side of the neck was lifted and an incision with a trocar (Innovative Research of America, Sarasota, USA) allowed minimally invasive insertion of 3-mm pellets with 0.5- or 5-mg tamoxifen released over 60 days. Tumor growth under tamoxifen treatment was monitored until an eightfold-increased tumor volume compared to the day of pellet implantation was reached. Tumor volume was determined as (length × width2)/2. Studies were performed in parallel as such that age- and litter-matched mice received the same drug treatment at the same time.

Materials

Pellets containing tamoxifen and a placebo were provided by Innovative Research of America, located in Sarasota, USA. 1A Pharma (Oberhaching, Germany) provided the metizole, while Zoetis (Delemont, Switzerland) provided the carprofen. Sigma-Aldrich, Taufkirchen, Germany supplied the stock solutions (1 mM) of E2, progesterone, testosterone, tamoxifen, (Z)-endoxifen, (Z)-4-OH-tamoxifen, and fulvestrant. PBS was used to prepare the E2-BSA (Sigma-Aldrich) stock solution. As shown in Figures 5 and 6, stock solutions were serially diluted to the final concentrations applied. A stock solution (10 mM) of paroxetine (Sigma-Aldrich) was prepared by dissolving it in DMSO, which was then aliquoted and kept frozen.

RESULTS:

BK channel subunit expression in human BC and prognosis

We conducted a gene expression analysis of BKα and its subunits in matched healthy samples and tumor tissue using The Cancer Genome Atlas (TCGA) database. The expression of BKα and other auxiliary BK subunits, encoded by KCNMA1, did not differ in tumor compared to healthy tissue. On the other hand, the BKβ1 and BKβ2 mRNA levels were down-regulated in breast tumor tissue, while the BKγ1 subunit, which is encoded by LRRC26, was up-regulated (Figure 1a). Age, intrinsic molecular subtype (PAM50 status), progesterone receptor or ER status, tumor stage, or menopausal state were not associated with these outcomes (data not shown). KCNMA1 expression values were either divided by the median or by subgroup-specific gene expression cut-offs inferred

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in order to ascertain a relationship between gene expression and the outcome of breast cancer from assessments of decision trees. When analyzing all available ER-positive or ER-negative patients (n = 1,711, Figure 1b), patients with increased KCNMA1 expression (above median) had an overall favorable outcome. However, this beneficial effect was reversed in the subgroup of patients treated with tamoxifen (n = 492), who had worse outcomes with high KCNMA1 expression (cut-off >12.14). This suggests that KCNMA1 status is a response modifier in the anti-hormone tamoxifen used in ER-positive cancer therapy (Figure 1c). Notably, the TCGA database did not provide RFS analyses or data on tamoxifen use, hence these analyses were not included in this dataset. The expression of BKβ subunits did not exhibit significant correlations with RFS in corresponding survival statistics, and the Breast Mark database did not contain gene probes for the BKγ subunit LRRC26.

Murine MMTV-PyMT WT cells demonstrated paxilline-sensitive, outwardly-rectifying currents (original tracings in Figure 4a and mean current density/voltage relationships in Figure 4b), as observed with the human breast cancer cell line. This was inferred from a significant block of approximately 50% of the conductance density by paxilline (inset in Figure 4b). The paxilline-sensitive fractions of conductance density between WT and KO breast cancer cells did not reach statistical significance, but paxilline did not affect the whole cell currents in MMTV-PyMT BK KO cells (Figure 4c), which is consistent with a contribution of BK channels to the paxilline-sensitive currents in MMTV-PyMT WT cells. In conclusion, in murine MMTV-PyMT WT cells, BK channels accounted for almost 50% of the outward currents. Approximately 100 pS·pF−1 This assumed BK current fraction was more than ten times smaller than that in human MDA-MB-453 breast cancer cells (compare open and closed bar in Figure 4b's insert).

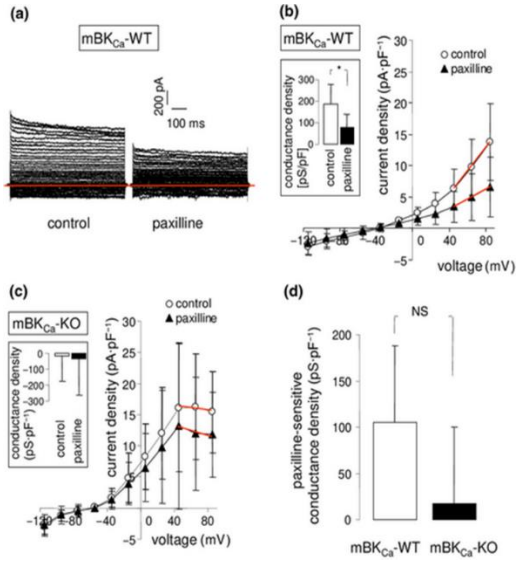

FIGURE : 3 Murine breast cancer cells functionally express BK channels. (a) Representative whole-cell current tracings from a murine MMTV-PyMT tumour-derived WT breast cancer cell before (left) and during (right) bath administration of the BK channel blocker paxilline. (b,c) Relationships between the mean current densities (±SD, n = 7–8) and the clamp voltage recorded as in (a) before (control) and during paxillinesuperfusion in murine (b) MMTV-PyMT tumour-derived WT and (c) BK KO breast cancer cells. Inserts in (b,c) show the mean (±SD, n = 7–8) conductance densities recorded in the absence (control) and presence of paxilline. (d) Mean paxilline-sensitive conductance density of MMTV-PyMT tumour-derived BK WT (left) and BK KO BC cells (right). Conductances were calculated for the outward currents elicited by voltage sweeps between +40 and +80 mV (as indicated by the red lines in (b,c). *P ≤ .05, significantly different as indicated, NS, not significantly different as indicated (in d, P = .062); two-tailed t-test.

Anti-hormones stimulate tumor growth in a way that is dependent on BK.

Anti-oestrogens are known to prevent the effects of oestrogens both in vitro and in vivo. Examples of these include (Z)-tamoxifen, its active metabolites (Z)-4-OH-tamoxifen and (Z)-endoxifen, and fulvestrant. We measured cell counts and the Ki-67 index in the presence and absence of increasing quantities of these substances in MMTV-PyMT cells to investigate whether the existence of BK channels affected the anti-

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proliferative properties of anti-oestrogens. For every anti-oestrogen examined, the proliferation rate (Figure 7a) and Ki-67 index (data not shown) decreased at high concentrations (1 μM). Nevertheless, BK-positive MMTV-PyMT breast tumor cells showed stimulating rather than inhibitory effects at low concentrations (10 nM) of tamoxifen and its active metabolites, highlighting the fact that various dosages of tamoxifen, (Z)-4-OH-tamoxifen, and Through either ERα or BKα, (Z)-endoxifen exert distinct effects on cell growth. All treatments considerably (or at least by trend) boosted the relative rate of cell proliferation (Figure 7b) and the Ki-67 index (data not shown) in the ERα-negative and BKα/γ1-positive MDA-MB-453 cell line. Additionally, BKγ1-positive MDA-MB-157 cells that were negative for both BKα and ERα did not respond to the anti-oestrogenic therapy. On the other hand, we observed that the proliferative effects of anti-oestrogens were highly similar in MCF-7 (Figure 7b) and MMTV-PyMT WT cells (Figure 7a). This observation was in line with the fact that these two cell types had very similar ERα/BKα/γ1 status. In particular, the proliferation rates of MCF-7 were decreased by (Z)-4-OH-tamoxifen, (Z)-endoxifen, and fulvestrant at 100 nM (results not shown) and 1 μM, while lower concentrations (1 and These anti-oestrogens (at 10 nM) had the opposite effect. Thus, the positive ERα and BKα status of MCF-7 cells, respectively, may be partially responsible for the anti-versus pro-proliferative effects of anti-oestrogens. To examine the effects of functional BKα channel expression on the breast tumour-depleting activities of tamoxifen in vivo, 12-week-old WT mice were orthotopically allotransplanted with MMTV-PyMT WT or BK KO cells. At a tumour diameter of 5 mm equivalent to 62.5 mm3, mice were ovariectomized to decrease endogenous sex hormone production followed by implantation of a 5 mg per 60-day release tamoxifen or placebo pellet. For methodological control, tamoxifen metabolism was analysed with 0.5 mg per 60-day and 5 mg per 60-day release pellets in ovariectomized WT mice in order to confirm that tamoxifen, as well as ER and BK modulating tamoxifen derivatives were generated (Figure S5). Survival under tamoxifen treatment was defined as the time to reach an eightfold-increased tumour volume compared to the start of endocrine therapy (Figure 7c). The endocrine effect of 5 mg per 60-day tamoxifen was assayed by its endometrial agonist activity within the tamoxifen-treated groups, revealing an increased uterus weight, compared with those in mice receiving the corresponding placebo treatments (Figure 7d). Of note, the MMTV-PyMT BK KO tumour growth was clearly inhibited by tamoxifen, whereas in MMTV-PyMT WT cell recipients, perhaps due to the opposing tumour-promoting and tumour-supressing effects of tamoxifen on BKα and ER (Figure S6), respectively, tamoxifen did not prolong the relative survival time (Figure 7e). At another level of interaction, long-term administration of tamoxifen decreased BK mRNA expression in breast cancer cells (Figure S7). Overall, our in vivo data suggest that tamoxifen, in addition to its effects via genomic signalling controlled by the ER, acts through BK channels to promote breast cancer, thereby suggesting that this channel may be involved in breast cancer onset, development and the response to endocrine treatment.

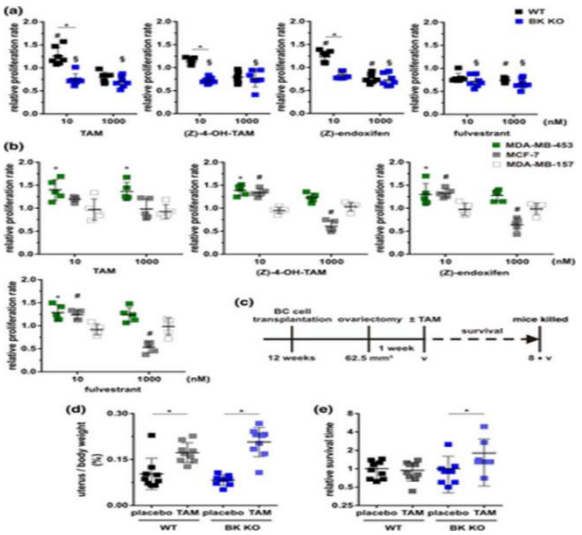

FIGURE : 4 Expression of BK channels limits success of anti-hormone therapy in vitro and in vivo. (a,b) Following a 72-h serum starvation period, anti-oestrogens were studied at the time point of serum re-stimulation for 72 h. (a) (Z)-Tamoxifen (TAM) and its active metabolites (Z)-4-OH-TAM and (Z)-endoxifen consistently reduced the proliferation rate of both BK-proficient and BK-deficient mouse tumour cells at 1 μM. Contrary to the clinically intended anti-proliferative effects, stimulating effects were observed at a lower dose of 10 nM only in BK positive MMTV-PyMT breast tumour cells. Fulvestrant inhibited the proliferation at all concentrations tested in both MMTV-PyMT WT and BK KO cells (n = 7 per

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genotype and condition. *P ≤ .05, significantly different as indicated; #P ≤ .05, for MMTV-PyMT WT cells, §P ≤ .05, for MMTV-PyMT BK KO cells, significantly different from corresponding control groups at 0 h (data not shown); two-way ANOVA and Sidak's test. (b) The ERα-negative, BKα/γ1 double-positive MDA-MB-453 cell line responded with higher relative proliferation rates to tamoxifen and its derivatives, and fulvestrant. ERα/BKα/γ1 triple-positive MCF-7 cells behaved differently from the MDA-MB-453 cell line showing either an increase or decrease in proliferation at low (10 nM) or high (1 μM) concentrations of the anti-oestrogens. Notably, none of the tested anti-oestrogens modulated the proliferative response of ERα/BKα double-negative and BKγ1-positive MDA-MB-157 cells; n = 5 per genotype. *P ≤ .05 for MDA-MB-453 cells. #P ≤ .05, for MCF-7 cells, significantly different from ctrl conditions at 0 h (data not shown); two-way ANOVA and Sidak's test. (c) Experimental timeline of MMTV-PyMT WT or BK KO cells allotransplanted into BK WT recipients, which underwent ovariectomy when the size of the tumours reached 62.5 mm3 and tamoxifen (TAM; a total of 5 mg per 60-day release) or placebo pellet implantation (details of tamoxifen metabolism are provided in Figure S5). Survival was defined as an eightfold increase in tumour volume (v) as compared to the initial size of the tumours at ovariectomy (n = 9 mice per genotype). (d) Endometrial agonism of the SERM tamoxifen was confirmed by increased uterus weight, normalized to the body weight of MMTV-PyMT WT and BK KO tumour-bearing mice. *P ≤ .05, significantly different as indicated; one-way ANOVA and Tukey's test. (e) Relative survival time of MMTV-PyMT BK KO tumour-bearing mice was increased with a low dose tamoxifen therapy that failed in BK channel-positive tumour-bearing mice. *P ≤ .05, significantly different as indicated; Mann–Whitney test.

Discussion :

Here, we provide new research on the role played by the endogenous BKα channel complex in the initiation and spread of breast cancer. Breast cancer initiation and progression depend on appropriate BK channel expression and activity, as demonstrated by our combined functional analyses of spontaneously occurring tumors in the MMTV-PyMT BK KO mouse model, tumor cells derived from it, and in vivo propagated breast cancer cell allotransplants lacking BK protein. Previous research has shown that different cancer entities exhibit altered BK channel expression or activity (Huang & Jan, 2014). BK has also been linked to malignant cell proliferation in breast cancer, which has been linked to a high tumor stage and a poor prognosis (Mound et al., 2013; Oeggerli et al., 2012). We verified that the expression profile of BK channel subunits in human breast tissue is abnormal. concerning the particular subunits KCNMB1, KCNMB2, and LRRC26 in cancer tissues (Figure 1a). It is noteworthy that different patient groups exhibited opposing characteristics in the relationship between tumor BKα mRNA expression and prognosis. While RFS was specifically worse in tamoxifen-treated patients with high KCNMA1 expression compared to those with lower expression levels, low KCNMA1 expression was associated with shorter RFS when all available ER-positive/negative breast tumour patients were considered, irrespective of treatment (Figure 1b). When taken as a whole, these results might suggest a clinical relationship between BKα and how well an anti-oestrogenic medication works. In light of the ongoing debate regarding the distinction between tamoxifen-treated ER-positive patients and breast cancer overall, we concentrated on our preclinical BK models to gain a better understanding of the interaction between BK, anti-oestrogenic therapies and the development of tumors. Preclinical evidence from two mouse models of breast cancer makes it abundantly evident that BK channel activity is essential for the development and spread of tumors (Figures 2 and S1). Tumor formation induced by the MMTV-PyMT oncogene occurred on a hybrid FVB/N × C57BL/6 × Sv129 background with shorter latency and at multiple foci within the mammary gland of all experimental mice within 82 days, in contrast to the FVB/N-based MMTV-cNeu model (Guy, Cardiff, & Muller, 1992). Because of this, the MMTV-PyMT model showed significantly greater rates of tumor formation than the MMTV-cNeu model (where BK channel blocking was linked to a reduced overall anti-tumour impact over time in all tumour-prone BK KO mice). But interruption of BK channels considerably raised TFS and OS in both models (Figures 2 and S1). While genetic variations and distinct tumorigenic pathways may account for some of the differences between the two mouse strains and murine breast cancer models (Ali et al., 2014; Brouxhon et al.,

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2014; Lifsted et al., 1998; Otsuka et al., 2018), our data suggest that the BK genotype plays a role in the differences in the kinetics of tumor appearance and progression in both models. Transplanting tumor cells provided confirmation of this substantial dependence. In comparison to BK WT transplants, tumor development was postponed when BC BK KO cells were grown in BK-proficient recipients (Figure 2h). As of yet, it is unknown if BK has an influence on OS or the macroscopically obvious metastatic potential of the disease (Figures 2i and S2). indicating that the role of BK channels may be greater at the onset of breast cancer than it is in the local or distant progression of the disease. Further research is necessary to understand the molecular underpinnings of the possible antagonistic functions of BK in non-malignant microenvironmental cell types and breast cancer cells, as well as the potential significance of this channel as a risk factor for various breast cancer subtypes and in the context of particular oncogenic signaling pathways. Numerous endogenous and exogenous variables, including as tamoxifen, oestrogens, hypoxia, ROS, and Hu et al. (2012), have been shown to affect the BK channel (Rottin, Fancher, Asano, Widlanski, & Dick, 2014; Tang, Garcia, Heinemann, & Hoshi, 2004). But the molecular processes that underlie this flexibility is not known. Finding mechanisms that avoid the traditional pharmacological interactions between ER signaling and anti-oestrogen inhibition is of great interest, given the therapeutic failure rate of breast cancer patients, of which one-third do not respond to adjuvant TAM treatment. Ultimately, we demonstrated that serum containing growth factors and sex hormones at physiological amounts was necessary for the BK-stimulated proliferation of human and mouse breast cancer cells. Regarding the latter, E2 coupled to membrane-impermeable BSA exhibited effects akin to those of non-conjugated E2, suggesting that traditional ER signaling was not the mechanism behind the observed pro-proliferative impact. Treatment with fulvestrant and the anti-oestrogens tamoxifen and its active metabolites 4-OH tamoxifen and endoxifen showed the anticipated anti-proliferative effect Low quantities (10 nM) of tamoxifen and its metabolites produced an opposite impact at high concentrations, which was an increase in cell proliferation, and this effect was obviously dependent on the presence of BK channels. Thus, we speculate that, especially at low (non-therapeutic) plasma doses, increased expression of BK channels in the tumor may overcome the anti-proliferative effects of tamoxifen treatment. Furthermore, the local concentrations of tamoxifen and its derivatives in the tumor may differ from the desired systemic drug concentration for tamoxifen's therapeutic effects (Gong et al., 2013). As a result, the routine use of anti-hormone therapies for the treatment of ER- and BK-positive breast cancer may have unpredictable results. At the clinical level, a number of findings could suggest that the patients' elevated BK channel tumorlevels reaction to the medical intervention. For instance, low available levels of anti-oestrogen and insufficient local tissue drug concentrations may be the cause of the transient worsening of the clinical status during the first weeks of tamoxifen treatment of advanced breast cancer, known as the "tamoxifen flare" (Plotkin, Lechner, Jung, & Rosen, 1978; Reddel & Sutherland, 1984). (Jordan, 1982; Kisanga et al., 2004). Similarly, in individuals with low amounts of active tamoxifen metabolites, increased expression of BK channels may influence the effectiveness of treatment because of an inherent impairment in tamoxifen metabolism (Brauch, Murdter, Eichelbaum, & Schwab, 2009; Murdter et al., 2011; Schroth et al., 2009). As a result, more research should be done to determine whether the BK channel state contributes to decreased tamoxifen efficacy or failure. ER expression levels that are variable in the MMTV-PyMT model (Lin et al., Tumor heterogeneity involving endocrine resistant cells that benefit from up-regulating BK and may further counteract anti-estrogenic growth control through down-regulating ER, respectively. In ER-negative/BK-positive MDA-MB-453 cells, this connection is consistent with the pro-proliferative pattern that occurs after exposure to tamoxifen metabolites. Strong evidence for the idea that BK channels stimulate cell growth and modulate the effects of anti-estrogens independently of ER signaling has been found in the observation that ER-negative/BK-negative MDA-MB-157 cells clearly lack growth induction, while BK-positive cell lines without (MDA-MB-453) or with (MCF7) ER expression exhibited a proliferative phenotype in response to low-dose anti-estrogens. Breast cancer cells with BK mRNA expression showed some tamoxifen sensitivity in our preclinical mouse model (Figure S7). Consequently, relationships involving tamoxifen, ER signaling, and BK channels

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appear to to be extremely flexible and dynamic, which may impact prognostic and predictive value at various levels and during various phases of the disease. The second significant crucial factor of BK channel activity seems to be the auxiliary BK channel subunit BKγ1. Its frequent and high expression in human breast cancer tissue and cell lines, such as MDA-MB-453, prostate cancer cells, and MMTV-PyMT-driven murine breast cancer models all support this (Yan & Aldrich, 2010). This subunit, when co-expressed with BKα, causes a significant change in the voltage-dependent half-maximal activation of BK, of −145 mV, which permits channel activation in tumor cells that are not excitable (Yan & Aldrich, 2010). Our research supported the notion that BKα and BKγ1 expression together was required to support a pro-proliferative phenotype of breast cancer cells from MDA-MB-453 human and MMTV-PyMT mouse models (Figure 5b–e). On the other hand, the simultaneous deletion of BKα and BKγ did not provide an additive anti-proliferative impact, indicating that BKγ alone mediates the pro-proliferative activities of BKγ in breast cancer cells through the K+ flow through BKα. Tumor aggressiveness has been linked to epigenetic DNA methylation of the LRRC26 gene, which has been linked to loss of BKγ1 expression in TNBC (Miyagawa et al., 2018). However, more research is required to clarify the function of BKα/γ1 complexes for various breast cancer subtypes. Our data, on the other hand, support a growth-enhancing effect of BKγ1 in hormone-sensitive breast cancer cells. Furthermore, this concerns the function of accessory subunits BKβ1-4 and BKγ2-4 for the BKα channel-mediated K+ The current study did not examine transport or cell proliferative activity in breast cancer cells, possibly because of their poor tumor expression. Overall, our research supports the idea that BK and BKα/η1 may both be appropriate pharmaceutical targets in the treatment of breast cancer by offering significant new insights into the in vivo biological significance of both proteins in the initiation and progression of breast cancer.

Conclusion :

Potential application in medicine

Given the pleiotropic functions of BKα and the anticipated multiple adverse effects upon global channel inhibition, we propose that targeting BKα/γ1 complexes associated with cancer and epithelial cells may present a suitable opportunity to develop tolerable treatments that could be helpful in conjunction with anti-hormonal treatment (Bentzen, Olesen, Ronn, & Grunnet, 2014; Nardi & Olesen, 2008; Yang et al., 2017). In order to control for unfavorable (pro-proliferative) consequences, the KCNMA1 status in breast cancer patients should be assessed in the context of anti-hormonal therapy, according to the combined experimental results and clinical association studies. Our discoveries regarding the interaction among BK channel subunits, steroid hormone pathways, and clinically utilized anti-estrogens are anticipated to facilitate subsequent investigations on ion channel-dependent dys-/functions as significant indicators or adjusters of illness as well as potential treatment areas for breast cancer**.**

CONFLICT OF INTEREST

None

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