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KEYNOTE (GREEN)

Targeting T-type channels in cancer: What is on and what is off?

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Over the past 20 years, various studies have demonstrated a pivotal role of T-type calcium channels (TTCCs) in tumor progression. Cytotoxic effects of TTCC pharmacological blockers have been reported *in vitro* and in preclinical models. However, their roles in cancer physiology are only beginning to be understood. In this review, we discuss evidence for the signaling pathways and cellular processes stemming from TTCC activity, mainly inferred by inverse reasoning from pharmacological blocks and, only in a few studies, by gene silencing or channel activation. A thorough analysis indicates that drug-induced cytotoxicity is partially an off-target effect. Dissection of on/off-target activity is paramount to elucidate the physiological roles of TTCCs, and to deliver efficacious therapies suited to different cancer types and stages.

Keywords: T-type Ca^{2+} channels; Pharmacological blockers; Repurposing; Cancer cells; Chemotherapy; On/off-target effects; Ca^{2+} signaling; Apoptosis; Autophagy

Repurposing of TTCC blockers for cancer

Deregulation of Ca^{2+} signaling as a means to overcome the G1 checkpoint of the cell cycle is an oncogenic hallmark.[1] Overexpression of plasma membrane Ca^{2+} channels in cancer cells includes store-operated channels, transient receptor potential channels, second messenger-operated channels, and voltage-activated Ca^{2+} channels (Ca_v channels).[2] The latter, which open (activate) by membrane depolarization and close (deactivate) by membrane repolarization, can adopt a refractory non-conducting state from both the activated and deactivated states (inactivate). Voltage-dependent transitions between the open and closed states make these channels especially suited to coupling membrane potential changes experienced along the cell cycle to Ca^{2+} signaling.

Ca_v3 is the least-homologous subfamily of Ca_v channels, displaying only an ~25% amino acid sequence identity with the Ca_v1 and Ca_v2 subfamilies. Differences in sequence are translated to differences in structure and function, with Ca_v3 channels activating and inactivating at significantly lower potentials compared with other Ca_v channels.[3] These features enable the presence of Ca_v3-mediated currents at membrane potentials around the resting value of most cell types; thus, their activity is physiologically relevant in the context of nonexcitable membranes.[4] Given that the number of Ca_v3 channels at the plasma membrane at steady potentials is predictably low even at its peak, their contribution to Ca^{2+}-activated cellular processes must be through the formation of signaling complexes, rather than by globally raising cytosolic Ca^{2+} levels. In line with this, Ca_v3 channels associate with different K^+ channel types at the nano- or microdomain level to regulate neuronal output.[5–6] Furthermore, Ca_v3-K^+ channel complexes are located within caveolae, from where they activate ryanodyne receptors to raise Ca^{2+} sparks in vascular smooth cells.[7–8]

The Ca_v3 subfamily (TTCCs) has three members, $Ca_v3.1$, $Ca_v3.2$ and $Ca_v3.3$, which were identified at the molecular level in 1998 and 1999.[9–11] The expression of TTCCs is linked to the

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cell cycle, increasing during the G1–S transition.[12–17]The presence of TTCCs in cancer cells was initially reported in cultured retinoblastoma Y79 cells.[18] In that study, the expression of $Ca_v3.1$ and $Ca_v3.2$ was reduced upon cell differentiation into neuronal or glial phenotypes, suggesting a role for TTCCs in cell cycle progression (but see[19–22]). Thus, the notion that TTCC pharmacological blockers used in the cardiovascular or neurological fields could have a chemotherapeutic value was consolidated through *in vitro* studies, which showed that tetralol, 3,4-dihydroquinazoline, and diphenylpiperazine derivatives not only inhibited proliferation, but also induced apoptotic cancer cell death.[23] Some of these studies included the analysis of $Ca_v3.1$ or $Ca_v3.2$ gene knockdown, which also led to cell cycle arrest and apoptosis, with the notable exception of work by Ohkubo *et al.*, who reported that overexpression of $Ca_v3.1$ reduced proliferation and, conversely, silencing of $Ca_v3.1$ enhanced proliferation of MCF-7 breast cancer cells.[24]

Having identified TTCCs as relevant chemotherapeutic targets, a necessary step is to understand their role in cancer physiology and the mechanisms by which their inhibition leads to cytostatic or cytotoxic effects. A simple rationale is that either blocking or silencing plasma membrane Ca^{2+} channels prevents a route for Ca^{2+} entry; thus, both actions are expected to inhibit Ca^{2+}-dependent pathways supporting cell proliferation or survival. However, viability reduction by TTCC pharmacological blockers is reported only at concentrations several times above the reported half maximal inhibitory concentrations (IC_{50}s) for TTCCs. The use of supramaximal concentrations increases the risk of off-targeting, which has been demonstrated for most TTCC blockers (Table 1). Furthermore, some of the effects described for these compounds are compatible with the activation of Ca^{2+}-dependent processes, rather than with a reduction of cytosolic Ca^{2+}.[4] Here, we summarize current knowledge of the signaling pathways activated or inhibited by pharmacological blockers of TTCCs in different cell settings, and how they compare with those altered by TTCC gene silencing. Gene knockdown strategies based on the introduction of specific short-interfering RNAs (siRNAs) or short hairpin RNAs (shRNAs) are not devoid of off-target effects. Seed region sequences in the sense strand can mimic natural miRNAs, often repressing several off-target transcripts.[25] Nevertheless, coincidental effects of pharmacological and gene targeting of TTCCs reinforce the specificity of both approaches.

TABLE 1

Off-target effects of TTCC blockers.

Concentration	Reported effect	Cell/tissue type	Refs
Mibefradil			
10 μM	Inhibits volume-activated Cl^- channels	Pulmonary artery endothelial cells	176
10 μM	Inhibits Ca^{2+}-activated Cl^- channels		
3.4 μM (IC_{50})	Blocks L-type channels	Spinal motoneurons	177
3 μM (IC_{50})	Blocks L-type channels	Atrial cardiomyocytes	178
1.4 μM (IC_{50})	Blocks Cav1.2 (L-type channels)	CHO cells (heterologous expression)	179
1.1 μM (Kd)	Blocks voltage-gated K^+ channels (Kv)	Coronary arterial smooth muscle cells	180
1.9 μM (IC_{50})	Blocks Kv channels	B lymphocytes	181
2.3 μM (IC_{50})	Blocks background K^+ channels		
0.5–2 μM	potentiation of Ca^{2+}-activated K^+ channels		
0.8 μM (Kd)	Blocks Kv1.5 channels	CHO cells (heterologous expression)	162
1.4 μM (IC_{50})	Blocks HERG K^+ channels	Mammalian cells (heterologous expression)	182
11.8 μM (IC_{50})	Blocks Kv7.1 channels		
0.7 μM (IC_{50})	Blocks HERG K^+ channels	Myoblasts	183
0.3 μM (IC_{50})	Blocks Kv channels		
5.6 μM (IC_{50})	Blocks KIR2.0 K^+ channels		
0.5 μM (IC_{50})	Blocks K(ATP) K^+ channels	Adrenal fasciculata cells	161
10 μM	Blocks Eag1 (Kv10.1) channels	HEK293 cells (heterologous expression)	184
55.1 μM (IC_{50})	Activates TrpM7 channels	HEK293 cells (heterologous expression)	165
1 μM	Blocks different voltage-gated Na^+ channels	HEK293 cells (heterologous expression)	185
NNC-55-0396			
50 μM	Activates TrpM7 channels	HEK293 cells (heterologous expression)	165
0.1 μM (IC_{50})	Blocks Kv channels	Coronary arterial myocytes	186
Niguldipine			
0.4 μM (IC_{50})	Blocks L-type channels	Vascular myocytes	187
0.8 μM (IC_{50})	Blocks bTREK-1 background K^+ channels	Adrenal zona fasciculata cells	164
Verapamil			
0.6 μM (IC_{50})	Blocks L-type channels	Coronary arterial myocytes	188
15.5 μM (IC_{50})	Blocks L-type channels	HEK293 cells (heterologous expression)	179
4.2 μM (IC_{50})	Blocks Cav1.2 (L-type) channels	HEK293 cells (heterologous expression)	189
5.2 μM (IC_{50})	Blocks TRESK background K^+ channels	Trigeminal ganglion neurons	190
3 μM (IC_{50})	Blocks different Kv channels	Hippocampal neurons	191
0.1 μM (IC_{50})	Blocks HERG K^+ channels	HEK293 cells (heterologous expression)	192

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TTCC-mediated Ca^{2+} entry engages the CaM/CaMKII pathway: A first positive feedback mechanism with several possible outcomes

The main Ca^{2+} transducer calmodulin (CaM) regulates cell cycle progression by association with cyclin-dependent kinase (CDK) complexes[26–27] and by triggering phosphorylation/dephosphorylation events.[28] Multifunctional CaM-dependent protein kinase II (CaMKII) activates signaling pathways directly or indirectly, such as the small GTPase Ras-mitogen-activated protein kinases (RAF/RAS/MAPKs), the stress-activated MAPK p38, the serine/threonine protein kinase B (AKT), and the nonreceptor tyrosine kinase c-Src pathways, involved in the regulation of transcription factors that control the expression, assembly, or stability of cyclin/CDK complexes.[28–29] Among the phosphatases, calcineurin (CaN) is also implicated in the G1–S transition by initiating a pathway that leads to the accumulation of cyclin D1[30] and/or by dephosphorylation of nuclear factor of activated T cells (NFAT), which results in its translocation to the nucleus.[31] In addition to proliferation, the biochemical pathways emanating from activation of CaMKII trigger other cell fate decisions, such as survival/death and differentiation/stemness.[32–33]

TTCC-mediated Ca^{2+} entry activates CaMKII, which successively phosphorylates $Ca_v3.2$ channels heterologously expressed in HEK293 cells and native $Ca_v3.2$ channels in adrenal zona glomerulosa cells.[34] Such phosphorylation increases the open probability of $Ca_v3.2$ channels, thus implying a positive feedback loop. The $Ca_v3.2$–CaMKII pathway appears to be ubiquitous: incubation of rat striatal slices with the TTCC blockers mibefradil (at 5 μM) or $NiCl_2$ (at 100 μM, a concentration that blocks mainly $Ca_v3.2$ channels) significantly decreased Thr286 autophosphorylation of CaMKIIα[35]. $Ca_v3.1$ channels were also shown to support phosphorylation of CaMKIIα upon transfection in tsA-201 cells and in neurons, in which CaMKII has a role in synaptic plasticity.[36] In this work, CaMKIIα activation triggered by 50 mM KCl could be prevented by mibefradil (1 μM) and by Ni^{2+} (300 μM) as TTCC blockers.

Hence, several known CaMKII targets relevant for cancer pathophysiology might be activated by Ca^{2+} influx through TTCCs, including kinases, phosphatases, G-proteins, and transcription factors (Fig. 1). As commented earlier, expected consequences of TTCC block or gene silencing would be inhibition of these pathways.

TTCCs and the RAF/RAS/MAPK pathway: A second positive feedback mechanism for cancer cell proliferation and survival?

Of the above-mentioned possibilities, the existence of a TTCC–extracellular signal-regulated kinase (ERK) axis has been reported in different settings, although the sign of the regulation appears to depend on cell type/context and channel isoform. Choi *et al.* showed that activation of $Ca_v3.1$ transiently expressed in HEK293 cells triggered small G-protein Ras (RAS) signaling, and that this signal was transferred to ERK by Raf kinase (RAF) and MAPK kinase (MEK) via the MAPK pathway (Table 2).[37] In a mouse model of mechanical hyperalgesia, Chen *et al.* concluded that $Ca_v3.2$-dependent activation of ERK in paraventricular thalamus modulated acid-induced chronic muscle pain.[38] However, in rat neonatal cardiomyocytes, aldosterone and angiotensin II-induced $Ca_v3.1$ expression resulted in a time-dependent activation of serine-threonine phosphatase 2A (PP2A), and subsequent dephosphorylation of MEK1/2 and its downstream target cAMP-responsive element-binding (CREB).[39] The use of TTCC pharmacological blockers added confusion to this issue: Li *et al.*, associated the halt of the cell cycle induced by 4.5 μM mibefradil with a persistent increase of pERK1/2 in hepatocellular carcinoma cells. However, neither shRNA silencing nor overexpression of $Ca_v3.1$ had observable effects on the proliferation rate.[40] Huang *et al.* found that application of mibefradil or NNC-55-0396 to leukemia cells induced the decrease of pERK1/2 in a concentration-dependent manner, except for NNC-55-0396 at 10 μM, which induced a robust phosphorylation of ERK1/2 that was dependent on Ca^{2+} release from the endoplasmic reticulum (ER).[41]

Importantly, the relationship between TTCCs and MAPK is bidirectional: activation of ERK was shown to enhance the trafficking of heterologously expressed $Ca_v3.1$ channels to the plasma membrane.[42] Of note, RanBPM, a scaffold and c-Raf interacting protein that might activate[43] or inhibit[44] ERK signaling, was found to interact with the I-II linker of $Ca_v3.1$ and increase its expression in the plasma membrane.[45] Activation of MAPK might also increase the transcriptional expression of TTCCs: in neonatal cardiomyocytes, application of 17β estradiol upregulated $Ca_v3.2$ mRNA through phosphorylation of ERK1/2.[46] Regardless of mechanistic considerations, such cross-regulation could be especially relevant in the context of cancer cells with hyperactive MAPK signaling. The RAF/RAS/MAPK pathway has a pivotal role in cancer progression by supporting cancer cell proliferation, survival, and migration.[47] This pathway is especially relevant in cutaneous melanoma, which frequently harbor gain-of-function mutations in B-RAF (~40–50%) and N-RAS (15–20%).[48] Many patients with melanoma experience an initial improvement with B-RAF- or MEK-targeted therapies, but resistance to treatment or secondary malignancies inevitably occur. Several mechanisms can contribute to recurrence, including the reactivation of the MAPK pathway at different levels or the compensatory activation of the phosphoinositide 3-kinase (PI3K)-AKT pathway,[49] the unfolded protein response (UPR),[50] and (macro)autophagy.[50] Interestingly, melanoma biopsies bearing B-RAF(V600E) mutations displayed higher immunolabeling for $Ca_v3.1$ compared with wild-type biopsies, and a positive association was found between $Ca_v3.1$ and microtubule-associated protein light chain 3 (LC3),[51] a key component of autophagosomes.[52] Moreover, two recent reports indicate that the expression of TTCC transcripts is increased in B-RAF- and N-RAS-mutant melanoma cells adapted to MAPK inhibitors, and that TTCC targeting with mibefradil or shRNA-mediated gene silencing might reverse resistance to MAPK inhibitors.[53–54] Thus, establishment of a positive feedback between TTCCs and the MAPK pathway might support tumor progression and chemotherapeutic resistance in the melanoma paradigm, with the puzzling involvement of autophagy.[55]

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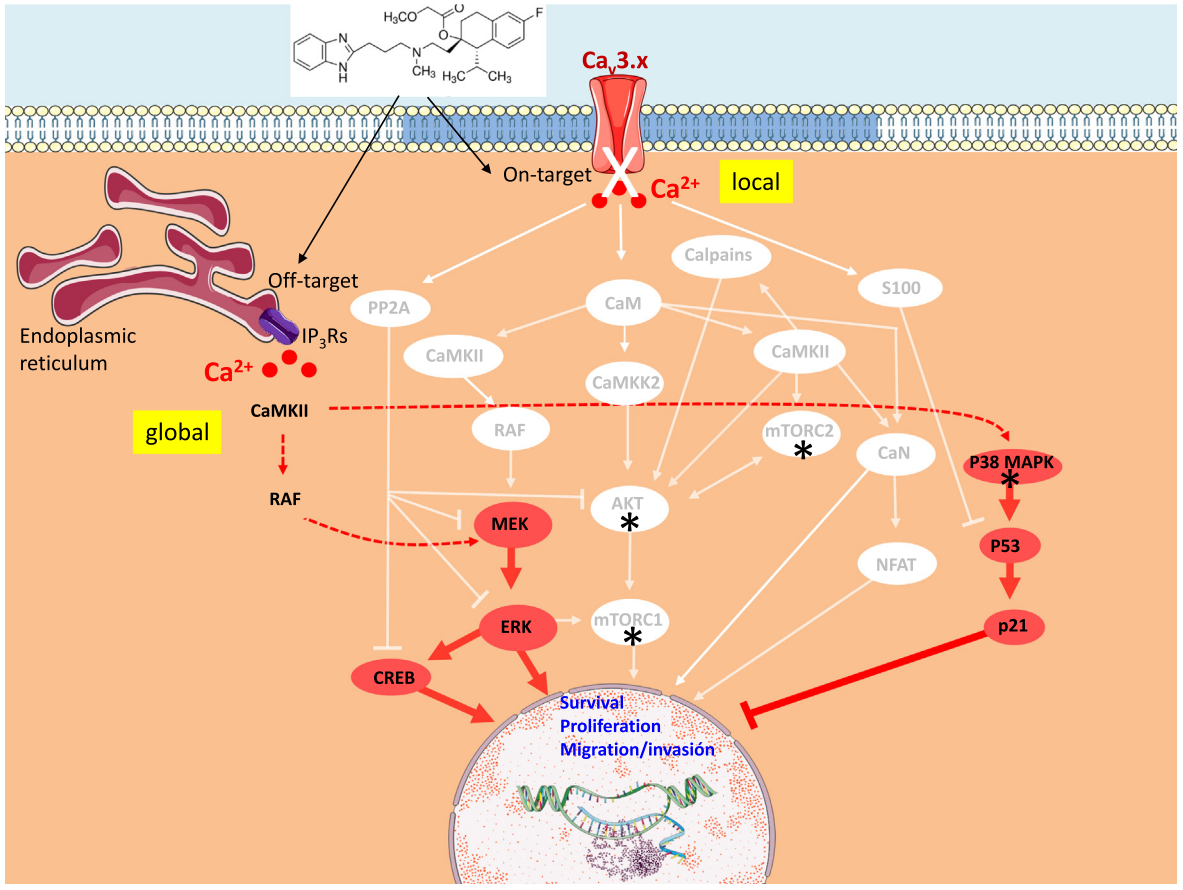

FIGURE 1

On-target vs off-target effects of T-type calcium channel (TTCC) blockers used in cancer cells. Selective inhibition of TTCCs (indicated by white cross on inner mouth of channel) inhibits all Ca^{2+}-dependent pathways in their signaling domain. Switched-off effectors/pathways are represented by white ellipses/arrows. Most of these effects have been observed by application of TTCC pharmacological blockers and, in a few cases, have been confirmed by specific gene knockdown of $Ca_v3.1$ and/or $Ca_v3.2$ channels (indicated by black asterisks). However, mitogen-activated protein kinase (MAPK) kinase (MEK), extracellular signal-regulated kinase (ERK), and the stress-activated p38 MAPK/p53 pathway have been shown to be activated by application of TTCC pharmacological blockers (mainly tetralol derivatives) at cytotoxic concentrations (red ellipses/arrows). These Ca^{2+}-dependent pathways might be activated by Ca^{2+} release from the endoplasmic reticulum (ER), a reported off-target effect for tetralol (putative routes are shown by discontinuous red arrows). Assuming the location of TTCCs in membrane microdomains, consistent with their role in signal transduction, on-target effects would be local. By contrast, off-target effects by intracellular Ca^{2+} mobilization could have global implications. Arrow tips indicate whether the modulation is positive (pointed) or negative (blunt-end). Chemical formula is that of mibefradil.

The stress-activated p38 MAPK pathway is inhibited by TTCC gene silencing but activated by TTCC blockers

The p38 mitogen-activated serine-threonine kinases (p38 MAPKs) regulate inflammatory and stress responses. Major p38 MAPK substrates are the transcription factors activator protein 1 and tumor-suppressor P53.[56] Activation of the p38 MAPK pathway occurs via a three-tiered kinase cascade initiated by activation of G-protein-coupled, cytokine, or tyrosine kinase receptors.[57] Alternative activation of p38 MAPK includes the Ca^{2+}-CaM/CaMKII axis, as shown in skeletal muscle,[58] osteocytes,[59] and colon cancer cells.[60] Although not exempt from complexities, accumulating evidence indicates that Ca^{2+} entry through TTCCs might lead to p38 MAPK activation. Dziegielewska *et al.* compared the effects of 10 μM mibefradil with those of $Ca_v3.1$ silencing in colon carcinoma HCT116 and adenocarcinoma CaCo2 cells.[61] siRNA-mediated knockdown of $Ca_v3.1$ reduced cell proliferation and induced caspase-3/7-dependent apoptosis, similar to treatment with mibefradil. Of note, mibefradil effects were mediated by p38 MAPK phosphorylation of P53 at Ser-392, but the implication of p38 MAPK was not examined in gene-silencing experiments. Opposite conclusions can be drawn from the study of the mechanisms for neurotoxicity of local anesthetics, known to be mediated by heightened Ca^{2+} influx[62] and p38 MAPK activation.[63] Application of lidocaine in SH-SY5Y neuroblastoma cells increased the expression of $Ca_v3.1$ channels in parallel to the concentration of cytosolic Ca^{2+}.[64] In addition, stable knockdown of $Ca_v3.1$ reduced the levels of intracellular Ca^{2+} and phosphorylated p38, while attenuating lidocaine toxicity. A similar inference can be built for $Ca_v3.2$ channels from the application of radiofrequency electro-

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TABLE 2

Interaction of TTCCs with Ca^{2+}-dependent signaling effectors/pathways.

Evidence of interaction	Feedback	Cell/tissue type	Refs
CaMKII			
Co-immunoprecipitation of $Ca_v3.2$ II-III loop and CaMKIIαc	Activates CaMKII phosphorylated $Ca_v3.2$ at Ser1198 increasing its open probability	HEK293 cells (heterologous expression)	34
	Activates CaMKII (by Angiotensin II) promoted phosphorylation of $Ca_v3.2$ at Ser1198	Adrenal zona glomerulosa cells	
Mibefradil (5 μM) or $NiCl_2$ (100 μM) decreased autophosphorylation of CaMKIIα at Thr286		Striatal slices	35
Co-immunoprecipitation of $Ca_v3.1$ (C terminus) and CaMKIIα		tsA-201 cells (heterologous expression)	36
Activates $Ca_v3.1$ (by 50 mM KCl)-induced phosphorylation of CaMKIIα (Thr286), prevented by mibefradil (1 μM) or $NiCl_2$ (300 μM)			
RAS/RAF/MAPK			
Activates $Ca_v3.1$ (by 150 mM KCl)-induced RAS and ERK transient activation		HEK293 cells (heterologous expression)	37
Acid-induced activation of $Ca_v3.2$ induced ERK1/2 phosphorylation (pERK1/2)		Paraventricular thalamus	38
Angiotensin II/aldosterone-induced $Ca_v3.1$ expression promoted MEK1/2 dephosphorylation through PP2A activation		Neonatal ventricular cardiomyocytes	193
Application of mibefradil (4.5 μM) increased pERK1/2 levels		Hepatocellular carcinoma cells	40
	17β estradiol upregulated $Ca_v3.2$ mRNA through phosphorylation of ERK1/2	Neonatal ventricular cardiomyocytes	46
Application of mibefradil (2.5–10 μM) or NNC-55-0396 (2.5, 5 μM) decreased pERK1/2 levels		MOLT-4 leukemia cells	41
Application of NNC-55-0396 (10 μM) increased pERK1/2 levels			
Co-immunoprecipitation of $Ca_v3.1$ III loop and c-Raf-interacting protein RanBPM		Rat brain	45
	Expression of RanBPM increased $Ca_v3.1$ expression in plasma membrane	HEK293 cells (heterologous expression)	
	ERK activation enhanced $Ca_v3.1$ trafficking to plasma membrane	Ovarian cells and murine cardiomyocytes cell line (HL-1)	42
	Immunolabeling for $Ca_v3.1$ was associated with gain-of-function $BRAF^{V600E}$ mutation	Melanoma biopsies	51
	$Ca_v3.1$ mRNA upregulated in BRAF-mutant cells resistant to MAPK inhibitors	Melanoma cells	53
	$Ca_v3.1$ and $Ca_v3.2$ mRNAs upregulated in BRAF- and NRAS-mutant cells resistant to MAPK inhibitors	Melanoma cells	54
p38 MAPK			
Application of mibefradil (10 μM) promoted phosphorylation of p53 at Ser392 mediated by p38 MAPK activation		HCT116 colon carcinoma	61
$Ca_v3.1$ gene knockdown reduced phosphorylation of p38 MAPK		SH-SY5Y neuroblastoma	64
$Ca_v3.2$ activation (by RF-EMF) induced p38 MAPK phosphorylation		Metastatic breast cancer cells	65
AKT			
Mibefradil (10 μM) reduced AKT phosphorylation at Ser473; $Ca_v3.1$ gene silencing reduced AKT phosphorylation at Ser473		GBM cell lines	75
Mibefradil (10 μM) reduced AKT phosphorylation at Ser473		Ovarian cancer cells	76
Mibefradil (5 μM) reduced AKT phosphorylation at Ser473		Glioma stem cells	77
$Ca_v3.1$ gene knockdown reduced AKT phosphorylation at Ser473		Prostate cancer cells	78
	PTEN deficiency or gene knockdown (implying hyperactivity of AKT) associated with increased $Ca_v3.1$ transcript levels; PTEN overexpression reduced $Ca_v3.1$ transcript levels	Melanoma cells	53

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TABLE 2 (*CONTINUED*)

Evidence of interaction	Feedback	Cell/tissue type	Refs
Co-immunoprecipitation of $Ca_v3.2$ and CaN		HEK293 cells (heterologous expression)	91
Pressure overload failed to boost NFAT activation in $Ca_v3.2$-knockout mice		Heart ventricle	
Co-immunoprecipitation of $Ca_v3.2$C terminus and CaN NFAT-binding domain		HEK293 cells (heterologous expression)	99
Kurtoxin (1 μM), nisoldipine (3 μM), or efonidipine (10 μM) prevented CaN–NFAT activation	Csx/Nkx2.5 (downstream of CaN-NFAT) upregulated $Ca_v3.1$ and $Ca_v3.2$ transcripts	Neonatal ventricular myocytes	101
		Pulmonary vein cardiomyocytes	102
$Ca_v3.1$ knockout reduced nuclear translocation of NFAT		T helper cells	104
PP2A/CREB			
Aldosterone-induced expression of $Ca_v3.1$ increased PP2A and PP1 activities, abrogated by mibefradil (1 μM); mibefradil also increased CREB phosphorylation		Neonatal ventricular cardiomyocytes	39
Calpains			
Oral administration of mibefradil prevented calpain-II upregulation and decreased infarct size		Left ventricular wall (myocardial infarction model)	122
NNC-55-0396 (10 μM) prevented calpain-I activation and long-term depression triggered by type-I metabotropic glutamate receptor stimulation		Hippocampal Schaffer collateral-CA1 synapses	124

magnetic fields (RF-EMFs) to breast cancer brain metastasis. Sharma *et al.* reported that antitumor effects of patterned RF-EMFs were dependent on the activation of stress pathways.[65] Notably, p38 MAPK activation was found to be mediated by Ca^{2+} influx through $Ca_v3.2$ channels and ensuing activation of CaMKII, and $Ca_v3.2$ gene silencing (but not that of $Ca_v3.1$ or $Ca_v3.3$) curtailed the effects of EMFs on tumor growth *in vitro* and *in vivo*.

Collectively, data from gene silencing of $Ca_v3.1$ or $Ca_v3.2$ support the prevalence of a TTCC–CaMKII–p38 MAPK axis in cancer cells, opposing tumorigenesis, such that its inhibition facilitates tumor growth. By contrast, treatment with mibefradil exerts cytotoxic effects by activating the p38 MAPK pathway. Although both approaches lead to the same conclusions regarding the proapoptotic role of p38 MAPK, the opposite effects of TTCC pharmacological block and gene silencing on the pathway require attention. How does preventing a route of Ca^{2+} entry activate p38 MAPK? A possible explanation is through inhibition of Ca^{2+}-dependent phosphatases for which p38 MAPK is a substrate, such as PP2A[66] and PP2C.[67] Instead, p38 MAPK dephosphorylation by TTCC gene silencing suggests that p38 MAPK activation by treatment with mibefradil is an off-target effect, owing to cytosolic Ca^{2+} elevation (Fig. 1).

TTCCs and the AKT pathway: A third positive feedback mechanism favoring tumor progression and chemoresistance

A role for TTCCs in tumorigenesis/tumor progression might also be rooted in the activation of a prosurvival AKT pathway. In cancer cells, AKT can be activated by CaMKII and Ca^{2+}-CaM-dependent protein kinase kinase 2 (CaMKK2), independently of phosphoinositide 3 kinase (PI3K) activity.[68–70] These alternative activation routes might compensate the low availability of nutrients or trophic factors inherent to tumor growth, as well as conferring resistance to pharmacological compounds targeting components of the PI3K pathway upstream of AKT.[70] Thus, active AKT can phosphorylate different downstream effectors, which trigger signaling chains culminating in the activation of prosurvival/antiapoptotic transcription factors.[71–72] Mammalian target of rapamycin complex 1 (mTORC1) is the nodal point at which signals generated by growth factors, nutrients, and metabolic status concur, acting as a gateway to cell growth and proliferation.[73] This pathway is modulated by diverse inputs, including RAS (a dual PI3K and MAPK activator) and mTORC2 (an AKT activator) as positive regulators, and phosphatase and tensin homolog (PTEN) as an inhibitor.[71,74]

Valerie *et al.* showed that treatment with 10 μM mibefradil or siRNA-mediated knockdown of $Ca_v3.1$ or $Ca_v3.2$ reduced the clonogenic potential and induced caspase-3/7-dependent apoptosis in GBM cell lines, marked by inhibition of antiapoptotic survivin/BIRC5 and BAD proteins.[75] Treatment with mibefradil also reduced phosphorylation of the mTORC2 subunit Rictor and AKT, the latter being mimicked by knockdown of $Ca_v3.1$. Similar results were later obtained in ovarian cancer cell lines treated with 10 μM mibefradil, which synergized with carboplatin to reduce cancer cell viability *in vitro* and tumor growth *in vivo*.[76] In this study, it was concluded that mibefradil reduced

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AKT phosphorylation and survivin expression via activation of Forkhead box O transcription factors. Again, direct involvement of TTCC isoforms was examined by silencing of $Ca_v3.1$ or $Ca_v3.2$, which induced apoptosis in parallel to a reduction in survivin mRNA and protein levels. More recently, reverse phase protein arrays revealed inhibition at multiple levels of the AKT–mTORC1 pathway by 5 μM mibefradil in glioma stem cells (GSCs). Inhibition of this pathway was evidenced by increased phosphorylation of the upstream negative effectors liver kinase B1 and Tuberin/TSC2, and decreased activation of AKT, mTORC1, and the downstream effector 4EBP1. shRNA-mediated $Ca_v3.2$ knockdown also reduced GSCs proliferation and increased cell death, but the involvement of the AKT pathway was not studied.[77] Furthermore, $Ca_v3.1$ silencing induced G1–S cell cycle arrest and reduced the migration and invasion capabilities of prostate cell lines. These effects, mediated by AKT dephosphorylation, were prevented to a large extent by ectopic expression of AKT.[78] Remarkably, an inverse relationship between PTEN and $Ca_v3.1$ expression was recently reported in melanoma cells,[53] indicating that the AKT pathway promotes the transcription of these channels. Together, TTCC-mediated AKT phosphorylation and the repression of TTCC expression by PTEN activity indicate a feedback relationship between TTCCs and the AKT pathway that could have a significant role in melanoma progression and therapeutic resistance, akin to the relationship between TTCCs and the MAPK pathway.[55]

TTCC blocker mibefradil activates the p53–p21 pathway to induce cell cycle arrest and apoptosis

The assembly of the tumor-suppressor p53 transcription factor into functional oligomers, as well as its activation and stability, can be regulated positively or negatively by local Ca^{2+} elevations activating CaM-CaMKII[79–80] or members of the S100 family of EF-hand Ca^{2+}-binding proteins.[81–84] Evidence for the involvement of TTCCs in the p53 pathway is pharmacological: treatment with 3 μM mibefradil or $Ca_v3.1$ knockdown reduced the proliferation of several esophageal cancer cell lines.[85] In this work, it was concluded that mibefradil reduced cell proliferation via p53-dependent upregulation of cyclin-dependent kinase inhibitor p21, a protein that inhibits the activity of cyclin-CDK1, -CDK2, and -CDK4/6 complexes, thus halting G1–S progression.[86] In addition, 10 μM mibefradil was reported to induce cell cycle arrest and apoptosis in colon cancer cells through p38 MAPK phosphorylation of p53,[61] a pathway the activation of which is more compatible with cytosolic Ca^{2+} rise rather than with Ca^{2+} channel block (Fig. 1).

TTCCs and the calcineurin/NFAT pathway: A relationship demonstrated in different cell types but unknown in cancer cells

Downstream effectors of CaM other than the CaMK cascade include serine/threonine phosphatase CaN[31] and nitric oxide synthase (NOS).[87] The best-characterized CaN substrates are the four NFAT Ca^{2+}-responsive isoforms (NFAT1–NFAT4). Ca^{2+} influxes through L-type channels (LTCCs),[88–90] transient receptor potential channels (TRP),[90] and TTCCs[91] have all been proposed to induce cardiac hypertrophy via CaN/NFAT or CaMK/histone deacetylase (HDAC) circuits. In contrast to LTCCs or TRP channels, the expression of TTCCs in adult hearts is restricted to pacemaker cells, but widens to cardiomyocytes upon cardiovascular disease or injury.[92–93] It has been long known that TTCCs are upregulated in experimental models of cardiac hypertrophy,[94–95] and that remodeling of excitation–contraction coupling of hypertrophied ventricular myocytes depends on TTCC expression.[96] Accordingly, the positive chronotropic effect of aldosterone (a hypertrophic stimulus with a relevant role in cardiac pathophysiology) was linked to increased expression of $Ca_v3.2$[97] and of both $Ca_v3.1$ and $Ca_v3.2$ in neonatal ventricular cardiomyocytes.[39] However, TTCC isoforms appear to have opposite roles in cardiac hypertrophy, which is antagonized by $Ca_v3.1$ via activation of NOS3-cGMP-dependent protein kinase type I (PKG1),[98] and promoted by $Ca_v3.2$ via activation of CaN.[91] Huang *et al.* shed light on a direct but complex interaction between $Ca_v3.2$ and CaN in neonatal cardiomyocytes.[99] According to their work, $Ca_v3.2$ channels regulate the CaN/NFAT pathway by both sustaining Ca^{2+} influx and direct binding to the NFAT-binding domain of CaN. This regulation is double edged: at sub-mM Ca^{2+} concentrations, the cross-regulation is reciprocally inhibitory; by contrast, at supra-mM Ca^{2+} concentrations likely to be achieved in caveolae microdomains, CaN dissociates from $Ca_v3.2$ and dephosphorylates NFAT, resulting in NFAT nuclear translocation and downstream gene activation. This model is supported by the colocalization of caveolin and $Ca_v3.2$,[100] and explains how activation of the CaN/NFAT pathway would be prevented by systolic Ca^{2+} oscillations occurring outside the designed signaling platforms. The above studies posit that is possible to modulate cardiac hypertrophy through the modulation of TTCC expression or function. Indeed, Horiba *et al.* demonstrated that TTCC pharmacological block with scorpion peptide kurtoxin or the dihydropyridines efonidipine and nisoldipine prevents CaN/NFAT3 activation and limits the hypertrophic growth of mouse ventricular myocytes.[101] Recent work showed that increased TTCC expression can also be the cause of ethanol-induced atrial fibrillation, which can be prevented to a large extent by kurtoxin or mibefradil administration to binge-drinking rats.[102] At the cellular level, acute exposure of pulmonary vein cardiomyocytes (PVCs) to ethanol activated the CaN/NFAT/cardiac transcription factor Csx pathway by PKC-mediated inhibition of glycogen synthesis kinase 3β. In turn, Csx/Nkx2.5 upregulated $Ca_v3.1$ and $Ca_v3.2$ channels and enhanced PVC automaticity. This finding suggests that NFAT-dependent transcriptional upregulation of TTCCs represents a fourth positive feedback between TTCC-mediated Ca^{2+} entry and cell-fate determinant signaling pathways. In a different setting, Lin *et al.* provided convincing evidence for a role of $Ca_v3.2$ channels in promoting CaN/NFAT/Sox9-dependent tracheal chondrogenesis, based on ectopic expression of recombinant channels, use of $Ca_v3.2$-knockout mice and pharmacological block of TTCCs with 5 μM NNC-55-0396.[103] The $Ca_v3.1$ isoform has a part in NFAT activation in a mouse model of experimental autoimmune encephalomyelitis: T helper cells from $Ca_v3.1$-knockout mice showed reduced nuclear translocation of NFAT and reduced secretion of NFAT-dependent cytokines, resulting in a milder phenotype.[104]

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Apart from controlling cell growth and differentiation, the NFAT transcription factor family regulates diverse cellular functions involved in cancer progression, such as cell survival, proliferation, migration, and angiogenesis.[105–106] Two classical inhibitors of CaN widely used as immunosupressors, cyclosporin A and tacrolimus, have shown anticancer activity *in vitro* and *in vivo*.[107–109] However, their long-term use in autoimmune conditions or to prevent transplant graft rejection has been associated with an increased risk of malignancy.[110–112] These compounds inhibit all NFAT isoforms and other CaN substrates and, thus, tumor- or isoform-specific NFAT inhibition is postulated to be a more efficient chemotherapeutic approach. Hence, investigating whether TTCC blockade can have inhibitory activities on specific NFAT isoforms and NFAT-mediated cell proliferation or invasion, appears a well-founded proposal.

The $Ca_v3.1$/PP2A/CREB axis opposes cardiac hypertrophy and could oppose tumorigenicity

PP2A and, to a lesser extent, phosphatase 1 (PP1) are other Ca^{2+}-dependent serine/threonine phosphatases that might be activated by Ca^{2+} influx through TTCCs (Table 2). Among the targets of PP2A are proteins of oncogenic signaling cascades, such as RAF, MEK, and AKT, in which PP2A might act as a tumor suppressor, and also the CREB transcription factor.[113] As for CaN, most data on PP2A are from *in vitro* models of cardiac hypertrophy. Ferron *et al.* observed that application of aldosterone on rat neonatal cardiac myocytes increased the expression of TTCCs (principally $Ca_v3.1$) and induced PP2A/PP1 activity, which was associated with higher levels of proapoptotic markers. In addition, mibefradil at 1 μM decreased PP2A activity and PP2A-induced CREB dephosphorylation, reducing aldosterone-dependent cell death. These results were confirmed in a long-term stenosis-induced cardiac hypertrophy model, in which the re-expression of TTCCs was associated with the reduction of CREB-mediated gene expression and increase of apoptotic markers.[39] In conclusion, the referred work identified the $Ca_v3.1$/PP2A/CREB pathway as an alternative route to $Ca_v3.1$/NOS3/PKG1 for limiting cardiac hypertrophy, thus reinforcing the observation that highly homologous TTCC isoforms might have opposite roles in cardiac pathophysiology.

Decreased activity of the PP2A trimeric complex through phosphorylation or methylation of its catalytic subunit is a recurrent observation in many types of cancer.[114] In agreement, CREB is overexpressed and/or overactivated in different tumor types,[115] and its persistent phosphorylation has been associated with the development of melanoma resistance to RAF, MEK, and ERK inhibitors.[116] The therapeutic potential of small-molecule activators of PP2A has been investigated in lung adenocarcinoma cells, which showed reduced viability and resistance to tyrosine kinase, MEK, or AKT inhibitors.[117–119] In the same line of evidence, pharmacologically promiscuous metformin inhibited lung adenocarcinoma cell growth and invasion partially by activating PP2A.[120] However, other work points to a protumor role of PP2A in GBM, whereby PP2A activity mediated the dormancy of tumor stem-like cells exposed to hypoxic conditions and predicted poor patient survival.[121]

TTCC blockers inhibit calpains

There is indirect evidence for an involvement of calpains, Ca^{2+}-dependent cysteine proteases, in TTCC-mediated Ca^{2+} signaling. Sandmann *et al.* reported that long-term treatment with the LTCC blocker amlodipine attenuated calpain-I upregulation and reduced myocardial infarction, whereas mibefradil prevented calpain-II upregulation and decreased infarct size in a rat model of cardiac infarction.[122] In addition, mixed L/TTCC blockers (nifedipine, verapamil, and flunarizine, all at 50 μM) prevented calpain-mediated death of macrophages triggered by hypochlorous acid, a reactive oxygen species (ROS) generated at inflammation sites.[123] More recently, Zhu *et al.* linked TTCCs with fear memory through calpain-I activation. The authors determined that blocking TTCCs with 10 μM NNC-55-0396 suppressed the long-term depression elicited by metabotropic glutamate receptor activation in the CA1 layer of murine hippocampus, which is known to progress through the CaMKII/calpain-I/PP2A/AKT pathway.[124] Thus, although the activation of calpains by TTCCs has not been demonstrated by specific gene silencing, the reported actions of TTCC blockers on calpains are compatible with Ca^{2+} channel targeting (Fig. 1).

Targeting TTCCs as a means of inhibiting calpain might be of therapeutic interest. Calpains might promote tumor progression and chemotherapeutic resistance, and high expression of components of the calpain system can be considered negative prognostic markers in several types of cancer.[125]

How inhibition of TTCCs can affect macroautophagy

In melanoma and glioblastoma cells, the expression of $Ca_v3.1$ channels correlates with that of autophagosomal markers.[126–127] Furthermore, induction of HIF-1α[128–129] or HIF-2α[130] in hypoxic environments, well known to promote (macro)autophagy, upregulate $Ca_v3.2$ channels at the transcriptional level. Thus, the expression of TTCCs are somehow tied to autophagy, although additional evidence is required to elucidate the nature of this relationship at the molecular and functional levels.

The involvement of TTCCs in multiple signaling pathways involving mTORC1, a nodal hub for controlling the balance of anabolic and catabolic processes, also suggests these proteins as targets for autophagy deregulation, as already been demonstrated in cardiomyocytes[131] and cancer cells.[127–133] A paradigmatic example is that of the AKT pathway. $Ca_v3.1$ transcripts are reportedly upregulated in melanoma cells with an exacerbated AKT pathway because of loss-of-function mutations in PTEN. By reducing AKT phosphorylation, TTCC gene silencing or pharmacological block are expected to enhance autophagy through mTORC1 inhibition, as shown for AKT small-molecule inhibitors.[134–135] In addition, coincident actions of mibefradil and $Ca_v3.1$ gene silencing suggest that Ca^{2+} entry through TTCCs activates CaMKK2. This connection could have broad implications for cancer cell physiology, because CaMKK2 and mTORC2 are involved in the promotion of autophagy via mTORC1-dependent and independent mechanisms.[136–138]

Often, the precise contribution of TTCCs to Ca^{2+}-dependent pathways and their intricate relationships with autophagy remain unclear. $Ca_v3.1$ channel transcripts are increased in BRAF- and NRAS-mutant cells, which display an overactive MAPK pathway,

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and further increase in mutant cells resistant to MAPK inhibitors.[55] The dependency of BRAF- and RAS-mutant cells on autophagy for survival within the tumor microenvironment, and their heightened susceptibility to autophagy inhibitors, are well documented.[139–140] Yet, we have only a few glimpses of the mechanisms of autophagy induction in these cells, overcoming the hyperactivation of antiautophagic mTORC1. Activation of the MAPK pathway can promote autophagy in bladder cancer cells by ERK1/2-mediated inhibition of Beclin-1 negative regulators, thus facilitating the formation of the autophagosome initiation complex.[141] In addition, ERK1/2-dependent phosphorylation of Gα-interacting protein has been shown to induce autophagy in colon cancer cells.[142] Over the longer term, ERK1/2-dependent phosphorylation of CREB[143] could also potentiate autophagy by induction of autophagy-related genes (Atgs).[144–145] However, after the assumption that the balance is tipped toward autophagy induction, it remains to be clarified whether selective targeting of $Ca_v3.1$ inhibits or activates ERK1/2 (Table 2).

From the perspective of other signaling pathways, the consequences of TTCC block on autophagy are also a conundrum because of the different impact of pathway ramifications on the catabolic process. The p38 MAPK pathway has a dual role as a positive or negative regulator of autophagy by activating different downstream targets depending on the nature, strength, and duration of the stimuli.[146] Adding to the confusion, data indicate that, whereas TTCC gene knockdown prevents p38 MAPK activation, the pharmacological blocker mibefradil exerts the opposite effect.

TTCC-mediated activation of Ca^{2+}-dependent phosphatases also alters the autophagic status of cells. CaN phosphatase might induce autophagy by dephosphorylating transcription factor EB, enabling its nuclear translocation and the induction of lysosomal biogenesis and autophagy-related proteins[147] upon lysosomal Ca^{2+} release.[148] However, TTCC-mediated activation of CaN has only been related to dephosphorylation of NFAT, the nuclear action of which is eminently prohypertrophic in differ-

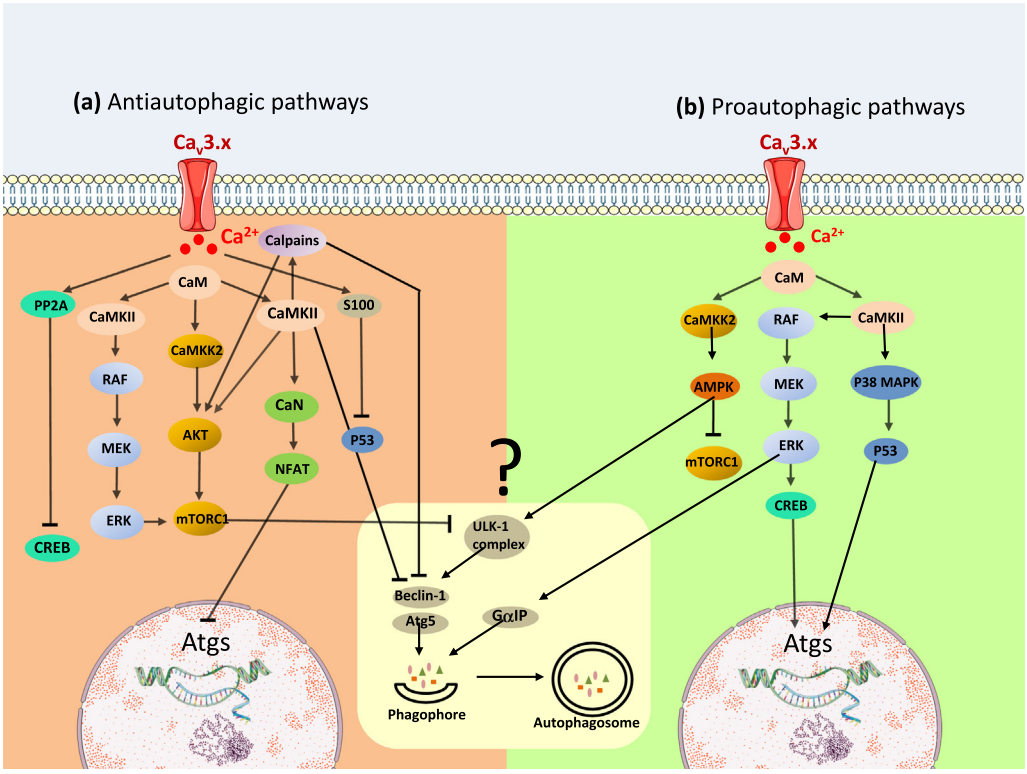

FIGURE 2

T-type calcium channel (TTCC)-mediated Ca^{2+} entry might activate antiautophagic and proautophagic signaling downstream of calmodulin (CaM). **(a)** Transcription of Atg autophagy-related genes (Atgs) (encoding the molecular machinery responsible for autophagy initiation and elongation) is repressed by nuclear factor of activated T cells (NFAT). In addition, activation of mammalian target of rapamycin complex 1 (mTORC1) by extracellular signal-regulated kinase (ERK) and serine/threonine protein kinase B (AKT) pathways inhibits the Unc-51-like autophagy activating kinase 1 (ULK-1) complex, the kinase activity of which activates the autophagy initiator Beclin-1/Vps34 complex, essential for phagophore formation. Other negative inputs come from activation of calpains 1/2, which might promote degradation of Beclin-1 and Atg5, a protein required for phagophore elongation. **(b)** ERK1/2-dependent phosphorylation of cAMP-responsive element-binding (CREB) promotes its nuclear translocation and subsequent activation of Atgs. Similarly, p38 MAPK-dependent nuclear translocation of p53 promotes the expression of Atgs. Other proautophagic effects are post translational: AMP kinase (AMPK) inhibits mTORC1 and directly activates ULK-1, whereas GαIP phosphorylation by ERK has been shown to induce autophagic sequestration. On-target effects of TTCC block are expected to promote autophagy by inhibiting pathways in (a) and to inhibit autophagy by inhibiting pathways in (b). The figure combines a selection of relationships identified by inverse reasoning in different studies. Some effectors and relationships have been omitted for clarity. Arrow tips indicate whether the modulation is positive (pointed) or negative (blunt-end).

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entiated muscle cells[149] and proproliferative in cancer cells.[106] By contrast, activation of PP2A phosphatase has unequivocal proautophagic effects through activation of proteins involved in the Unc-51-like autophagy activating kinase 1 complex.[150–151] Accordingly, PP2A inhibition or gene silencing resulted in autophagy inhibition in cortical neurons.[152] Again, however, the relationship of the pathway with autophagy can be double-sided: PP2A-mediated dephosphorylation of CREB, which promotes the expression of Atgs,[144–145] is predicted to impair the autophagic flux. TTCCs might also regulate autophagy through the activation of Ca^{2+}-dependent proteases. Calpains can negatively adjust autophagy by cleaving autophagy-related proteins, such as Beclin-1[153] or Atg5.[154] In addition, activation of calpains can lead to activation of the AKT pathway.[124,155] Judged by their ability to inhibit calpain-I and II, TTCC blockers are thus expected to enhance autophagy. Collectively, inhibition of local Ca^{2+} signaling downstream of TTCCs might exert pro- and antiautophagic effects at the transcriptional and post-translational levels, with unpredictable outcomes (Fig. 2).

TTCC pharmacological blockers can also deregulate autophagy through actions other than altering classical Ca^{2+} signaling cascades and, in some cases, rarely owing to TTCC targeting. At a 6 μM concentration, KYS05090, a 3,4-dihydroquinazoline with TTCC-blocking properties, induced autophagy and apoptosis in lung carcinoma A549 cells through generation of ROS and inhibition of glucose uptake.[133] ROS can induce macroautophagy at different levels and this interplay can promote tumor progression and survival of cancer cells during oxidative or metabolic stresses.[156] Together with other causal factors, mitochondrial Ca^{2+} overload by transfer from the ER through Ca^{2+}-dependent ryanodine (RyRs) or inositol triphosphate (IP_3Rs) receptors can increase the respiratory chain ROS production, which, in turn, can feed back on Ca^{2+} transfer from the ER to mitochondria.[157,158] Among the plasma membrane channels able to con-

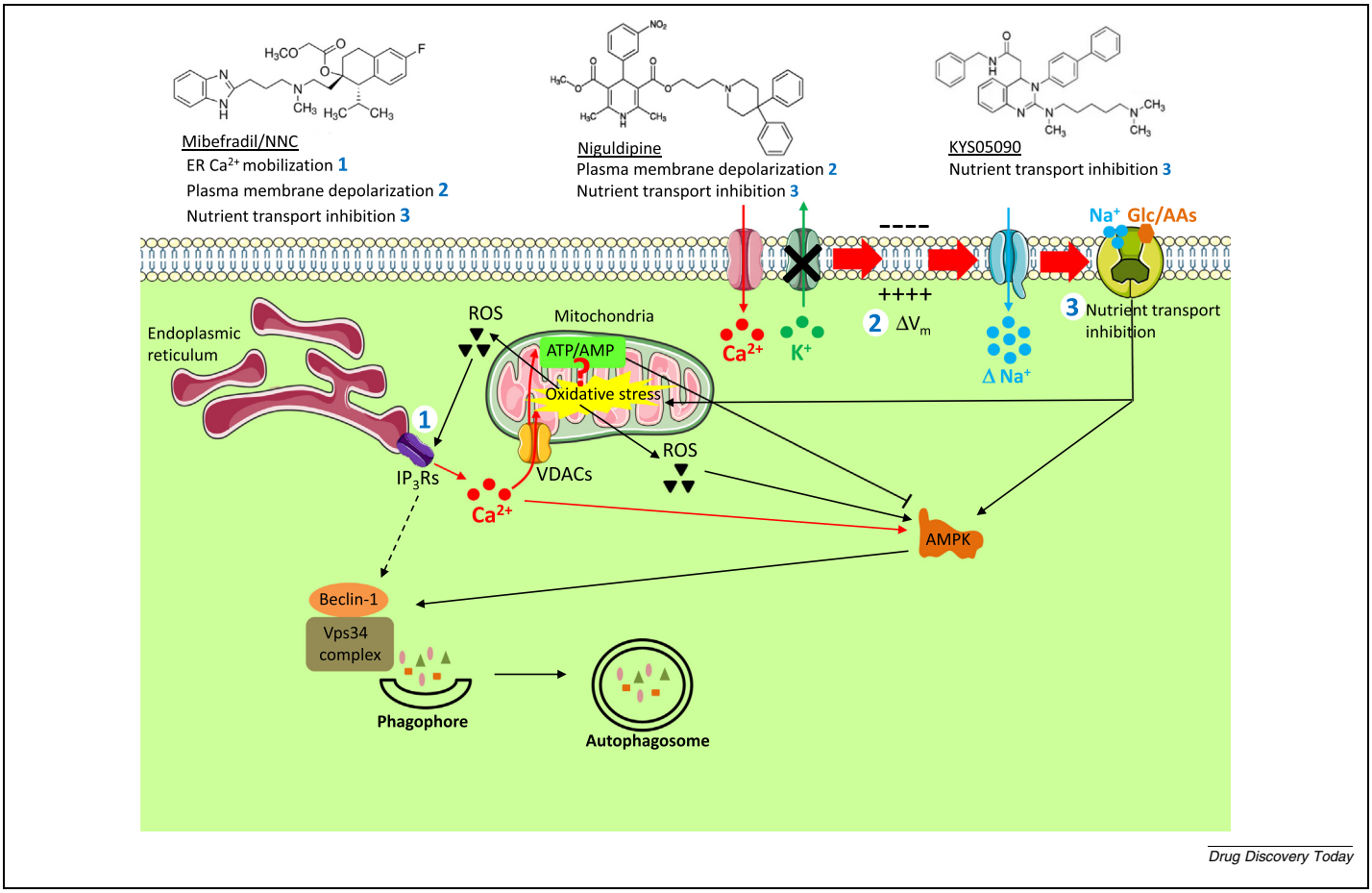

FIGURE 3

Off-target actions of T-type calcium channel (TTCC) blockers cytotoxic to cancer cells modulate autophagy initiation by different mechanisms. First, the tetralol derivatives mibefradil and NNC-55-0396 induce Ca^{2+} mobilization from the endoplasmic reticulum (ER) through inositol triphosphate receptors (IP_3Rs). In turn, activation of IP_3Rs might promote autophagy dually by releasing Beclin-1 and favoring the assembly of the Vps34 initiation complex, and by activating AMP kinase (AMPK) via Ca2 + -calmodulin-dependent protein kinase kinase 2 (CaMKK2). In addition, IP_3Rs can interact with voltage-dependent anion channels (VDACs) to fuel Ca^{2+} to mitochondria, with two possible effects, which might depend on stimuli duration/intensity: (i) increased activity of the tricarboxylic acid cycle and ATP/AMP ratio, inhibiting AMPK and autophagy initiation; and (ii) production of reactive oxygen species (ROS), which might exacerbate mitochondrial Ca^{2+} overload and activate AMPK. Second, tetralol derivatives and dihydropyridine niguldipine have been reported as membrane-depolarizing agents by as-yet unclear mechanisms, which could imply activation of Ca^{2+} channels and/or inhibition of K^+ channels. As a result of plasma membrane depolarization, both compounds have been shown to increase Na^+ entry through voltage-gated channels, in turn negatively affecting the function of Na^+-glutamate symporter and promoting the amino acid response, a known AMPK activator. Third, 3,4 dihydroquinazoline KYS05090 is reportedly an autophagy promoter by increasing ROS production and reducing glucose uptake, both activating AMPK and inducing macroautophagy.

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vey Ca^{2+} ions to RyRs are TTCCs,[8,159] the activity of which would then support autophagy through ROS generation. Thus, the effects of KYS05090 on ROS and autophagy reported by Rim *et al.* are consistent with TTCC enhancement or with an unidentified off-target action. More recently, Niklasson *et al.* screened a library of ion channel blockers on glioblastoma-initiating cells (GICs) and found that 10 μM concentrations of mibefradil and dihydropyridine niguldipine shared the ability with penitrem A, a mycotoxin that potently blocks high-conductance KCa channels, to induce cell death.[160] GIC death was preceded by increased cytosolic Na^+ concentration and reduction of Na^+-dependent transport, leading to nutrient starvation and activation of the related amino acid and unfolded protein responses. This sequence of events was initiated by membrane depolarization, which can be induced by KCa channel block or KCa inhibition through TTCC block, provided that both channels are expressed and open at sufficiently high levels to pose a significant contribution to the resting membrane potential. Of note, membrane depolarization by mibefradil and niguldipine can also be the consequence of actions reported on other types of K^+ channels[161–164] and TrpM7 channels[165] (Table 1). Although the autophagic status of GICs was not subject of this study, enhancement of macroautophagy by these drugs is a foreseeable outcome by virtue of its mechanistic links to the above-mentioned stress responses.[166–168] As another obvious off-target effect, tetralol derivatives have been shown to promote ER Ca^{2+} release in different cells by activation of IP_3Rs.[169,170] Ca^{2+} mobilization from the intracellular stores has been related to autophagy induction via activation of the CaMKK2/AMPK axis and via release of Beclin-1 upon IP_3R activation, both events favoring the assembly of the initiator complex Vps34.[171] Nonetheless, IP_3R activation might also inhibit basal autophagy by supplying mitochondria with Ca^{2+} necessary for the tricarboxylic acid cycle and subsequent ATP synthesis.[172,173] Moreover, it has been suggested that cancer cell bioenergetics would be dependent on ER-mitochondria Ca^{2+} fueling through enhanced activity or expression of IP_3R2 and IP_3R3 isoforms in growing tumors.[171,174] Off-target effects of TTCC blockers with repercussions on autophagy are summarized in Fig. 3.

Finally, only a few studies have analyzed the effect of TTCC gene knockdown on autophagy flux. It was reported that $Ca_v3.1$ or $Ca_v3.2$ knockdown in melanoma cell lines inhibited macroautophagy, based on the observation of increased LC3-II and P62/SQSTM1 protein levels.[132] By contrast, $Ca_v3.1$ knockdown led to reduced P62/SQSTM1 protein and mRNA levels in GBM cells, resulting in increased polyubiquitinated aggregates.[127] Downregulation of P62/SQSTM1 was shown to induce cargo-loading failure in myeloma cells, leading to accumulation of proapoptotic NBK/Bik proteins, which could be relieved by inhibition of autophagy initiation.[175] These results corroborate that TTCCs are necessary for the correct development of autophagy and additionally point to different roles of $Ca_v3.1$ and $Ca_v3.2$ in the process.

Together, the off- and on-target actions of TTCC blockers on multiple pathways are destined to exert both pro- and antiautophagic effects, such that the net outcome would be the promotion of defective autophagy, with catastrophic consequences for cell viability.

Concluding remarks

TTCCs engage in positive feedback loops with signaling cascades important for cell proliferation and cell fate, such as MAPKs, AKT/mTORC1, and CaN/NFAT. Establishment of these loops might warrant a switch-like activation of the pathways despite the low availability of TTCCs at the steady membrane potentials of nonexcitable cells.

Given the central role of TTCCs in Ca^{2+} signaling, a conspicuous question is the effectiveness and specificity of pharmacological targeting of TTCCs in the context of diseases characterized by their augmented expression or activity. Most available blockers display a preferential affinity for the nonconducting states of the channels. This feature is understood to help the intended activity-dependent block in hyperexcitable conditions, such as cardiac arrhythmias, epilepsies, or neuropathic pain. However, cytotoxicity of these compounds in cancer cells has only been reported at concentrations 5–40-fold higher than the reported half-maximal inhibitory concentrations for TTCCs calculated in electrophysiological experiments. Thus, the effects of pharmacological blockers used at high concentrations on cancer cells should be a mixture of on-target and off-target actions. Here, we compared the effects reported for TTCC blockers with the expected effects of Ca^{2+} channel block and preclusion of Ca^{2+} entry. Their pharmacological action has been studied in parallel to overexpression or gene-silencing studies in only in a few cases, a comparison bound to confirm the specificity of pharmacological block.

Globally, these data indicate that TTCCs are formidable chemotherapeutic targets: as main transducers of electrical into chemical signals in nonexcitable membranes, their activity can trigger several biochemical pathways and cellular mechanisms that determine cell fate and metabolism. Mounting evidence indicates a role for TTCCs in the activation of protumoral AKT, NFAT, and calpain pathways, and the inhibitory actions of existing TTCC blockers on these pathways are in line with TTCC block and the achievement of desired chemotherapeutic effects. However, available data also show that TTCC blockers inhibit antitumoral PP2A, and that their effects on the cancer-relevant MAPK and p38 MAPK pathways are essentially off-target. Given that all these signaling routes can have different levels of activity throughout the cell cycle, the net effect of TTCC blockers applied at toxic concentrations is unpredictable. Whereas toxicity of these compounds on 2D cancer cell cultures is overwhelming, one concern is the lack of knowledge of their long-term effects on *in vivo*-growing tumors, particularly from the viewpoint of development of resistance mechanisms. In conclusion, knowing what is on- and what is off-target becomes essential to harness the full potential of pharmacological compounds with the ability to modulate TTCCs, which, because of their voltage-dependence, have a counter-intuitive yet crucial role in cancer cell physiology.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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