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Targeting Ion Channel TRPM4

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Abstract: The transient receptor potential melastatin 4 (TRPM4) ion channel is ubiquitously expressed. Dysregulation and/or functional mutations of TRPM4 lead to several diseases. Within our studies, we screened for TRPM4 inhibitors and identified small molecules that block TRPM4 in the low µM range. Furthermore, we investigated the pathophysiology of TRPM4 in cardiac conditions, immune diseases and cancer using these novel inhibitors, molecular biology techniques and functional assays.

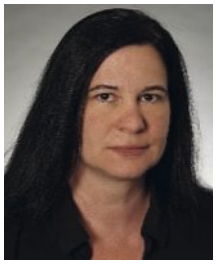
Keywords: Cancer · Cardiac disease · TRPM4 · Small-molecule inhibitors

Barbara Preti obtained her PhD from the University of Bern in 2022. She investigated the role of GPCRs and ion channels involved in neurodegenerative disorders, chronic pain, cardiac conduction alterations and cancer, with a focus on the adenosine A1 receptor and TRPM4 cation channel. Currently, she is Study Manager at Roche where she monitors and oversees *in vitro* ADME studies conducted at CROs in close collaboration with Roche.

Jean-Sébastien Rougier is interested in the regulation of cardiac ion channels. He obtained a PhD from the University of Lausanne in 2005 followed by two post-doctoral formations; one at the University College of London and one at the University of Bern. Since 2018 he is a lecturer at the Institute of Biochemistry and Molecular Medicine (IBMM) at the University of Bern.

Hugues Abriel focuses on channelopathies. He received an MD and a PhD from the University of Lausanne (1995). After two years at Columbia University in New York, he established his research group as SNSF-Professor (University of Lausanne). He is a full professor at the University of Bern and served as Director of the Department of Clinical Research, as Co-Director of the IBMM and at the SNSF research council (President). Since 2015, he is the Director of NCCR TransCure. Currently, he serves as Vice-Rector of Research at the University of Bern.

Martin Lochner and his lab synthesize pharmacological tool compounds and bioactive probes to investigate the function of ion channels, receptors and transport proteins. He obtained his PhD from the University of Basel (2003). After postdocs in total synthesis and ion channel biochemistry at the University of Cambridge, he joined the Chemistry Department of the University of Warwick in 2006 to establish his independent research career. He moved to the Department of Chemistry and Biochemistry of the University of Bern as an SNSF-Professor and NCCR TransCure PI (2010). Since 2016, he is a lecturer and research group leader at the IBMM.



Christine Peinelt works on the pathophysiology of ion channels. She received a PhD from Konstanz University in 2004. After a postdoctoral research stay at the Queen's Medical Center in Honolulu, she established her independent research group at the Institute of Biophysics at the Saarland University as a junior professor and received an Emmy-Noether grant. Since 2016, she is a full professor at the IBMM and a PI in the NCCR TransCure. Since 2017, she serves as Co-Director of Teaching at the IBMM, since 2018 in NCCR TransCure Management Committee and since 2021 as President of Life Science Switzerland Physiology.

1. Ion channel TRPM4

The transient receptor potential melastatin 4 (TRPM4) ion channel is a Ca^{2+}-activated channel that conducts monovalent ions into the cell. It is ubiquitously expressed and most prominent in the intestines, the prostate, the heart, the skin and immune cells.[1,2] TRPM4 is a homotetramer with a large cytosolic domain. TRPM4 monomers are characterized by the cytosolic N-terminal nucleotide-binding domain, and the C-terminal coiled coils add to the tetrameric assembly of the channel around a central ion-conducting pore. Ca^{2+} binding sites are located close to the cytosolic side of the membrane.[3–6] Numerous physiological and pathophysiological cellular functions have been reported for TRPM4 in immune, neuronal, cardiac and cancer cells.[7–10] However, the pharmacological toolbox for TRPM4 was limited previously.[11] In our project, we successfully screened for novel small-molecule inhibitors targeting TRPM4 in the low µM and high nM range. In addition, with these inhibitors, we assessed the pathophysiological functions of TRPM4 using biochemical and molecular biology approaches, and in cellular and whole organ assays.

1.1 *Discovering better TRPM4 Inhibitors*

For a long time, the TRPM4 field has been plagued by low potency and low selectivity tool compounds to study the cellular

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function and role of this ion channel in physiology and pathophysiology. The most commonly used TRPM4 inhibitor is 9-phenanthrol, which exhibits low activity (IC_{50} = 29 μM, Na^+ influx assay). Its additional lack of target selectivity and suboptimal physicochemical properties render it unsuitable for animal studies or studies in primary cell lines.[12–14] Despite these limitations, 9-phenanthrol has been used in numerous experiments to dissect the role of TRPM4 in diseases.[15,16] Other, weaker TRPM4 inhibitors, such as flufenamic acid and glibenclamide, have also been reported, but they have both well-documented off-target effects (flufenamic acid)[17] or other primary targets (glibenclamide).[18,19]

As there were no TRPM4 structures published at the outset of our project and very little to no information available with regards to the location of the modulator binding site(s), we decided to pursue a ligand-based virtual screening (LBVS) approach,[20] using the three weak TRPM4 inhibitors 9-phenanthrol, flufenamic acid and glibenclamide as input structures, to discover similar ligands in commercially available compound databases. LBVS was conducted using an in-house developed atom category eXtended Ligand Overlap Score (xLOS) method, which computes and ranks the 3D shape and pharmacophore similarity between input and database compounds.[21] A selection of these LBVS hit structures was purchased and assessed utilizing a fluorescence-based Na^+ influx assay. This *in vitro* assay was developed as part of our TRPM4 compound screening campaign and set up on a FLIPR (Fluorescent Imaging Plate Reader) platform, using a cell line stably expressing TRPM4, loaded with an intracellular Na^+-specific dye (Asante Natrium Green-II, ANG-II). Validation of the Na^+ influx assay showed that it could reliably measure the modulation activity of small molecules on TRPM4 function.[21] Activity evaluation of the LBVS hits uncovered chloroanthranilic amides **LBA** (IC_{50} = 1.6 μM) and **CBA** (IC_{50} = 1.5 μM) as almost 20-fold more potent TRPM4 inhibitors compared to 9-phenanthrol (Fig. 1).

This was followed by an extended structure–activity relationship (SAR) study, including synthetic modification of several positions and moieties, and bioisosteric replacement of functional groups of **LBA** and **CBA**. The SAR study yielded naphthyl analogue **NBA** as first sub-micromolar inhibitor of TRPM4 function (IC_{50} = 0.4 μM Na^+ influx assay, IC_{50} = 0.2 μM patch-clamp electrophysiology).[21]

Further biological evaluation of **CBA** by patch-clamp electrophysiology recordings on TRPM4 overexpressing HEK293 cell lines showed that it selectively inhibits TRPM4 over other TRP family members, including TRPM5, TRPM7, TRPM8, TRPV1 and TRPV6. Remarkably, at 10 μM concentration, CBA did not affect 17 different ion channels and membrane receptors, including $GABA_A$ and NMDA receptors, voltage-gated Ca^{2+} and K^+ channels, and showed <5% inhibition of dofetilide binding to the cardiac anti-target hERG (K_v 11.1 channel).

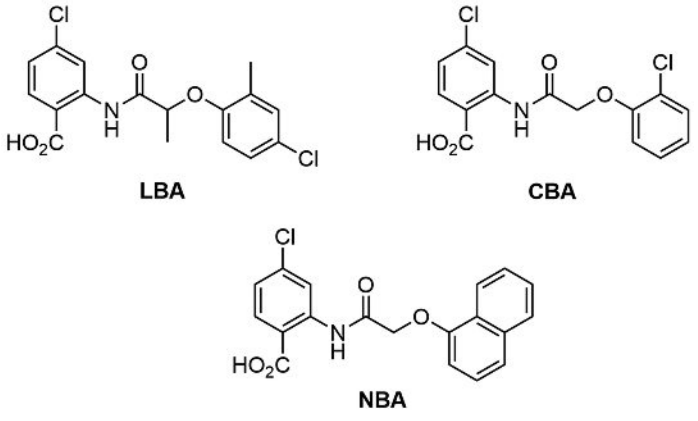


Fig. 1. Structures of TRPM4 inhibitors **LBA**, **CBA** and **NBA**.

Finally, we demonstrated that **CBA** can inhibit endogenous TRPM4 currents in prostate cancer LNCaP cells (*vide infra*). More interestingly, **CBA** showed restoration of functional expression of the A432T TRPM4 mutant, a loss-of-expression variant found in cardiac AVB patients.[22]

A second, more focused synthetic SAR study is currently ongoing on the **CBA** scaffold and has already generated congeners that are more potent TRPM4 inhibitors than **NBA**. These findings will be reported in due course.

1.2 *High-throughput Compound Screening*

Whilst we were synthetically optimizing the chloroanthranilic amides (**LBA**, **CBA**, **NBA**), we experienced some limitations with these compounds in cancer hallmark assays when using certain prostate cancer cell lines (*vide infra*). Therefore, we sought possibilities to identify novel TRPM4 modulator scaffolds.

In collaboration with the FAST (Facilitated Access to Screening Technologies) Lab at Novartis Pharma AG (Basel, Switzerland), we decided to undertake a high-throughput compound screen to identify novel TRPM4 modulator chemotypes. This open resource allows screening of up to *ca*. 50'000 non-proprietary drug-like compounds at the FAST Lab FLIPR-based high-throughput screening facility.

The first goal was to adapt our in-house TRPM4 Na^+ influx assay to a 384-well format and optimize the assay conditions to screen as many compounds as possible in a short period of time. This assay optimization included, for instance, the adjustment of ionomycin concentration in the stimulus buffer, the stimulus buffer salt composition and the concentration of Ca^{2+} in the sodium-free buffer in order to achieve the best signal-to-noise ratio. Ionomycin induces the influx of external Ca^{2+} into the cell, causing an increase in $[Ca^{2+}]_i$, which in turn activates TRPM4. Due to availability and performance issues, the Na^+-selective dye used previously was changed to membrane-permeable ION Natrium Green (ING-II AM) intracellular Na^+ fluorescent indicator.

After having optimized the TRPM4 screening assay for a high-throughput format, we tested 9'825 compounds from the Novartis public library at 7 μM single concentration and found 134 compounds that showed a TRPM4 inhibitory activity of at least 30% (corresponding to a hit rate of 1.36%). For 129 of these primary hits full dose-inhibition curves were run at eight different concentrations, using the same high-throughput FLIPR assay format. Only 53 of these compounds showed dose-dependent inhibitory activity, seven displaying IC_{50} values lower than 1 μM. Careful analysis of the most active compounds revealed that a significant number produced a high baseline in the assay due to intrinsic fluorescence, or a small dose-response curve amplitude. These compounds were therefore not considered further. Fig. 2 shows the top ten TRPM4 inhibitors **1**–**10** discovered in the high-throughput screen, devoid of these issues.

Overall, the scaffolds of TRPM4 inhibitors **1**–**10** are very diverse. Compounds **4** and **6** are anthranilic amide derivatives, very similar to **LBA** and **CBA**, which we discovered in the LBVS (*vide supra*). What is more, compounds **2**, **5** and **10** might act as covalent inhibitors. Compound **10**, in particular, is a close analogue of the selenium-containing anti-inflammatory, anti-oxidant and cytoprotective drug ebselen. It has been shown that ebselen is a cysteine-reactive inhibitor (Scheme 1) of the *mycobacterium tuberculosis* transpeptidase Ldt_{Mt2} and of SARS-CoV-2 protease M^{pro}.[23,24]

Analysis of the human TRPM4 sequence and recent cryo-EM structures (PDB IDs 5WP6, 6BQR, 6BQV, 6BWI)[3,4,6] revealed that one TRPM4 subunit contains more than twenty free cysteines. Of particular note is a Cys-6aa-Cys-6aa-Cys motif (aa, amino acid) in helix S3, which is part of the S1-S4 voltage sensor domain of TRPM4. We have tested ebselen on TRPM4 but found no inhibitory activity. We are currently unable to explain

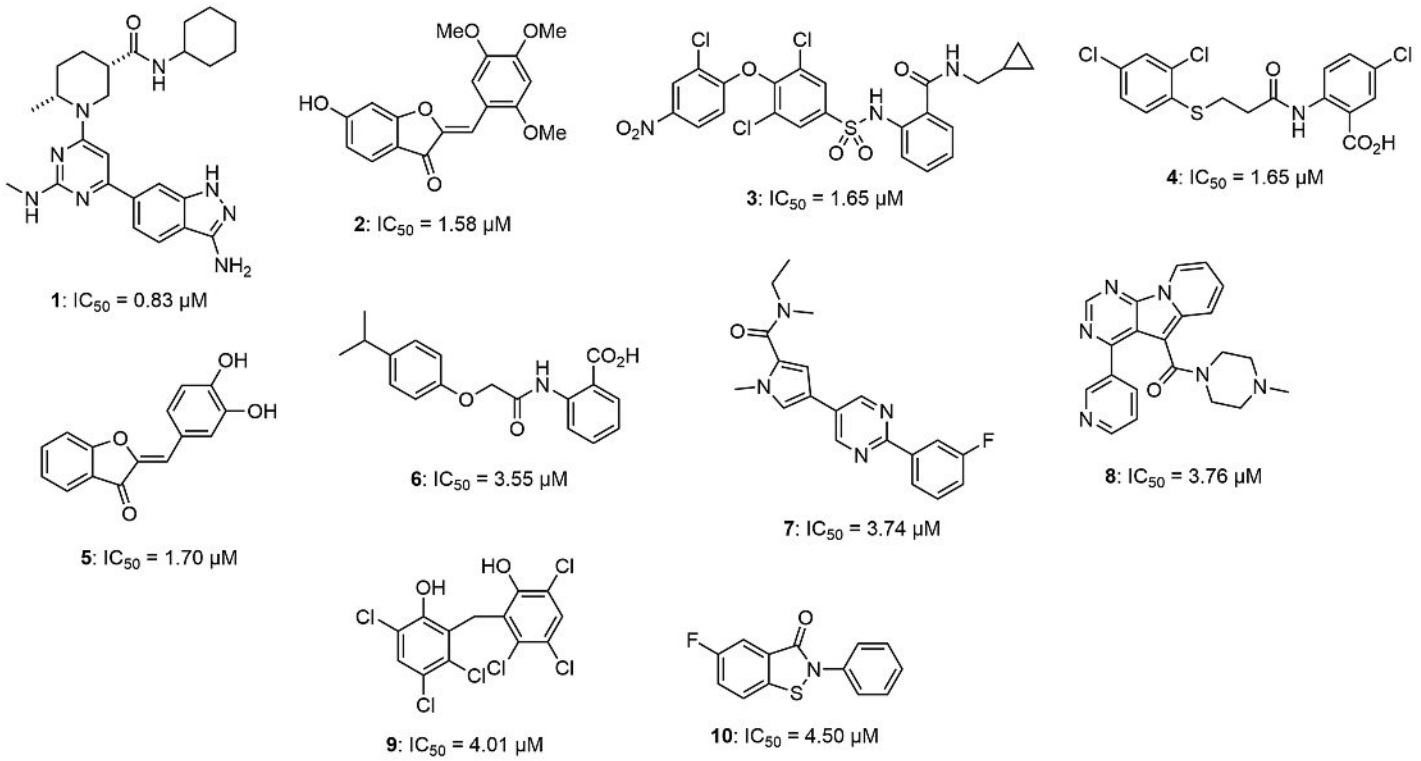
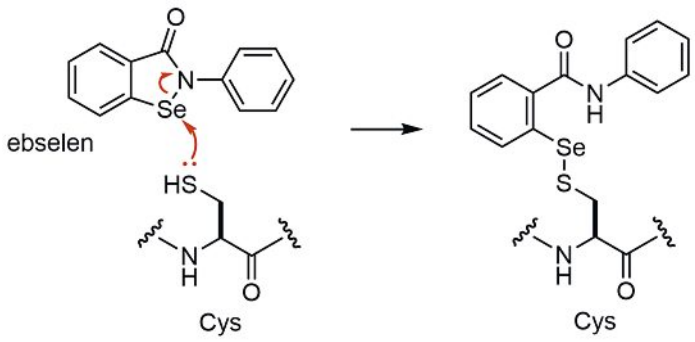


Fig. 2. Ten most potent TRPM4 inhibitor hits discovered in high-throughput screen of Novartis FAST Lab public library.



Scheme 1. Proposed covalent modification of cysteine residues by ebselen.

this result and assume that **10** blocks TRPM4 by a different, non-covalent mechanism.

2. TRPM4 Pathophysiology

2.1 *TRPM4 in the Heart*

In 2009, the first mutation of the human *TRPM4* gene has been linked to cardiac bundle branch block.[25] Since then, many clinical and experimental studies have linked *TRPM4* mutations to other conduction disorders such as Brugada syndrome, atrioventricular block, and right bundle branch block.[26–28] Moreover, TRPM4 has also been suggested to be a potential drug target in myocardial infarction due to its presence in non-cardiomyocyte populations (endothelial cells, immune cells, granulocytes, macrophages, and fibroblasts,) playing a key role in the genesis of inflammatory signaling pathways and scar tissue formation occurring during and after the infarct of the heart.[29,30] However, the role of TRPM4 channels in the proper electrical conduction of the heart remains unclear and still subject to debate.

Surprisingly, *TRPM4* mutants found in patients with cardiac disorders such as atrioventricular block or Brugada syndrome, when expressed in heterologous expression systems, were found to cause either a gain-of-function or a loss-of-function of TRPM4 channels.[10,27,31] A possible explanation of this intriguing observation may be related to the supernormal excitability and conduction mechanism, a phenomenon described in atrioventricular conduction.[32,33] It is proposed that TRPM4 activity may fine-tune the resting membrane potential of cardiac cells, which is a crucial parameter for the proper generation of cardiac action potentials. Any variation of the resting membrane potential will reduce the amount of available cardiac voltage-gated sodium channels ($Na_v1.5$) required to generate the cardiac action potential and its propagation through the myocardium. Knowing that under physiological conditions, the ion flux passing through the TRPM4 channel at the resting membrane potential leads to depolarization, TRPM4 gain-of-function mutants may shift the resting membrane potential to more positive values. Opposite to this mechanism, TRPM4 loss-of-function mutants would hyper-hyperpolarize the resting membrane potential. Overall, in both cases, the number of responding voltage-gated sodium channels dependent on TRPM4 activity would be drastically affected.

To address the question of TRPM4's role in cardiac conduction, we first took advantage of a new knock-out (KO) *Trpm4*$^{-/-}$ mouse model (Fig. 3).[34] The *Trpm4*$^{-/-}$ mice showed no increase in mortality or alteration of Mendelian genetic transmission. Investigating 12 weeks old male mice, no major alteration of the *in vivo* surface electrocardiogram was observed.[34] Surprisingly, resting membrane potential measurements performed on freshly isolated cardiomyocytes showed no alterations.[34] However, cardiac action potential recordings on isolated cardiomyocytes highlighted a significant decrease in the upstroke velocity, suggesting a reduction in sodium current mediated by $Na_v1.5$ channels, which may lead, under specific physiological conditions, to a decrease in cardiac excitability.[34] Sodium current density quantification using wild-type and *Trpm4*$^{-/-}$ cardiomyocytes confirms a reduction of 20% of this conductance without any major alteration of its main biophysical properties.[34] Although other studies using other *Trpm4*$^{-/-}$ mouse lines and study designs did not observe similar findings,[35,36] our observations raise two questions:

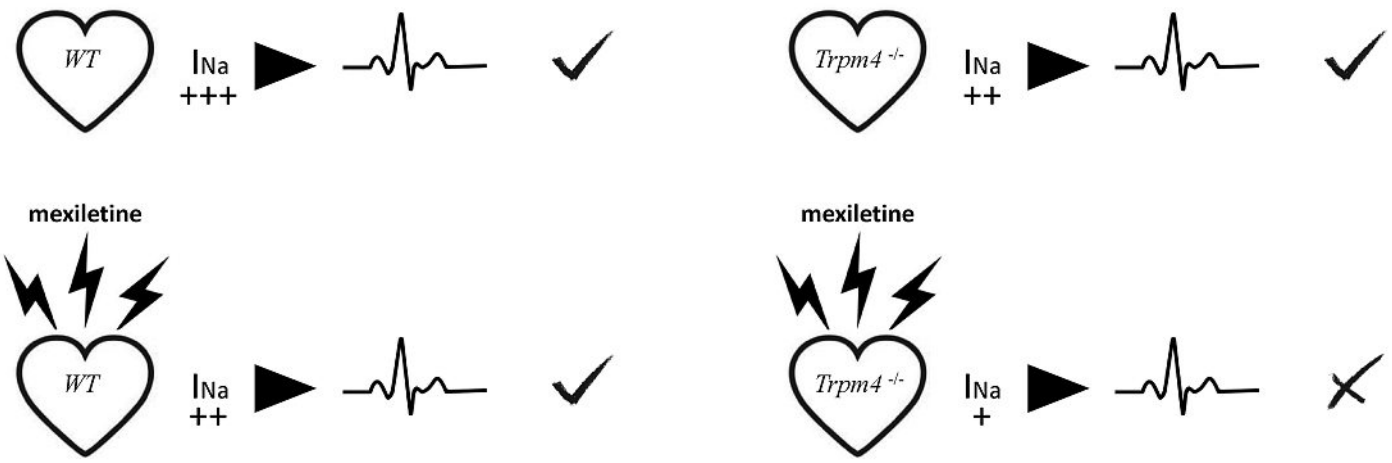


Fig. 3. Carton summarizing the findings concerning the role of TRPM4 in cardiac function. KO of the *Trpm4* gene in mouse heart *(Trpm4$^{-/-}$)* decreases the sodium current (I_{Na}) and may lead to cardiac conduction alteration under specific stress of the heart (*e.g.* mexiletine treatment).

1) Which study design (*e.g.* isolated myocytes vs whole tissue, perforated-patch clamp vs microelectrode or whole-cell recordings) should be used to investigate the role of TRPM4 in the heart?
2) Knowing that a recent publication suggests that the effect of Trpm4 KO is strain-dependent, which strain of the *Trpm4$^{-/-}$* mouse model should be used for further investigation?[37]

We further challenged the cardiac excitability and conduction using the sodium channel blocker mexiletine to investigate this observed decrease of sodium current under basal conditions without significant ECG alteration. In 20 to 30% of the cases, Brugada syndrome patients harbor a loss-of-function mutation in the *SCN5A* gene, coding for the $Na_v1.5$ channel. The ECG sign of Brugada syndrome is often concealed due to sufficient remaining sodium current, allowing the generation of the cardiac action potential. However, the typical Brugada ECG pattern can be unmasked with potent sodium channel blockers, such as mexiletine, leading to a further decrease of the sodium current, which in turn triggers the ECG perturbation *in fine*. Pseudo-ECG recordings on Langendorff-perfused, explanted mouse hearts showed that under perfusion of mexiletine, the alteration of the ECG pattern is more pronounced for *Trpm4$^{-/-}$* mouse hearts compared to the control group.[34] Overall, these results suggest that under stress conditions, patients with loss-of-function TRPM4 channels may develop cardiac conduction alterations that are, in fact, due to a decrease in sodium current.

In parallel to these electrophysiological approaches, co-immunoprecipitation experiments performed in a heterologous expression system, overexpressing TRPM4 and $Na_v1.5$, demonstrated that both proteins physically interact. This observation suggests a potential co-regulation as already observed for $Na_v1.5$ and the cardiac potassium channel Kir2.1.[34,38,39] Further investigations are required to understand the molecular determinants of this interaction and regulation. Nevertheless, this interaction also raises the question concerning the potential functional coupling between these two channels ($Na_v1.5$ and TRPM4), as suggested recently between wild-type and mutant $Na_v1.5$ channels.[40]

2.2 *TRPM4 in Prostate Cancer*

TRPM4 is up-regulated in several types of cancer and reports include prostate, colorectal, breast, and cervical cancer as well as large B-cell lymphoma.[7] Fig. 4 shows an overview of TRPM4 RNA expression levels in different types of cancer from the Human Protein Atlas.[41] Data from the human protein atlas are based on the Cancer Genome Atlas (TCGA) project that collects and analyzes multiple human cancer samples.[42]

In prostate cancer (PCa), TRPM4 mRNA expression levels are most prominently elevated. TRPM4 expression levels depend on cancer stages and clinical parameters. TRPM4 is upregulated upon transition from androgen-sensitive to androgen-insensitive PCa that characterizes cancer progression from an early- to a late-stage cancer.[43] In addition, TRPM4 is upregulated in cancerous tissue[44] and prostatic intraepithelial neoplasia (PIN)[45] compared to benign glands. TRPM4 is associated with the risk of biochemical recurrence after radical prostatectomy.[44] Analysis of a tissue microarray from 210 PCa patients demonstrates a correlation between TRPM4 protein expression and local and metastatic progression of PCa.[46]

Several studies from different groups demonstrate that TRPM4 contributes to cancer hallmarks in PCa, including increased proliferation, migration, and cell-cycle shift.[45–48] TRPM4 expression in PCa is up-regulated by micro RNA-150[48] and by loss of function of the tumor suppressor p53 that is associated with cancer progression.[49] Remarkably, not only plasma membrane TRPM4 but also TRPM4 in intracellular vesicles may add to its phenotype in migration.[50] Down-regulation of TRPM4 in PCa cells reduces exocytosis, and upregulation of TRPM4 may thus contribute to polarized exocytosis in metastasis.

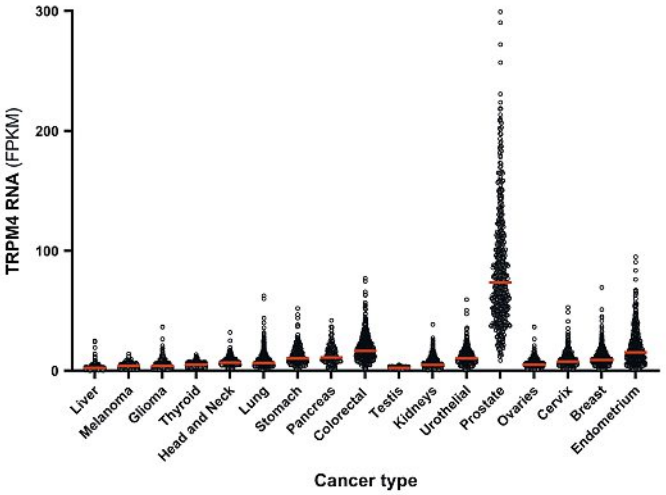


Fig. 4. TRPM4 RNA expression levels in different types of cancer. RNA sequencing data are reported as median FPKM (fragments per kilobase of transcript per million reads mapped). Each individual sample is illustrated as a circle on the graph; the median is indicated as red line. All data generated by the Cancer Genome Atlas,[42] figure adapted from the Human Protein Atlas.[41]

2.3 *Compounds in Cancer Hallmark Functions*

Various groups have demonstrated a role of TRPM4 in the pathophysiology of PCa as well as in other types of cancer. In a first study, describing the screening and identification of **CBA**, **LBA** and **NBA** as TRPM4 inhibitors, our results from an androgen-sensitive PCa cell line (LNCaP) looked already promising. For **CBA**, we determined an IC_{50} of 1.1 ± 0.3 μM to inhibit endogenous TRPM4 currents, and the block was almost 100%.[21] The next step was to investigate the role of TRPM4 in androgen-intensive PCa cells with these new inhibitors. We generated CRISPR/Cas KO clones in DU145, representing androgen-insensitive PCa cells. As shown before, KO of TRPM4 reduced migration, viability and proliferation of cancer cells and resulted in a cell-cycle shift. In addition, we identified a role of TRPM4 in cell adhesion. However, **CBA**, **LBA** and **NBA** failed to reduce cancer hallmark functions in a TRPM4-specific manner.[46] There may be different reasons for this. 1) With **CBA**, **LBA** and **NBA**, we block the current of TRPM4 but no other mechanisms that may be involved, such as the interaction of TRPM4 with other proteins.[7,51] 2) In contrast to electrophysiological experiments in Ringer solutions, our inhibitors fail to block TRPM4 due to the stickiness of **CBA**, **LBA**, and **NBA**, presumably to medium components. 3) The block of TRPM4 is insufficient to reduce TRPM4-associated cancer hallmark functions. Indeed, in DU145 cells, TRPM4 is only 65% blocked at a concentration of 50 μM CBA. **NBA** blocked 88% of the endogenous TRPM4 currents in DU145, and 50 μM of **LBA** blocked 85%.[46] It is unclear why **CBA**, **LBA** and **NBA** failed to completely block TRPM4 in DU145. In colorectal cancer (CRC), TRPM4 appears not to be upregulated *per se*, but in CRC tissue, TRPM4 protein expression levels correlate with the number of tumor buds (disseminated single CRC cells or small clusters of up to five CRC cells, which are often found in the tumor microenvironment with infiltrative growth patterns) and invasive tumor growth.[52] Interestingly in CRC cells, **CBA**, **LBA** and **NBA** almost completely inhibited TRPM4 currents. Already with 10 μM of inhibitor, TRPM4 currents were 92% blocked with **CBA**, 98% with **LBA** and 97% with **NBA**. In CRC cells, inhibitors reduced cell proliferation and prevented the TRPM4-specific shift in the cell cycle. In addition, in a CRC cell line derived from a late-stage cancer patient (Colo-205) that highly expresses TRPM4, **NBA** reduced cell viability. In CRC cells, we re-transfected TRPM4 KO cells with either TRPM4 wild-type or a dominant-negative mutant, non-permeable to Na^+. Our data show a recovery of the viability and cell cycle shift in cells expressing functional TRPM4, while the pore mutant was not able to rescue this phenotype.[52,53] All this points to TRPM4 conductivity as an underlying mechanism for the contribution of TRPM4 to cancer hallmarks. In both PCa and CRC cells, Na^+ current through TRPM4 is a negative feedback mechanism for store-operated Ca^{2+} entry (SOCE).[45,49] Changes in SOCE have been previously shown to affect cancer hallmark functions in PCa.[54] Thus, decreased SOCE that is caused by increased TRPM4 activity may be the underlying mechanism for altered cancer hallmark functions in PCa and CRC cells.

Future investigations in tumor spheroids from prostate cancer cells (Fig. 5) and PCa and CRC organoids from patients will help to validate TRPM4 as a therapeutical target in cancer.

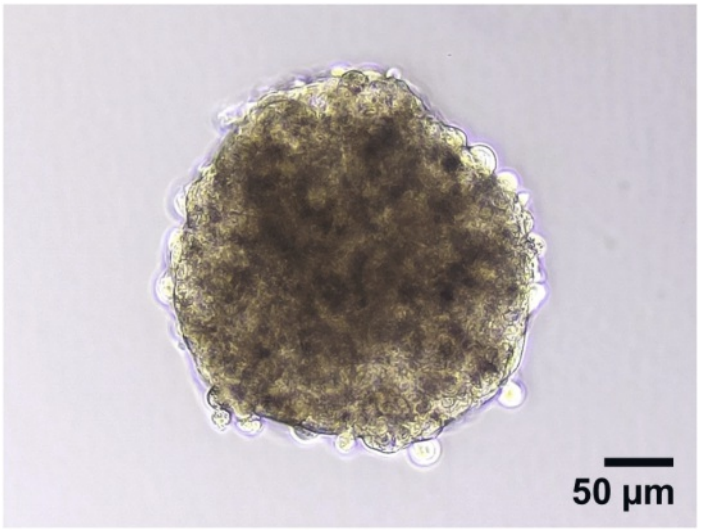


Fig. 5. Multicellular DU145 tumor spheroid. A total of 5000 cells were seeded in a round-bottom ultra-low attachment microplate and cultured for six days (representative image).

3. Conclusions

The virtual screening approach and subsequent synthetic SAR study allowed us to identify more potent and more selective TRPM4 inhibitors **LBA**, **CBA** and **NBA** than previously used in the field. These compounds are valuable pharmacological tools to investigate the role of TRPM4 in cardiac pathophysiology, prostate and colorectal cancers. The high-throughput compound screen, conducted at Novartis FAST Lab, yielded several novel TRPM4 inhibitor chemotypes and scaffolds that can be used as starting points for synthetic optimizations.

Although, the cardiac characterization of the *Trpm4*$^{-/-}$ mouse model confirms the important role played by TRPM4 for its electrical function, further investigations must be performed to better characterize this mouse model at different ages of both sexes. Finally, due to the functional consequences of TRPM4 variants leading to either gain-of-function or loss-of-function, a new TRPM4 mouse model overexpressing this channel may be important to fully understand the role of this channel in cardiac physiology and pathophysiology. The new TRPM4 inhibitor NBA will be a valuable tool in these future studies.

In androgen-insensitive PCa cells, inhibitors failed to reduce TRPM4-mediated cellular functions, possibly due to an incomplete block of TRPM4 currents. In CRC cells, inhibitors almost entirely block TRPM4 currents and reduce cancer hallmark functions in a TRPM4-specific manner. Further experiments will show if TRPM4 inhibitors affect organoids from certain PCa and CRC patient subgroups and if drugs that block TRPM4 currents may add to personalized medicine in the future.

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