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REVIEW

# The $Ca^{2+}$-activated chloride channel ANO1/TMEM16A: An emerging therapeutic target for epithelium-originated diseases?

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**KEY WORDS**

$Ca^{2+}$-activated $Cl^-$ channels (CaCCs);
ANO1;
TMEM16A;
$CaCC_{inh}$-A01;
$T16A_{inh}$-A01;
Drug target;
Cancer;
Cystic fibrosis

**Abstract** Anoctamin 1 (*ANO1*) or *TMEM16A* gene encodes a member of $Ca^{2+}$ activated $Cl^-$ channels (CaCCs) that are critical for physiological functions, such as epithelial secretion, smooth muscle contraction and sensory signal transduction. The attraction and interest in ANO1/TMEM16A arise from a decade long investigations that abnormal expression or dysfunction of ANO1 is involved in many pathological phenotypes and diseases, including asthma, neuropathic pain, hypertension and cancer. However, the lack of specific modulators of ANO1 has impeded the efforts to validate ANO1 as a therapeutic target. This review focuses on the recent progress made in understanding of the pathophysiological functions of CaCC ANO1 and the current modulators used as pharmacological tools, hopefully illustrating a broad spectrum of ANO1 channelopathy and a path forward for this target validation.



*Abbreviations:* Ang II, angiotensin II; ANO1, anoctamin-1; ASM, airway smooth muscle; BBB, blood−brain barrier; CaCCs, $Ca^{2+}$ activated chloride channels; CAMK, $Ca^{2+}$/calmodulin-dependent protein kinase; CF, cystic fibrosis; CFTR, cystic fibrosis transmembrane conductance regulator; DRG, dorsal root ganglion; EGFR, epidermal growth factor receptor; ENaC, epithelial sodium channels; ER, endoplasmic reticulum; ESCC, esophageal squamous cell carcinoma; FRT, fisher rat thyroid; GI, gastrointestinal; GIST, gastrointestinal stromal tumor; GPCR, G-protein coupled receptor; HNSCC, head and neck squamous cell carcinoma; HTS, high-throughput screening; ICC, interstitial cells of Cajal; IPAH, idiopathic pulmonary arterial hypertension; MAPK, mitogen-activated protein kinase; NF-$\kappa$B, nuclear factor $\kappa$B; PAH, pulmonary arterial hypertension; PAR2, protease activated receptor 2; PASMC, pulmonary artery smooth muscle cells; $PIP_2$, phosphatidylinositol 4,5-bisphosphate; PKD, polycystic kidney disease; TGF-$\beta$, transforming growth factor-$\beta$; VGCC, voltage gated calcium channel; VRAC, volume regulated anion channel; VSMC, vascular smooth muscle cells; YFP, yellow fluorescent protein.

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## 1. Introduction

$Ca^{2+}$-activated chloride channels (CaCCs) are a heterogeneous group of $Cl^-$ channels that can be synergistically activated by intracellular calcium and voltage. The CaCCs are found in almost all species ranging from invertebrates to mammals, and the ubiquitous expression of CaCCs indicates a variety of functions important for physiology, including regulation of epithelial $Cl^-$ secretion, excitability of neuronal and cardiac cells, smooth muscle contraction and nociception[1–3].

A subtype of CaCCs was first described in *Xenopus* oocytes nearly 40 years ago and its molecular identity was debated until in 2008 when three independent laboratories reported that anoctamin-1 (ANO1) or transmembrane protein 16A (TMEM16A) underlies the molecular basis of a subgroup of CaCCs[4–6]. The *Ano1/Tmem16a* gene encodes a 986-amino-acid protein that belongs to anoctamin family consisting of 10 members (ANO1−ANO10) in mammals. ANO1, as a CaCC, is primarily expressed in epithelial cells, smooth muscle cells and sensory neurons. ANO2, also as a CaCC, is expressed in the olfactory sensory neurons[7], photoreceptor synaptic terminals[8], hippocampal pyramidal neurons[9], thalamocortical neurons[10], and inferior olive neurons[11] in the brain. Other ANOs family members including ANO6 and ANO7 were onetime considered to be CaCCs[12], but evidence shows that ANOs3−7 neither generate $Ca^{2+}$-activated $Cl^-$ currents nor traffic into membrane, indicating that they are endoplasmic reticulum proteins[13]. More studies reveal that ANOs3−7 and ANO9 are linked to the $Ca^{2+}$-dependent membrane phospholipid scramblases that are responsible for translocation of phospholipids[14–16]. It is generally accepted that ANO1 and ANO2 are two members of the CaCC subfamily, whereas ANO3−ANO10 have been debated for their functions as CaCCs or $Ca^{2+}$-dependent membrane phospholipid scramblases, or other physiological proteins[17,18]. In addition, an integral membrane protein bestrophin-1 (BEST1) encoded by the *BEST1* gene also functions as a CaCC. BEST1 is predominantly expressed in retinal pigment epithelium[19], non-neuronal tissue, peripheral and central neurons[20,21].

ANO1 was initially thought to have eight transmembrane domains, possessing multiple protein isoforms generated by alternative splicing of four segments (a, b, c, and d) located at the C-terminus and the first intracellular loop[5]. ANO1 splice variant lacking segment b, for instance, increases the calcium sensitivity, whereas deletion of the segment c decreases apparent $Ca^{2+}$ sensitivity and increases voltage-dependent activity of ANO1 channel[22,23]. The X-ray structure of a $Ca^{2+}$-activated lipid scramblase ANO1 from fungus *Nectria haematococca* (nhANO1) shows a homodimer with each subunit containing a ten-transmembrane $\alpha$-helices[24]. Several critical amino acid residues for $Ca^{2+}$ sensitivity and ion selectivity are subsequently found[25,26]. Recently, a high-resolution cryo-EM structure reveals an overall structure of mouse ANO1 similar to nhANO1[27,28], exhibiting two $Ca^{2+}$ binding sites within the inner vestibule of the pore, and $Ca^{2+}$ binding triggering conformational changes of $\alpha$-helix rendering the pore conductive (Fig. 1[26–29]). The mechanisms underlying ANO1 activation and modulation are beginning to emerge. It has been shown that calmodulin, protons, cell volume and thermal stimuli can regulate the channel activation[30,31], and phosphatidylinositol 4,5-bisphosphate ($PIP_2$) regulates the activation and desensitization of ANO1[32,33].

ANO1 channel appears to be preferentially activated by local rise of intracellular $Ca^{2+}$ release from the endoplasmic reticulum (ER) $Ca^{2+}$ stores, which may represent a general mechanism of ANO1 activation[34]. ANO1 expression is regulated by multiple signaling cascade pathways such as mitogen-activated protein kinase (MAPK), nuclear factor $\kappa$B (NF-$\kappa$B) and transforming growth factor-$\beta$ (TGF-$\beta$) in pathological functions[35]. Several recent reviews have substantially covered the molecular basis, structure and pathophysiological functions of ANO1[17,35–37]. In this review we will mainly focus on the aspect of channelopathies and pharmacological validation of ANO1 as an emerging therapeutic target.

## 2. Pharmacological modulation of ANO1 channel

Validating ANO1 as a therapeutic target requires specific modulators that can serve as essential tools for understanding the channel pharmacology. Up to now, many ANO1 modulators have been reported, and unfortunately most of them are lack of potency and efficacy. Therefore, there exists a great need for discovery of more selective and potent modulators, inhibitors in particular, which can be used for ANO1 target validation.

### 2.1. Inhibitors

Some broad-spectrum blockers, such as NFA, FFA, DIDS, NPPB and 9AC, have been used as tools for understanding functions of CaCCs (Table 1[38–84]). These small molecules can block endogenous CaCCs in *Xenopus* laevis oocytes[39,43,45,85] and are also known to non-specifically modulate other channels, such as inhibition of volume regulated anion channel (VRAC) and Kv4 by NFA and DIDS and activation of $Ca^{2+}$ activated $K^+$ channel by NFA and FFA or potentiation of TRPV1 channel by DIDS[2,38]. $CaCC_{inh}$-A01 was identified to inhibit CaCC current in 2008 from screen of 50,000 compounds using human intestinal epithelial HT29 cell that highly express endogenous CaCCs without obvious effects on intracellular $Ca^{2+}$, $Ca^{2+}$/calmodulin-dependent protein kinase (CAMK) or cystic fibrosis transmembrane conductance regulator (CFTR)[49]. $CaCC_{inh}$-A01 as an ANO1 inhibitor has been commonly used in many investigations for the role of ANO1 in interstitial cells of cajal (ICC), cardiac fibroblast and rod bipolar cells of retina[86–88], and channelopathies, including cancer, hypertension, nociception, diarrhea and high glucose induced renal cyst growth[51,53–55]. $CaCC_{inh}$-A01 is shown to inhibit cancer cell proliferation through increasing the ubiquitination of ANO1 and facilitating ER-associated, proteasomal degradation of ANO1, while other ANO1 inhibitors, such as $T16A_{inh}$-A01 and digallic acid, are shown to have no effect on cancer cell proliferation[89–91]. Similarly, $CaCC_{inh}$-A01 inhibits proliferation of cardiac fibroblast, but not another ANO1 inhibitor $T16A_{inh}$-A01[87]. $CaCC_{inh}$-A01 reduces upregulation of ANO1 expression and attenuates brain infarct size and neurological deficits after ischemic stroke whereas $T16A_{inh}$-A01 shows no effect[56]. That ANO1 protein expression is reduced by $CaCC_{inh}$-A01 but not $T16A_{inh}$-A01 may partially explain why the two ANO1 inhibitors show different effects on cell proliferation and ischemic stroke. In addition, intrathecal

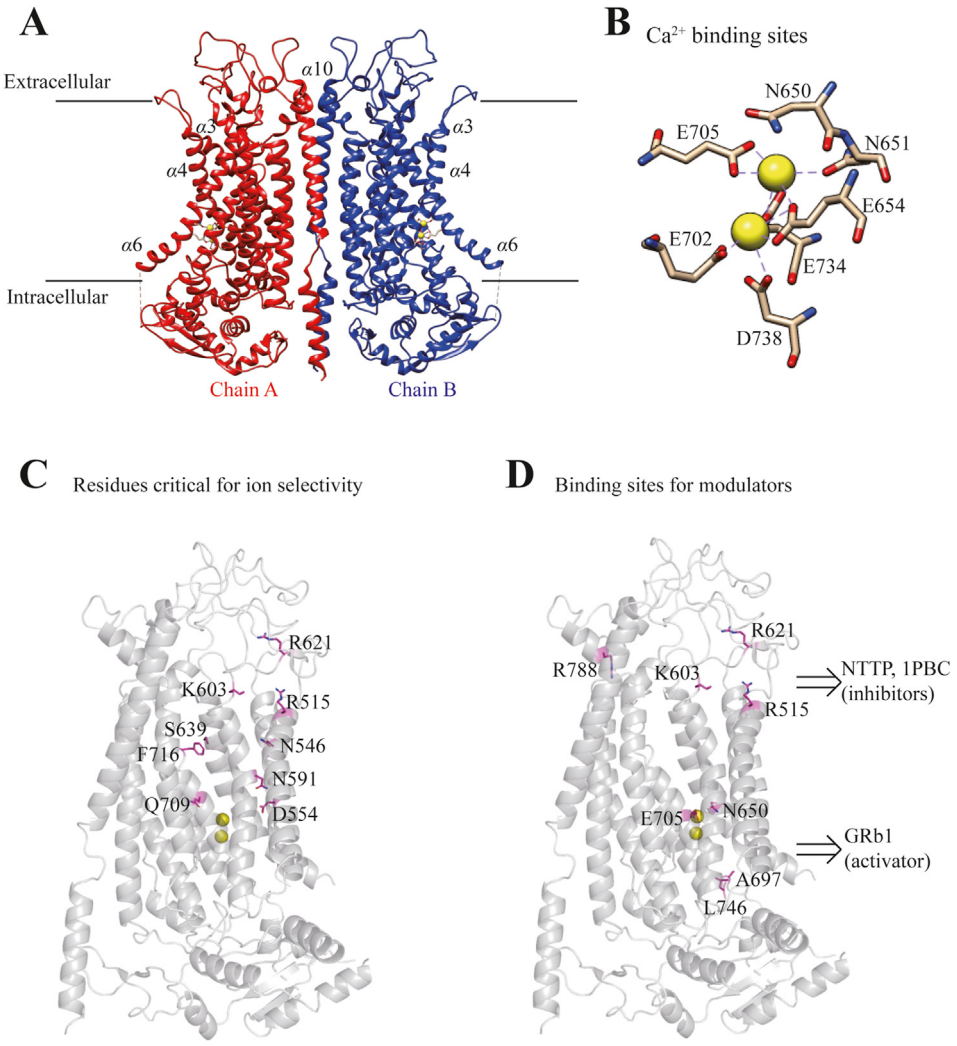
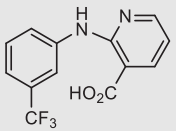
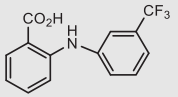
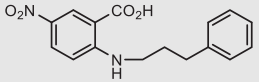
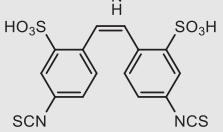
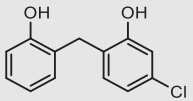
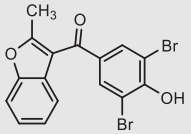
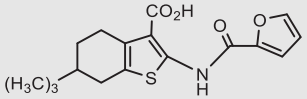
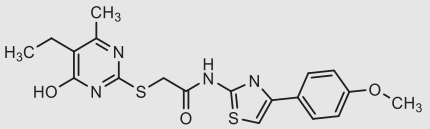
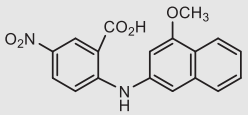



**Figure 1** The molecular structure of ANO1/TMEM16A channel. (A) The $Ca^{2+}$-bound structure of mANO1 channel in dimer (chains A and B), and 2 yellow filled circles for $Ca^{2+}$ in each monomer containing 10 transmembrane $\alpha$ helices. (B) $Ca^{2+}$ binding sites formed by residues N650, N651, E654 from $\alpha$6, E702, E705 from $\alpha$7, and E734, D738 from $\alpha$8[28,27]. (C) Residues critical for ion selectivity including R515 from $\alpha$3, N546, D554 from $\alpha$4, N591, V599 from $\alpha$5, K603, R621 from $\alpha$5−6 linker, S639 from $\alpha$6, and Q709, F716 from $\alpha$7[26,28]. (D) Putative binding sites, R515 from $\alpha$3, K603, R621 from $\alpha$5−6 linker, and R788 from $\alpha$8, for ANO1 inhibitors NTTP and 1PBC[26]; and N650 from $\alpha$6, A697, E705 from $\alpha$7, and L746 from $\alpha$8 for ANO1 activator GRb1[29]. The structure is regenerated based on the cryo-EM structure of ANO1 channel (PDB 5OYB)[27]. The residue number labeling is based on the sequence of mTMEM16A (ac) isoform (UniProt Q8BHY3.2).

injection of $CaCC_{inh}$-A01 reduces tactile allodynia and thermal hyperalgesia and also decreases ANO1 upregulation after spinal nerve injury[54].

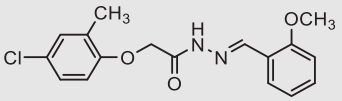
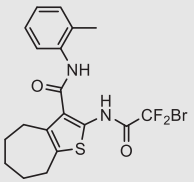
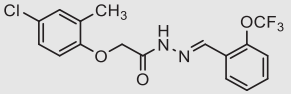
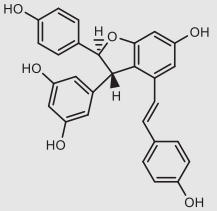
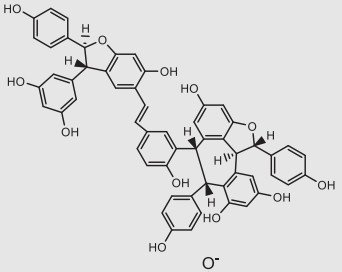
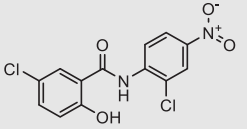
Since the identification of $CaCC_{inh}$-A01, several ANO1 inhibitors have been screened out using the iodide-sensitive yellow fluorescent protein (YFP)-based high throughput screening (HTS) assay[50,65,67]. $T16A_{inh}$-A01 was reported to inhibit human ANO1 current expressed in Fisher rat thyroid (FRT) cells with an $IC_{50}$ of 1 μmol/L and had no effect on CFTR current[50]. However, our previous study showed that $T16A_{inh}$-A01 at 10 μmol/L only inhibits mouse ANO1 current expressed in CHO cells about 28% at +80 mV[38], and it may because that the efficacy of $T16A_{inh}$-A01 on ANO1 inhibition dependents on splice variants of ANO1 and intracellular calcium[92]. Nevertheless, $T16A_{inh}$-A01 has been used as a pharmacological probe to investigate the role of ANO1 in pancreatic ductal adenocarcinoma cells[93], and also physiological and pathological functions of ANO1 in different tissues, including contraction of vesical smooth muscle, iodide release from thyrocyte, initial waveform modulation in retinal rod bipolar cell and melatonin secretion in pineal glands[52,88,94−96] (Table 1). $T16A_{inh}$-A01 attenuates the interleukin-13 (IL-13) induced increase of ANO1 expression and secretion of mucin in human nasal polyp epithelial cells from chronic rhinosinusitis patients, cultured human bronchial epithelial cells and goblet cells in the guinea pig asthma model[59,97], suggesting that $T16A_{inh}$-A01 may be useful for treatment of hypersecretion in asthma and other inflammatory airway diseases. In addition, several studies show that ANO1 inhibitor $T16A_{inh}$-A01 exerts therapeutic effect on cancer, neuropathic pain and eosinophilic esophagitis[58,98−100].

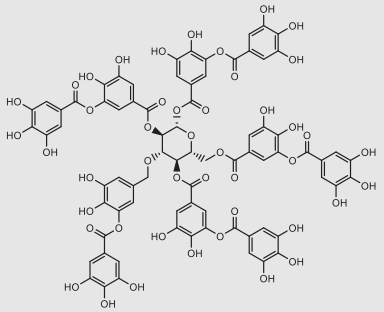
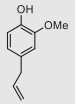
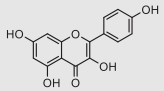
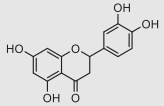
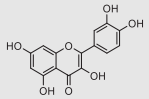
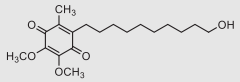
**Table 1** Pharmacology of CaCC ANO1/TMEM16A inhibitors.

| Inhibitor | Structure | $IC_{50}$ (μmol/L) | Test assay | Selectivity | Effect | Ref. |
|---|---|---|---|---|---|---|
| NFA |  | 7−37 | *Xenopus* oocytes<br>mANO1-CHO | KCa channel (+)<br>Kv4 channel (−)<br>VRAC (−)<br>ANO6 (−)<br>$[Ca^{2+}]i$ (+) | Anti-cancer,<br>Anti-asthma | 38−42 |
| FFA |  | 14−35 | *Xenopus* oocytes<br>mANO1-CHO | KCa channel (+)<br>$[Ca^{2+}]i$ (+) | NR | 38,39 |
| NPPB |  | 22−68 | *Xenopus* oocytes<br>mANO1-CHO | K+ channel (−)<br>$[Ca^{2+}]i$ (+)<br>VRAC (−) | Anti-cancer,<br>Antinociception | 38,39,43,44 |
| DIDS |  | 11−550 | *Xenopus* oocytes<br>mANO1-CHO | Kv4 channel (−)<br>VRAC (−)<br>$[Ca^{2+}]i$ (+)<br>TRPV1 (+)<br>xBest2a (/) | Anti-cancer,<br>Antinociception | 38,39,44−47 |
| Dichlorophen |  | 5.5 | ANO1-HEK293 | ANO2 (−)<br>CFTR (/)<br>ENaC (/) | Anti-asthma | 48 |
| Benzbromarone |  | 10 | ANO1-HEK293 | ANO2 (−)<br>CFTR (/)<br>ENaC (/) | Anti-asthma | 48 |
| $CaCC_{inh}$-A01 |  | 1−7.8 | HT-29<br>hNO1-FRT<br>mANO1-CHO | hBest1 (−, 7 μmol/L)<br>ANO2 (−)<br>CFTR (/)<br>VRAC (−)<br>$[Ca^{2+}]_i$ (/)<br>xBest2a (/) | Anti-cancer,<br>Anti-hypertension,<br>Anti-diarrhea,<br>Antinociception,<br>Anti-high glucose induced renal cyst growth,<br>Anti-ischemic stroke induced BBB intergrity | 38,47,49−56 |
| $T16A_{inh}$-A01 |  | 1 | hANO1-HEK293 | ANO2 (−)<br>CFTR (/)<br>$[Ca^{2+}]_i$ (/)<br>hBest1 (/)<br>VGCC (−, 50 nmol/L)<br>xBest2a (/) | Anti-cancer,<br>Anti-hypertension,<br>Anti-Asthma,<br>Antinociception,<br>Anti-eosinophilic esophagitis, | 38,47,50,<br>52,54,<br>57−60 |
| MONNA |  | 1.27 | hANO1-HEK293<br>xANO1 (0.08 μmol/L) | VRAC (−)<br>ANO2 (−)<br>mBest1 (/)<br>hBest1 (/)<br>mCLC2 (/) | Anti-cancer,<br>Anti-itch,<br>Anti-hypertension,<br>Anti-polyfertilization | 47,61−65 |

(*continued on next page*)

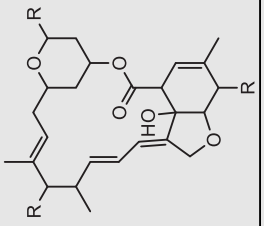
**Table 1** (*continued*)

| Inhibitor | Structure | $IC_{50}$ (μmol/L) | Test assay | Selectivity | Effect | Ref. |
|---|---|---|---|---|---|---|
| Ani9 |  | 0.08 | hANO1-FRT | hCFTR (/)<br>xBest2a (/)<br>ANO2 (/)<br>CFTR (/)<br>ENaC (/)<br>VRAC (/)<br>ANO6 (slower activation)<br>xBest2a (/) | Anti-cancer,<br>Anti-polyfertilization | 47,57,65,66 |
| **10bm** |  | 0.03 | hANO1-FRT | ANO2 (−, 0.4 μmol/L)<br>CFTR (/) | NR | 67 |
| Ani9-5f |  | 0.02 | hANO1-FRT | ANO2 (/)<br>CFTR (/) | Anti-cancer | 68 |
| Dimer *trans*-ε-viniferin (TV) |  | 1.1 | HT-29 | CFTR (−) | Anti-diarrhea | 69 |
| Tetramer, γ-2-viniferin (RV) |  | 12.3 | HT-29 | CFTR (−) | Anti-diarrhea | 69 |
| Niclosamide |  | 0.1−2.4 | hANO1-HEK293T | CFTR (/)<br>ANO6 (−)<br>$[Ca^{2+}]_i$ (−) | Anti-asthma | 42,66,70 |

| | | | | | | |
|---|---|---|---|---|---|---|
| Tannic acid |  | 6–25 | hANO1-FRT<br>mANO1-CHO | CFTR (/)<br>ENaC (/)<br>hBest1 (−, 15 μmol/L)<br>ANO2 (−) | Anti-nociception,<br>Anti-cancer,<br>Anti-asthma | 38,50,62,71–73 |
| Eugenol |  | 150 | hANO1-FRT<br>T84 cell | CFTR (/)<br>$[Ca^{2+}]_i$ (/)<br>Nav1.8 (−)<br>TRPV1 (−)<br>Kv1.5 (−)<br>VGCC (−) | Anti-diarrhea,<br>Local analgesia | 74–76 |
| Galangin |  | 4.5–9.7 | mANO1-CHO | NR | Anti-cancer | 77,78 |
| Luteolin |  | 9.37 | hANO1-FRT<br>mANO1-CHO | ANO2 (−, 120 μmol/L)<br>L-type $Ca^{2+}$ channel (−)<br>$[Ca^{2+}]_i$ (/) | Anti-cancer | 77–79 |
| Quercetin |  | 13.7 | mANO1-CHO | NKCC1 (+)<br>$Na^+$-$K^+$-ATPase (+)<br>CFTR (+)<br>K+ channel (+)<br>TRPM7 (−) | Anti-cancer,<br>Attenuation of GI tract motility,<br>Increasement of intestinal $Cl^-$ secretion | 78,80 |
| Idebenone |  | 9.2 | hANO1-FRT | ANO2 (−)<br>CFTR (/)<br>$[Ca^{2+}]_i$ (/) | Anti-cancer,<br>Anti-renal cyst | 81,82 |
| Plumbagin |  | 12.46 | hANO1-FRT | CFTR (−)<br>$K^+$ channel (/)<br>$Na^+$-$K^+$-ATPase (/) | Anti-cancer,<br>Anti-diarrhea | 81,83 |

(*continued on next page*)

**Table 1** (*continued*)

| Inhibitor | Structure | $IC_{50}$ (μmol/L) | Test assay | Selectivity | Effect | Ref. |
|---|---|---|---|---|---|---|
| Avermectins |  | 0.15−1.32 | mANO1-CHO | $[Ca^{2+}]_i$ (/)<br>NR | Anti-cancer | 84 |

+, activation effect; −, inhibitory effect; /, no effect; NR, no report; NKCC, $Na^+/K^+/Cl^-$ co-transporter.

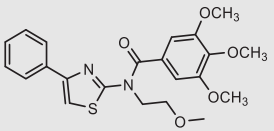
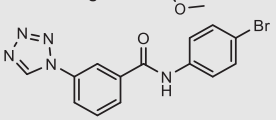
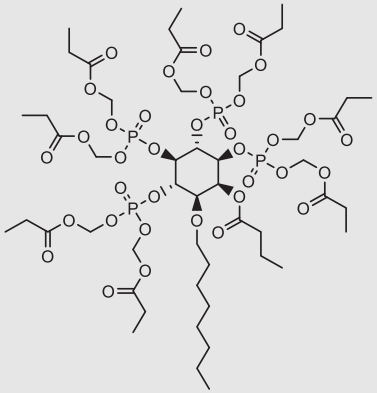
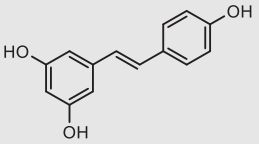
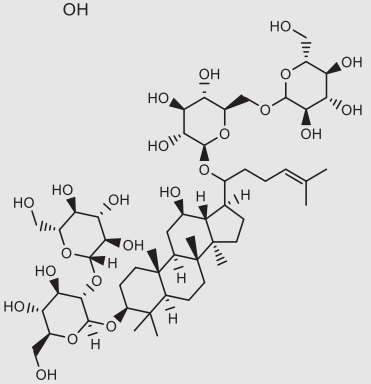
MONNA, the most potent blocker of anthranilic acid derivatives, blocks xANO1 and hANO1 with $IC_{50}$ of 0.08 and 1.27 μmol/L, respectively, without any effects on CFTR, CLC2 and BEST1[61]. Similar to ANO1 inhibitors $CaCC_{inh}$-A01 and $T16A_{inh}$-A01, MONNA concentration-dependently inhibits agonist-induced rodent vesical constriction through hyperpolarization of vesical smooth muscle cells[52,63]. MONNA also inhibits chloroquine-induced action potential discharge at itch nerve terminals and bouts of scratching[64], suggesting a role of ANO1 in pruritus. MONNA has also been used to investigate the role of ANO1 in blocking poly-fertilization[47].

Another small molecule Ani9 was also identified to inhibit hANO1 expressed in FRT cells with an $IC_{50}$ of 0.08 μmol/L using YFP-HTS assay (Table 1). Ani9 belongs to acetamides and shows a relatively high selectivity on ANO1 over ANO2 without inhibitory effects on CFTR, VRAC, epithelial sodium channels (ENaC) and intracellular $Ca^{2+}$ signaling at concentration of 30 μmol/L[65], whereas Ani9 can inhibit inward current and slow the time-dependent activation of ANO6[66]. The Ani9 derivative **5f** shows more potent inhibitory effect on ANO1 with an $IC_{50}$ of 20 nmol/L without activating on ANO2[68]. Ani9 as a novel potent and selective ANO1 inhibitor has also been used in several investigations for the physiological and pathological functions of ANO1, including hypertension, polyspermy block and cancer[47,57,63]. As one of the 2-acylamino-cycloalkylthiophene-3-carboxylic acid arylamides, **10bm** shows a potent inhibition on ANO1 current with an $IC_{50}$ of 30 nmol/L and exhibits dose-dependent inhibition on isometric smooth muscle contraction. The **10bm** compound also inhibits ANO2 with an $IC_{50}$ of 0.4 μmol/L without effect on CFTR[67]. At present, synthesized ANO1 inhibitors are only used as tools for preclinical studies (Table 1).

Many natural products from diverse plants have been found to inhibit ANO1. Tannic acid presented in the green tea and red wine was identified as a blocker for both ANO1 and ANO2 without effect on CFTR or ENaC, showing an inhibitory effect on arterial smooth muscle contraction and intestinal $Cl^-$ secretion[71]. Like the synthesized small molecule ANO1 inhibitors, tannic acid has also been used as a pharmacological tool for investigations of the biophysical feature and physiological functions of ANO1[101−103]. Series of flavonoids were recently identified to inhibit ANO1, exerting anticancer effects[77,78]. Natural products or synthetic analogues of natural products, including idebenone with anticancer activity[81], plumbagin with antidiarrhea[83] and matrine with anti-lung adenocarcinoma activity[104] have been shown to inhibit ANO1.

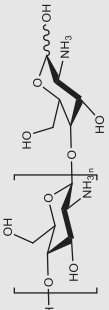
Several clinical drugs are recently found to have inhibitory effect on ANO1, including clarithromycin, benzbromarone, niclosamide, nitazoxanide and avermectins. Clarithromycin is a clinical antibiotic that was reported to decrease IL-13 induced ANO1 expression in goblet cells from asthma models[105]. Benzbromarone, a clinical drug for gout treatment, shows anti-asthmatic effect through inhibiting IL-13 induced mucin secretion and methacholine induced airway smooth muscle (ASM) contraction *via* the inhibition of ANO1[70,106]. Niclosamide and nitazoxamide are clinical anthelmintics that as potent ANO1 inhibitors can block ASM depolarization and contraction[70]. Avermectins are a type of macrocyclic lactones that are widely used as pharmaceuticals against roundworms in humans and animals and also for crop protection. Several avermectins, including avermectin B1, ivermectin, doramectin, selamectin and moxidectin, exhibit inhibitory effects on cancer cell proliferation

**Table 2** Pharmacology of CaCC ANO1/TMEM16A Activators.

| Activator | Structure | $EC_{50}$ (μmol/L) | Test assay | Selectivity | Effect | Ref. |
|---|---|---|---|---|---|---|
| Eact |  | 3 | hANO1-FRT | TRPV1 (+)<br>TRPV4 (+)<br>$[Ca^{2+}]_i$ (+)<br>ANO6 (+) | Pain,<br>Smooth muscle contraction | 66,109−111 |
| Fact |  | 6 | hANO1-FRT | NR | NR | 109 |
| INO-4995 |  | NR | hANO1-HEK293 | NR | NR | 112 |
| Resveratrol |  | 48 | mANO1-HEK293T | NR | Smooth muscle contraction | 113 |
| GRb1 |  | 38.4 | mANO1-HEK293 | NR | Smooth muscle contraction | 114 |
| Cinnamaldehyde | | 9.7 | ANO1-HEK293 | NR | Smooth muscle | 66 |

(*continued on next page*)

**Table 2** (*continued*)

| Activator | Structure | $EC_{50}$ (μmol/L) | Test assay | Selectivity | Effect | Ref. |
|---|---|---|---|---|---|---|
| | | | | | contraction | |
| Chitosan oligosaccharides |  | 74.5 μg/mL | mANO1-HEK293 | NR | Smooth muscle contraction | 115 |
| ETX001 | NR | 116 nmol/L | hANO1-FRT<br>sANO1-CHO | $[Ca^{2+}]_i$ (/) | Anti-CF | 116 |
| Melittin | $C_{131}H_{229}N_{39}O_{31}$ | 0.5–2 | hANO1-HEK293 | ANO6 (+)<br>PLA2 (+) | Phospholipid scrambling through ANO6 but not ANO1 | 66,117 |

+, activation effect; /, no effect; NR, no report.

and migration through inhibition of ANO1[84]. These drugs may have repurposing potential for dysfunctional ANO1 related diseases, such as asthma and hypertension.

It is noticeable that many compounds can act on ANO1 with limited selectivity, which imposes a big challenge for validation of ANO1 as a therapeutic target. The reasons for lack of identifying specific ANO1 modulators can be in multiple folds including chemical designs without guidance of target structure, use of an indirect YFP fluorescence-based HTS assay, and compound screens against cells expressing endogenous xANO1 in oocytes (for MONNA)[61], hANO1 in HT29 cells (for $CaCC_{inh}$-A01)[49], or heterogeneous hANO1 in FRT cells (for $T16A_{inh}$-A01, Ani9 and **10bm**)[50,65,67]. In addition, ANO1 and ANO2 subunits share 62% of sequence[4], which also presents the selectivity challenge. The recent solved cryo-EM structure of ANO1 may help understand the biophysical features and physiological functions of the channel and also can greatly assist to develop more specific ANO1 modulators[27,28]. A pharmacophore model based on three-dimensional quantitative structure−activity relationship (3D-QSAR) for predicting best target and compound interactions also appears to be a promising tool for virtual screening and enhancing rational design for novel potent and specific ANO1 modulators[107].

### 2.2. *Activators*

During the past almost 40 years since the identification of CaCC in 1982[108], only a few activators of ANO1 have been reported. Two ANO1 activators Eact and Fact are the first discovered using an HTS approach[109]. Eact and Fact are two different classes of chemicals that activate ANO1 through different mechanisms (Table 2[66,109−117]). Eact is an activator that induces ANO1 current in the absence of intracellular $Ca^{2+}$ with an $EC_{50}$ of 3 μmol/L. Several studies indicate that Eact might indirectly activate ANO1 through an intracellular $Ca^{2+}$ increase[110,111]. Fact is a potentiator that increases ANO1 current in a $Ca^{2+}$-dependent manner with an $EC_{50}$ of 6 μmol/L. INO-4995 is an 1-*O*-octyl-2-*O*-butyryl-*myo*-inositol 3,4,5,6-tetrakisphosphate octakis(propionoxymethyl) ester directly activates overexpressed ANO1 current but without effect on endogenous ANO1 currents in *Xenopus* oocytes, human airways and colonic cells[112].

A small molecule ANO1 potentiator ETX001 is recently shown to increase ANO1 current with an $EC_{50}$ of 116 nmol/L without effect on intracellular $Ca^{2+}$ signaling[116] (Table 2). ETX001 enhances fluid secretory response in human bronchial epithelial cells from cystic fibrosis (CF) patients, and increases airway mucus clearance in sheep CF models. Several compounds from traditional Chinese medicines, including resveratrol[113], ginsenoside Rb1 (GRb1)[114], GRb2[29], cinnamaldehyde[118] and chitosan oligosaccharides[115], were recently reported to activate ANO1 channel although their mechanisms of action remain elusive. Activators of ANO1 can be useful for validating ANO1 as a therapeutic target for treatment of disorders associated with ANO1 hypofunction, including Sjogren's syndrome, cystic fibrosis lung disease, and dry eye syndrome.

It should be mentioned that the relatively wide distribution of ANO1 may impose a liability concern or a challenge in developing organ- or tissue-specific therapy. Nevertheless, development of targeted drug delivery systems such as tissue-specific or selective organ targeting nanoparticles and controlled release of therapeutic agents may circumvent potential ANO1-originated complications[119]. In addition, target-related complications can also be minimized thought means of different drug formulations,

such as topical preparations, sublingual, buccal and rectal administrations.

## 3. Pathological functions and related diseases

Since ANO1 was identified as a member of CaCCs in 2008, its investigations have been focused on the distribution and expression in numerous organs and tissues, and its roles in pathological conditions and diseases (Fig. 2[51,98,120,121]).

### 3.1. Epithelial diseases

Epithelial cells are a group of tightly compressed cells that line the inside of all organs and function as a barrier between the inside and outside of an organ. Chloride ion is important for transepithelial secretion, whereas intracellular $Ca^{2+}$ is one of two major signals to modulate $Cl^-$ secretion[122,123], indicating an important role of $Ca^{2+}$ activated $Cl^-$ channels in regulating epithelial secretion. ANO1 is observed in many epithelial tissues, including but not limited airway, bronchus, salivary glands, pancreatic ductal cells and intestinal epithelium[1,4,124]. Numerous literatures not only identify native ANO1 expressions in multiple epithelia tissues but also demonstrate the pathological functions of ANO1 important for the process of fluid and electrolyte secretion[5,37,125–127]. Here, we review several epithelial diseases involved in ANO1 dysfunction.

#### 3.1.1. Asthma

Asthma is a prevalent inflammatory airway disease characterized by chronic inflammation, remodeling, and excessive constriction of the airway. The mechanism underlying asthma is complex and treatment for asthma is still challenging[128]. Hypersecretion of airway epithelium and hypercontraction of airway smooth muscle are two major contributions to asthma[129]. The $Ca^{2+}$ activated $Cl^-$ channel ANO1 as an anion channel plays a critical role in airway transepithelial mucus function. Mucus secreted by goblet cells is composed of water and mucus protein, which requires anion channel activity to instill $Cl^-$ and $HCO_3^-$ for ensurance of proper salination, hydration and pH of the mucus gel layers[130]. ANO1 is found robustly expressed in epithelial goblet cells and involved in mediating native $Ca^{2+}$ dependent $Cl^-$ current[131]. ANO1 is also highly permeable to $HCO_3^-$ at higher intracellular $Ca^{2+}$ levels, likely contributing to mucus release by providing a secretory pathway for $HCO_3^-$ that is an essential ingredient in mucus[132,133]. Downregulation of ANO1 markedly decreases epithelial mucus secretion[126,127], suggesting ANO1 inhibition beneficial for asthma. ANO1 expression is upregulated by cytokines IL-4, IL-13 and T helper type 2 that are asthmatic biomarkers, and upregulated ANO1 colocalizes to the apical plasma membrane of goblet cells along with the mucin MUC5AC[48,97,131]. In addition, ANO1 is also found abundantly expressed in the smooth muscle cells and plays a role in airway hyperreactivity[48]. ANO1 expression is upregulated both in epithelial cells and airway smooth muscle cells from asthmatic human patients and rodent models[41,48,127]. These investigations suggest that hyperactivity of ANO1 may underlie the pathogenesis of asthma, and inhibition of ANO1 channel activity may represent a therapeutic strategy for mucus hypersecretion in asthma.

The mechanism for ANO1 involving in asthma is unclear. A recent investigation shows that the GPCR/ANO1/VGCC axis contributes to the bronchial hyperresponsiveness in asthma[134]. The asthmatic inflammatory mediators, including 5-HT, histamine and thromboxane A2 binding to G-protein coupled receptors (GPCRs), activate $IP_3R$ to release $Ca^{2+}$ from sarcoplasmic reticulum for activation of ANO1 and voltage gated calcium channel (VGCC) that leads to $Ca^{2+}$ influx and subsequently depolarize the membrane potential and cause airway contraction both *ex vivo* ASM and *in vivo* asthmatic animals[134]. Downregulation of ANO1 by either pharmacological inhibitor $T16A_{inh}$-A01 or knockdown can inhibit the inflammatory mediator-induced $Ca^{2+}$-activated $Cl^-$ current and ASM contractile response[134]. Several reports show the involvement of ANO1 in mucus production or secretion[48,59,127], but the mechanistic insights into mucus production and secretion in airways and validation of the role ANO1 in asthma are required.

Huang and his colleges[48] adopted a HTS assay and identified benzbromarone, a clinical drug for gout treatment, as an ANO1 blocker. Benzbromarone shows anti-asthmatic effect through inhibiting IL-13-induced mucin secretion and methacholine-induced ASM contraction *via* inhibition of ANO1 function[70,106]. In addition, several ANO1 inhibitors, such as NFA[41], tannic acid[73], $T16A_{inh}$-A01[59], and niclosamide[70] have been shown to exert anti-asthma effects. However, the anti-asthma effects observed using those ANO1 inhibitors need to be further confirmed in some degree because of their relatively low potency and poor selectivity.

#### 3.1.2. Diarrhea

Diarrhea is characterized by an increase in the frequency of bowel movements with loose and watery stools and major causes of infectious secretory diarrhea include bacteria, viruses and parasites[135]. Intestinal $Cl^-$ channels are important for regulation of excessive mucus secretion induced by bacteria/virus-secreted enterotoxins through increasing apical membrane permeability, suggesting an involvement of $Ca^{2+}$ activated $Cl^-$ channel ANO1 in pathology of diarrhea. There is an increasing evidence that ANO1 plays an important role in the regulation of intestinal epithelial secretion and the pathogenesis of diarrhea based on the following observations: 1) ANO1 is expressed in intestinal epithelia and ANO1 gene knockdown reduces $Ca^{2+}$ activated $Cl^-$ secretion induced by muscarinic agonist carbochol[126]; 2) $Ca^{2+}$-dependent $Cl^-$ secretion increases through enhancement of ANO1 expression or activation of ANO1 current in the distal colon in response to the rotavirus nonstructural glycoprotein NSP4 that causes infantile gastroenteritis[136]; and 3) ANO1 expression is upregulated in animal models of diarrhea[137].

The increased activity of gastrointestinal (GI) tract is another important cause for diarrhea. ICC are mesenchymal cells located within the muscle layers in the GI tract and myenteric ICC serve as a pacemaker generating the bioelectrical slow wave potential that leads to contraction of GI smooth muscles in the GI tract[138]. Recent studies have found that ANO1 is also highly expressed in the ICC and mediates the slow wave current in the ICC[4,139,140]. The intracellular $Ca^{2+}$ rise from intracellular stores and VGCC in the ICC causes the generation and propagation of pacemaker potential that is also amplified by activation of ANO1 channels[138], suggesting that inhibition of ANO1 may reduce intestinal mobility for diarrhea. Indeed, pharmacological inhibition or gene silencing of ANO1 blocks slow wave in intestinal smooth muscle[141,142]. It is noted that several ANO1 inhibitors including $T16A_{inh}$-A01 and $CaCC_{inh}$-A01 exhibit different potency on blocking gastric and small intestinal slow waves. For instance, $CaCC_{inh}$-A01 blocks slow waves in the murine stomach at 5 μmol/L and the small intestine at more than 30 μmol/L[143]. The mechanism underlying

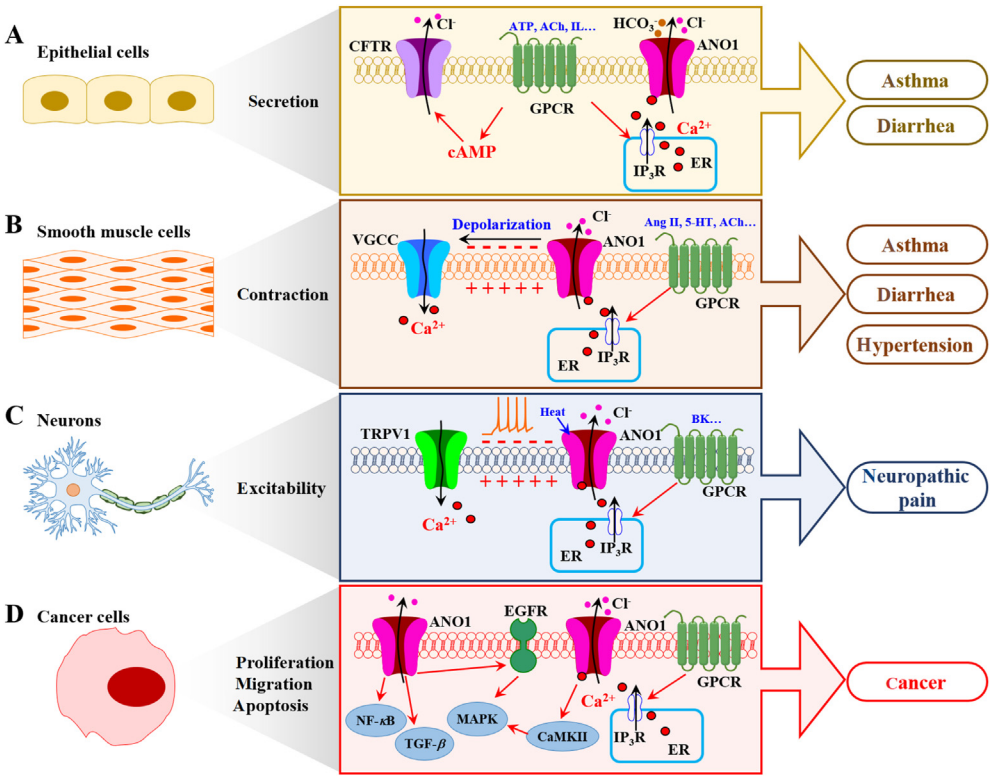



**Figure 2** Distribution of ANO1/TMEM16A in different tissues and its role in diseases. (A) In epithelial cells, ANO1 activation contributes to electrolyte and music secretion. Activation of GPCRs causes an increase in intracellular cAMP and $Ca^{2+}$, further inducing $Cl^-$ secretion through the activation of CFTR and ANO1. A crosstalk between CFTR and ANO1-dependent secretions also occurs for regulation of secretory signaling in the airway epithelia. ANO1 high expression or hyperactivity can cause inflammatory diseases such as asthma and diarrhea. (B) Outward flow of $Cl^-$ through the activation of ANO1 in smooth muscle cells causes depolarization and smooth muscle contraction. ANO1 high expression or hyperactivity is responsible for asthma diarrhea, and hypertension. (C) In DRG sensory neurons, activation of ANO1 by intracellular $Ca^{2+}$ or heat causes $Cl^-$ efflux and increases neuronal excitability for induction of neuropathic pain. The functional coupling between TRPV1 and ANO1 is also involved in nociception. (D) In cancer cells, ANO1 upregulation promotes cell proliferation and migration, whereas ANO1 downregulation induces apoptosis through multiple signaling pathways, including EGFR/MAPK signaling pathway[98], CaMKII/MAPK signaling pathway[51], TGF-$\beta$ signaling pathway[120] and NF-$\kappa$B signaling pathway[121]. Pharmacological activation of ANO1 by activators or potentiators may serve as a therapeutic strategy for treatment of CF, dry mouth and dry eye syndromes, and inhibition of ANO1 by inhibitors may be beneficial for ANO1 related channelopathies including asthma, diarrhea, hypertension, neuropathic pain and cancers.

these different sensitivities is not entirely clear, but several probabilities including other channels contributions, different splice variants and the local $Ca^{2+}$ concentrations may contribute to these differences[138]. Nevertheless, both GI epithelium and ICC are synergistically involved in the pathogenesis of diarrhea and downregulation of ANO1 in either type of the two cells may help mitigate diarrhea.

Rotavirus has been known to cause severe diarrhea in infants and young children, whereas red wine extracts with alcohol-free and $CaCC_{inh}$-A01 can prevent intestinal fluid loss in a neonatal mouse model of rotaviral diarrhea through inhibition of ANO1-mediated $Ca^{2+}$-activated $Cl^-$ secretion[53]. However, red wine extracts and $CaCC_{inh}$-A01 show no obvious effect on cholera toxin-induced diarrhea or CFTR current in cultured cells or intestinal absorption, suggesting the important role of ANO1 in diarrhea[53]. A recent study demonstrates that glucose and NSP4 synergistically increase the expression of ANO1 and $Ca^{2+}$-activated $Cl^-$ secretory in the mouse model of diarrhea[137]. Suppressing ANO1 expression in apical membranes of colonic epithelium decreases $Ca^{2+}$-activated $Cl^-$ secretion in chemical dextran sulfate sodium-induced chronic colitis in mice[144]. There are also observations that inhibition of ANO1 by non-specific ANO1 inhibitors including eugenol[74], shikonin[145], plumbagin[83], resveratrol dimer *trans*-$\varepsilon$-viniferin (TV) and tetramer $\gamma$-2-viniferin (RV)[69] can reduce water content in stools, but those observations should be confirmed with specific ANO1 inhibitors.

### 3.1.3. *Cystic fibrosis*

CF is an inherited disease of airway obstruction caused by mucus hypersecretion, mucus plugging and bronchoconstriction[146]. The dysfunction of CFTR $Cl^-$ channel is considered to be a major cause for CF as defective CFTR $Cl^-$ channel mutations are identified in CF patients[147]. It has been shown that small molecules can rescue dysfunctional CFTR mutation through increasing the number or open probability of CFTR $Cl^-$ channels. Unfortunately, these CFTR correctors or potentiators only exhibit limited efficacy for CF patients because of their multiple and different mutation sites of CFTR[148]. Therefore, an alternative strategy is CFTR-independent approach for treatment of CF by increasing the activity of ANO1 channel to promote mucus secretion[149]. This hypothesis is supported by observations that: 1) ANO1 is abundantly expressed in the airway goblet cells and upregulated in

inflammatory conditions[131]; 2) overexpression of ANO1 in CF human bronchial epithelia suppresses proinflammatory cytokine IL-8 secretion[150]; 3) specific knockdown of ANO1 in respiratory airways eliminates $Ca^{2+}$-and cAMP-activated $Cl^-$ secretion[151]; 4) *Ano1* gene knockout mouse exhibits abnormal trachea morphology, and also mucus obstruction and defective mucociliary clearance that presenting with a CF-like lung phenotyp[125,152].

Several studies demonstrate that ANO1 has a functional cross talk with CFTR through PSD-95/Dlg/AO-1 proteins[151,153]. Such an interaction between ANO1-mediated $Ca^{2+}$-activated $Cl^-$ secretion and CFTR-mediated cAMP-dependent $Cl^-$ secretion in airway epithelial cells strongly overlaps through a cAMP sensor protein termed exchange protein directly activated by cAMP and $Ca^{2+}$-sensitive adenylate cyclase type 1[154]. ANO1 expression and activity are deficient in CF patients and upregulation of ANO1 can improve mucus dynamics in CF mice[155]. These lines of evidence support the notion that ANO1 may represent an alternative therapeutic target to circumvent CFTR dysfunction in the airway epithelia of CF patients.

Some CFTR-independent drug candidates have been developed for potential CF therapy. Denufosol, a P2Y2 receptor agonist, promotes airway epithelial chloride secretion through activating CaCCs with P2Y receptors[156]. Unfortunately, denufosol failed in phase III trial because of its short half-life *in vivo*[147,156,157]. Recently, an ANO1 activator, ET000516-A-2 from pre-clinical study is expected to be evaluated in CF patients[147]. Another novel ANO1 potentiator ETX001 developed by Enterprise Therapeutics (Brighton, UK) was recently reported that it can enhance fluid secretion and improve mucociliary clearance in both primary CF bronchial epithelial cells and sheep model of CF-like airway disease[116]. These two candidates may bring us an exciting prospect for potential development of alternative CF therapy in the clinic.

#### 3.1.4. *Other epithelial diseases*

A recent study shows that ANO1 is overexpressed in mouse pancreatic tissue of acute pancreatitis model[158]. ANO1 promotes the pathogenesis of acute pancreatitis through activating the $IP_3R$/$Ca^{2+}$/NF-$\kappa$B/IL-6 pathway, and inhibition of ANO1 by $T16A_{inh}$-A01 reduces the pancreatic damage in acute pancreatitis mice[158]. ANO1 is also likely involved in other epithelial diseases, such as polycystic kidney disease (PKD), diabetic nephropathy and pulmonary fibrosis. The PKD is characterized by multiple bilateral renal cysts that gradually enlarge and can lead to a decline in renal function. ANO1 is found in human kidney and renal epithelial cell lines, and its expression is upregulated in the forskolin induced renal cyst model, autosomal dominant PKD patients and high-fat diet/streptozotocin-induced diabetic nephropathy mice[55,159,160], indicating a role of ANO1 in kidney disease.

ANO1 promotes renal cyst growth *via* induction of $Cl^-$ secretion and proliferation of cyst lining epithelium[159]. Inhibition of ANO1 by pharmacological inhibitors or gene knockdown significantly decreases glucose dependent cyst growth, reduces nephron numbers and also causes albuminuria and tubular damage[55,159,161]. Mechanistically, ANO1 drives the growth of renal cysts through enhancing $Ca^{2+}$ release from $IP_3R$ sensitive $Ca^{2+}$ stores[162] and lipid peroxidation also promotes renal cyst growth through activating ANO1[82]. In diabetic nephropathy, ANO1 deletion alleviates renal injury in diabetic mice through increasing nephrin expression, reducing the expression level of apoptosis related factors and also suppressing the activation of P38/JNK signaling pathway[160].

On the contrary, ANO1 activation aggravates renal injury by activating P38/JNK signaling pathway to promote podocyte apoptosis in diabetic nephropathy mice[160] and exacerbates inflammation *via* activating the TGF-$\beta$-SMAD3 pathway[163]. Downregulation of ANO1 by shRNA can inhibit apoptosis and promote the proliferation of lung fibroblasts in mouse model of idiopathic pulmonary fibrosis[163]. These observations suggest that inhibition of ANO1 may hold therapeutic potential for kidney and other epithelium-originated diseases.

### 3.2. *Cancers*

Epithelial cancer is also known as carcinoma that arises from epithelial tissues. Prior to identification as a CaCC, ANO1 was known as TMEM16A first described in 2003[164]. ANO1 was also named as DOG1 (gastrointestinal stromal tumor 1), TAOS2 (tumor amplified and overexpressed sequence 2) and ORAOV2 (oral cancer overexpressed 2) because of its overexpression in these cancers[35]. Investigations from ours and others show that ANO1 is overexpressed and involved in the pathogenesis of cancers especially originated from epithelial cancers, such as gastrointestinal stromal tumor (GIST)[165], head and neck squamous cell carcinoma (HNSCC)[166], prostate cancer[46], lung cancer[167], colon cancer[90], ovarian cancer[168], breast cancer[51], liver cancer[169], gastric cancer[120], esophageal cancers[170], pancreatic adenocarcinoma[171], salivary gland carcinoma[172] and glioblastoma[121] (Table 3[40,46,51,57,62,91,93,98,120,121,165–187]).

ANO1 upregulation in many kinds of cancers is related to its gene location at chromosome 11q13 that is frequently amplified in many malignant tumors[51,164] and amplification of 11q13 is associated with the increase of *ANO1* gene copy numbers[166]. Overexpression of ANO1 promotes proliferation and migration in multiple cancer cell lines[51,169,179], and ANO1 upregulation is associated with lower overall survival in patients with breast cancer, pancreatic cancer or gastric cancer[51,171,180] (Table 3). *ANO1* mRNA is also highly expressed in the blood of GIST patients[184] and patients with epithelial ovarian cancer, and the expression level of *ANO1* mRNA decreases after surgical removal of tumors[168], suggesting that detection of *ANO1* gene in blood may serve as a biomarker for early diagnosis of cancer.

Multiple signaling pathways have been shown to be involved in ANO1 modulation in cancer development (Table 3). In breast cancer and HNSCC, downregulation of ANO1 by knockdown or pharmacological inhibition inhibits cancer cell proliferation, induces apoptosis and reduces tumor growth through reducing epidermal growth factor receptor (EGFR) cell signaling pathways[51]. Studies have also shown that ANO1 can affect the progression of intestinal cancer, liver cancer, and pancreatic cancer through the EGFR pathway[169,171,187], as well as the modulation of glioma by NF-$\kappa$B signaling[121]. In prostate cancer, ANO1 can regulate TNF-$\alpha$ signaling to contribute to cell growth and apoptosis[57]. In epithelial ovarian cancer, silencing ANO1 can suppress cancer cell proliferation, migration and invasion as well as the growth of xenograft tumors through inactivation of PI3K/AKT cell signaling pathway[168]. In gastric cancer, ANO1 overexpression can promote tumor invasion and predict poor prognosis through affecting TGF-$\beta$ signaling function[120,180], that is also the target for ANO1 regulating cell proliferation, migration and invasion in esophageal squamous cell carcinoma (ESCC)[179]. In glioblastoma, ANO1 expression is regulated by CaMKII-$\beta$ and suppression of CaMKII-$\beta$ inhibits ANO1 mediated glioblastoma development[183], and CaMKII also plays a role in ANO1-mediated

**Table 3** ANO1 modulation, expression and function in cancer cell lines, xenograft tumors and human cancer tissues.

| Cancer type | High expression | | | Cell assay | | Inhibition | | Signaling pathways | Ref. |
|---|---|---|---|---|---|---|---|---|---|
| | Cell line | Human tissue | Clinical implication | Proliferation/ viability | Migration/ invasion | Tool | Xenograft tumor | | |
| Breast cancer | ZR75-1, HCC1954, MDA-MB-415 | + | Poor Prognosis (+) | + | NR | shRNA/$CaCC_{inh}$-A01 | Tumor growth (−) | 11q13 amplification, $Cl^-$ channel activity, Apoptosis, EGFR, CAMKII, AKT, MAPK | 51 |
| | YMB-1 | NR | NR | + | NR | siRNA/NFA | NR | Epigenetic regulation | 40 |
| | YMB-1, MDA-MB-453 | NR | NR | NR | NR | siRNA/$T16_{inh}$-A01 | NR | AKT, STAT3 | 173 |
| | SKBR3 | NR | Improved response to biological therapies (−) | + | NR | siRNA/$T16A_{inh}$-A01 $CaCC_{inh}$-A01 | NR | EGFR, HER2, STAT3 | 174 |
| | MCF-7, T47D | + | Shorter overall survival in ER + patients (+) | + | NR | shRNA/$T16A_{inh}$-A01 | Tumor growth (−) | EGFR/STAT3 signaling | 175 |
| HNSCC | UM-SCC1, T24 | + | Poor prognosis (+) | + | NR | shRNA/$T16A_{inh}$-A01 | Tumor growth (−) | MAPK, Ki67 | 176 |
| | HEp-2, SCC-25 | + | A marker for distal metastasis (+) | No effect | + | siRNA/NFA, DIDS, Fluoxetine | NR | 11q13 amplification | 166 |
| | UM-SCC1, T24 | High in primary tumor and low in metastatic tumor | A biomarker for metastasis (−) | NR | − | shRNA | Tumor growth (−), Metastatic development (+) | Promoter methylation, E-cadherin | 177 |
| | FaDu | NR | Poor prognosis (+) | + | NR | shRNA/$CaCC_{inh}$-A01 | Tumor growth (−) | 11q13 amplification, $Cl^-$ channel activity, Apoptosis, EGFR, CAMKII, AKT, MAPK | 51 |
| | Cal-33, OSC19, UM-SCC-1, FaDu | NR | Increased efficacy of biologic therapies (−) | OSC19 (+) | NR | siRNA/$T16A_{inh}$-A01 $CaCC_{inh}$-A01 | ANO1-overexpressing tumors were heavier than control tumors | EGFR, HER2, STAT3 | 174 |
| | OSC19, FaDu, UM-SCC-1 | Positively correlated with tumor size | Recurrence of cancer (+) | + | NR | shRNA | ANO1-overexpressing tumors were greater than control tumors | ERK, BIM, Apoptosis | 178 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| ESCC | KYSE30, KYSE510 | + | Lymph node metastasis and advanced clinical stage (+) | + | NR | siRNA | NR | 11q13 amplification | 170 |
| | KYSE410, KYSE30 | + | Poor prognosis (+) Advanced stage (+) | + | + | shRNA | NR | TGF-$\beta$ signaling, cell cycle | 179 |
| Prostate cancer | PC-3, LNCap, RWPE1 | + | NR | + | NR | siRNA/$T16A_{inh}$-A01, $CaCC_{inh}$-A01, MONNA, tannic acid | Tumor growth (−) | ERK, AKT | 62 |
| | PC-3, LNCaP | NR | NR | + | NR | siRNA/NFA | NR | Epigenetic regulation | 40 |
| | LNCaP, PC-3 | + | Clinical TNM stage (+) Gleason score (+) | + | + | shRNA/DIDS | Tumor growth (−) | NR | 46 |
| | PC-3 | NR | NR | + | NR | shRNA siRNA/$CaCC_{inh}$-A01, $T16A_{inh}$-A01, Ani9 | Tumor growth (−) | TNF-$\alpha$ signaling, apoptosis | 57 |
| Gastric cancer | AGS, BGC823 | + | Poor overall survival (+) TNM (+) Lymphnode metastasis (+) | No effect | + | shRNA | NR | TGF-$\beta$, E-cadherin | 180 |
| | AGS, BGC823 | Negatively related with miR381 | Poor prognosis (+) | No effect | + | siRNA | NR | Regulated by miR-381, TGF-$\beta$, E-cadherin | 120 |
| | AGS, SGC7901 | + | TNM stage (+) | NR | + | siRNA | Tumor metastasis (−) | Regulated by SP1 through MLL1 and H3K4 trimethylation | 181 |
| HCC | SMMC7721 | + | NR | + | + | siRNA | Tumorigenicity (−) | MAPK signaling, cell cycle | 169 |
| | HepG2, SMMC7721 | + | Tumor grade (+) | + | + | shRNA | Tumor growth (−) | PI3K/AKT-MAPK signaling pathway, apoptosis, cell cycle | 182 |
| Glioma | U87MG | + | Tumor grade (+) | + | + | siRNA | NR | NF-$\kappa$B signaling | 121 |
| | U251, U87MG | NR | NR | NR | + | shRNA/$T16A_{inh}$-A01 | NR | CaMKII-$\beta$ | 183 |
| Lung cancer | GLC82, NCI -H520 | + | NR | + | + | shRNA | Tumor growth (−) | NR | 167 |
| | H1299 | NR | NR | + | + | shRNA/$T16A_{inh}$-A01 | Tumor growth (−) | EGFR/MAPK signaling | 98 |

(*continued on next page*)

**Table 3** (*continued*)

| Cancer type | High expression | | | Cell assay | | Inhibition | | Signaling pathways | Ref. |
|---|---|---|---|---|---|---|---|---|---|
| | Cell line | Human tissue | Clinical implication | Proliferation/ viability | Migration/ invasion | Tool | Xenograft tumor | | |
| Pancreatic adenocarcinoma | BxPC-3, AsPC-1, Capan-1 | NR | NR | No effect | + | siRNA/T16A$_{inh}$-A01, CaCC$_{inh}$-A01, NS3728 | NR | NR | 93 |
| | AsPC-1 | + | Poor prognosis (+) Biomarker (+) | NR | + | shRNA | NR | Ligand-dependent EGFR signaling | 171 |
| GIST | GIST-T1, GIST-882 | NR | NR | No effect | NR | shRNA/T16A$_{inh}$-A01, NFA, NPPB | Tumor growth (−) | IGFBP-5, no effect on KIT | 165 |
| | NR | Cancer and PBMCs | Biomarkers (+) Tumor size (+) | NR | NR | NR | NR | NR | 184 |
| | GIST-T1, GIST882 | NR | NR | + | NR | T16A$_{inh}$-A01 CaCC$_{inh}$-A01 | NR | Cell cycle | 91 |
| Salivary gland carcinoma | NR | + | NR | NR | NR | NR | NR | NR | 172 |
| Ovarian cancer | SKOV3 | Cancer and PBMCs | Pathologic stage and differentiation (+) | + | + | siRNA | Tumor growth (−) | PI3K/AKT signaling | 168 |
| Colorectal cancer | SW620 | NR | NR | + | + | shRNA | NR | MAPK signaling, cell cycle | 185 |
| | DLD-1, HCT116 | Liver metastasis cancer tissue | Poor prognosis (+) | + | + | siRNA | NR | Regulated by miR-132 | 186 |
| | SW480 | + | Poor prognosis (+) | NR | + | siRNA | NR | EGFR signaling, regulated by miR-144 | 187 |

+, positive effect; −, negative effect; NR, no report. HCC, hepatocellular carcinoma; PBMCs, peripheral blood mononuclear cells; TNM, tumor, lymph nodes and metastasis.

tumorigenic properties of HNSCC and breast cancer[51]. It appears that ANO1 can regulate different signaling pathways in the same type of tumor or the same signaling pathway in different types of tumor.

Inhibition of ANO1 by gene silencing or pharmacological means can suppress cancer cell proliferation and migration, invasion and tumor growth[62,175,181]. Small molecules, such as $CaCC_{inh}$-A01, $T16A_{inh}$-A01, MONNA and Ani9, as well as natural products including avermectins and flavonoids exhibit anticancer effects through inhibition of ANO1 activity[57,62,78,84], suggesting that ANO1 may serve as a potential drug target for cancer therapy.

### 3.3. *Hypertension*

Hypertension or high blood pressure is a common condition in which the long-term force of the blood against the blood vessels is high enough that it may increase the risk of heart diseases and brain stroke. ANO1 is expressed in various smooth muscle cells of arteries and veins[188,189], suggesting a role of ANO1 in regulation of vasoconstriction. Activation of $Ca^{2+}$-activated $Cl^-$ current can lead to $Cl^-$ efflux after $Ca^{2+}$ influx and membrane depolarization, thus resulting in vasoconstriction and increase of blood pressure. Indeed, ANO1 expression and activity are upregulated in murine pulmonary arterial myocytes induced by chronic hypoxia, which contributes to pulmonary hypertension[60]. Similar results were also observed in monocrotaline-induced pulmonary hypertension rats[188], spontaneously hypertension rats[63], idiopathic pulmonary arterial hypertension patients[190,191], and high-flow-induced pulmonary arterial hypertension (PAH) rats[192]. Conversely, cell-specific knockout of ANO1 reduces blood pressure and attenuates hypertension in mice and spontaneously hypertensive rats[193–195].

A recent study shows that upregulation of ANO1 depolarizes pulmonary artery smooth muscle cells (PASMC) membrane potential, contributing vasoconstriction and the increased pulmonary vascular resistance in PAH rats[190]. Conversely, pharmacological inhibition or gene silencing of ANO1 reverse the membrane depolarizes of PASMC. For instance, a specific ANO1 inhibitor MONNA hyperpolarizes the rat coronary artery smooth muscle cell membrane potential and increases coronary flow[63]. Vascular smooth muscle cells (VSMC) are the stromal cells of the vascular wall and are responsible for regulating arterial tone, and blood pressure. Overexpression of ANO1 in healthy donor PASMC promotes the cell proliferation and produces an idiopathic pulmonary arterial hypertension (IPAH)-like phenotype. Pharmacological inhibition of ANO1 may reverse vasoconstriction and remodeling of pulmonary arteries in IPAH[190,196]. These investigations suggest the involvement of ANO1 in VSMC contraction and vascular remodeling for hypertension.

Circulating angiotensin II (Ang II) as a major contributor of the renin−angiotensin system is upregulated during hypertension, and Ang II is frequently used to establish hypertension models. It has been shown that Ang II significantly enhances ANO1 expression in human umbilical vein endothelial cells and endothelial-specific *ANO1* knockout significantly reduces Ang II-induced hypertension through ROS signaling pathway, whereas endothelial-specific transgenic ANO1 shows the opposite effect[195]. These studies suggest that inhibition of ANO1 function may be beneficial for hypertension, and ANO1 inhibitors, including $T16A_{inh}$-A01, MONNA, and Ani9 can inhibit agonist-induced vesical constriction and cause vasorelaxation[63,94].

### 3.4. *Nociception*

Nociception is a perception in response to painful or harmful stimuli, such as heat, cold, mechanical and chemical stimulus in the environment. Dorsal root ganglion (DRG) is a cluster of sensory neurons in the dorsal root of spinal nerves responsible for pain signal transmission[197]. ANO1 is mainly expressed in small diameter DRG neurons that are intimately involved in nociception[4,198], suggesting a modulatory role of ANO1 in pain sensation. It has been shown that an inflammatory mediator bradykinin, released from damaged tissues or applied exogenously can activate ANO1 through B2 receptors and PLC pathway, subsequently depolarizing membrane potential and markedly stimulating firings in DRG neurons, and pharmacological inhibition of ANO1 attenuates pain behaviors[44]. ANO1 expression is upregulated in the spinal cord and DRG neurons after spinal nerve injury, suggesting the involvement of ANO1 in development of neuropathic pain[54]. Protease activated receptor 2 (PAR2), also known as G-protein coupled receptor 11, has been identified to be involved in the pathogenesis of pain[199]. PAR2 and ANO1 are co-localized in DRG neurons, and their expressions are increased in rat pain model of chronic constriction injury[200].

ANO1 can be activated by temperatures over 44 °C, and silencing of ANO1 in DRG neurons significantly reduces nociceptive behavior in thermal pain model[198], inflammation and nerve-injury induced hyperalgesia or allodynia[54,201]. Thus, downregulation of ANO1 activity may present a potential therapeutic strategy for neuropathic pain. ANO1 as a potential target for pain is evidenced by observations that inhibition of ANO1 by small molecules NPPB, NFA, $T16A_{inh}$-A01 or $CaCC_{inh}$-A01 reduces capsaicin-induced inward current and action potential firing, and as well as pain-related behaviors[99,100]. Again, these ANO1 inhibitors are not specific and a key question such as whether ANO1 directly affects the excitability of nociceptors should be addressed.

### 3.5. *Others*

A recent study shows that ANO1 is expressed in mouse brain endothelial cells where ANO1 expression is upregulated after ischemic stroke induced by the middle cerebral artery occlusion[56]. Targeting ANO1 with inhibitor $CaCC_{inh}$-A01 or silencing attenuates blood−brain barrier (BBB) breakdown after ischemic stroke through decreasing intracellular adhesion molecule-1 *via* NF-$\kappa$B signaling pathway, suggesting that downregulation of ANO1 protects BBB disruption after ischemia stroke. It is supported by another study that ANO1 inhibitor $T16A_{inh}$-A01 or siRNA inhibits proliferation and migration of brain capillary endothelial cells that comprise BBB[202]. It would be interesting to see more studies that are designed to validate ANO1 as a therapeutic target for brain stroke.

## 4. Summary and perspectives

The CaCC ANO1 channel is expressed in a wide variety of epithelial cells, smooth muscle cells and neurons. Although abnormal expression or dysfunction of ANO1 is involved in the

pathology of many diseases, validating ANO1 as a therapeutic target still presents a big challenge. While a significant progress has been made for the distribution, expression, structure and pathophysiological functions of ANO1, there still exists an urgent need for selective modulators of the channel for target validation. Current available ANO1 modulators are also in preclinical stage without any treatments ready for clinical utility, which highlights the much-needed efforts in understanding the channel pharmacology and validation of ANO1 as therapeutic target. The recent structure of ANO1 solved by single-particle cryo-electron microscopy can provide a valuable model for the design of more potent and selective ANO1 modulators that can be used to help validate this emerging target for therapeutic potential of diseases, including cancer, inflammatory epithelial diseases, neuropathic pain and hypertension and cystic fibrosis lung disease.

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## Author contributions

Yani Liu, Zongtao Liu, and KeWei Wang wrote and edited the manuscript. Yani Liu and KeWei Wang contributed to manuscript revision and discussion of the content.

## Conflicts of interest

The authors declare that there is no conflict of interest.

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