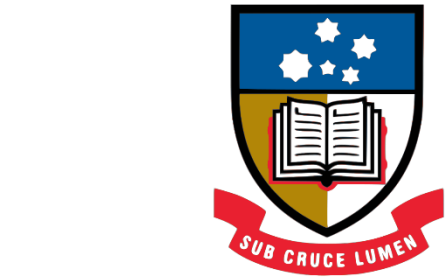

THE UNIVERSITY of ADELAIDE

Role of Aquaporin1 in Diseases and Drug Discovery

A thesis submitted in fulfilment for degree of

DOCTOR OF PHILOSOPHY

In

The Discipline of Physiology
Adelaide Medical School
The University of Adelaide

By

Pak Hin Chow

05.2.2020

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Thesis Abstract

Aquaporin-1 is a dual water and ion channel that mediates transport of water, glycerol, ions, and small molecules. AQP1 has reported to involve in a wide range of physiological functions and human diseases. At the cellular level, AQP1 promoted water movement across cell plasma membrane facilitates transepithelial fluid transport, cell migration, and cell volume change. Genetic knockdown studies of AQP1 showed impairment in fluid transportations, suggesting that pharmacologically targeting AQP1 can alter water movement in cell, which could be a useful approach for AQP1 related diseases. This thesis is aims to discover new pharmacological agents for both AQP1 water and ion channel, and test these agents in two diseases, cancer and sickle cell diseases. The first part of this thesis investigated the role of AQP1 in cancer migration. AQP1 expression is upregulated in many aggressive cancers, including colon, breast, and brain cancer. Overexpression of AQP1 in cancer is thought to facilitate cell migration, invasion and metastasis. Work here discovered a novel AQP1 ion channel blocker, 5-hydroxymethyl-furfural (5HMF). Results here showed that 5HMF impaired AQP1 ion channel conductance in AQP1-expressing oocytes, and significantly reduced cell migration and invasion in AQP1-expressing cancer cell lines. Work here also showed that KeenMind extracts from *Bacopa monnieri* blocked the AQP1 water channel and slowdown cancer cell migration in AQP1-expressing cell line. Both of the pharmacological agents did not induce any toxic effect to tested cell lines. In the last part of this thesis, the AQP1 ion channel was investigated for its role in sick cell disease. Mutated haemoglobin in red blood cell undergoes polymerization in deoxygenated conditions. Several novel anti-sickle agents were tested its effect on AQP1 ion channel activity. Work here showed that high efficacy agents impaired AQP1 ion channel activity, while low efficacy agents had no effect of AQP1 ion channel, suggesting that AQP1 ion channel is one of the factor that facilitate sickle cell formation. Work in this thesis paves the way for developing an

AQP1-based therapy and revealing a possible role of AQP1 ion channel in sickle cell diseases.

Declaration

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in my name in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in the future, be used in a submission in my name for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint award of this degree.

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Pak Hin Chow

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Thank you to my secondary supervisor, Associate Professor Joanne Bowen. I truly appreciate her support and guidance throughout my candidature. Our regular meetings, discussions, and email correspondence was of considerable value to me.

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I would also like to say a heartfelt thank you to my family for always believing in me and encouraging me to follow my dreams. Finally, I must express my very profound gratitude to my girlfriend Kei Lih Tan for providing me with unfailing support and continuous encouragement throughout my years of study and through the process of researching and writing this thesis. This accomplishment would not have been possible without them. Thank you.

Publications Arising From This Thesis

First author:

Chow PH, Kourghi M, Pei JV, Nourmohammadi S & Yool AJ (2019) 5-hydroxymethyl-furfural and structurally related compounds block the ion conductance in human aquaporin-1 channels, and slow cancer cell migration and invasion. *Molecular Pharmacology* (under review)

Co-author:

Kourghi M, Pei JV, De Ieso ML, Nourmohammadi S, **Chow PH** & Yool AJ (2017) Fundamental structural and functional properties of Aquaporin ion channels found across the kingdoms of life. *Clinical and Experimental Pharmacology and Physiology*, vol. 45, no.4, pp. 401-409.

De Ieso ML, Pei JV, Nourmohammadi S, Smith E, **Chow PH,** Kourghi M, Hardingham, JE & Yool AJ (2019) Combined pharmacological administration of AQP1 ion channel blocker AqB011 and water channel blocker Bacopaside II amplifies inhibition of colon cancer cell migration. *Scientific Reports* ,vol 9, pp 12635.

Chapter 1: Aquaporins and Cancer - A systematic review

1.1 Brief introduction

Aquaporins (AQPs) are a family of integral proteins that mediate the transport of water, glycerol, ions, and other molecules (King et al., 2004). AQP1, originally named channel like integral protein of 28 KDA (CHIP28), was first identified in red blood cells and the epithelium of proximal tubules (Denker et al., 1988a, Preston and Agre, 1991a). The mammalian AQP1 is highly expressed in secretory epithelia, including exocrine glands (Burghardt et al., 2006), choroid plexus (Boassa and Yool, 2005), and the ocular ciliary epithelium (Hamann et al.,1998). Therefore, AQP1 is linked to numerous pathologies including tumor metastasis (De Ieso and Yool, 2018a), fluid dysregulation (Nielsen et al., 2007), and traumatic injury (Badaut et al., 2014). Genetic knockdown studies of AQP1 provided insights into the development of AQP1-based therapy. Mice with AQP1 deficient showed significantly reduced ability to concentrate urine (Schnermann et al., 1998). In these mice, normal absorption of water filtered from glomerulus was impaired (Ma et al., 1998a); the water permeability at loop of Henle was reduced (Chou et al., 1999); the renal counter-current multiplication and exchange systems that responsible for concentrating urine at the loop of Henle was also impaired (Pallone et al., 2000). Thus, inhibiting AQP1 water channel is predicted to increase water diuresis by a mechanism that is distinct from traditional salt transport-blocking diuretics, which suggests the therapeutic potential of AQP1 inhibitor in fluid related diseases. Pharmacologically targeting AQP1 is unlikely to produce minor side effects. Studies on patients missing endogenous AQP1 have reported symptoms of diuresis exclusive of other abnormalities (King et al., 2001), suggesting the clinical benefits would outweigh the side effect. This thesis is focusing on two AQP1 related diseases, cancer and sickle cell disease. Chapter 1 is an

introductory chapter, introducing the role of AQPs in cancer.

Combined Systematic Review and Transcriptomic Analyses of Mammalian Aquaporin Classes 1 to 10 as Biomarkers and Prognostic Indicators in Diverse Cancers

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1.2 Abstract

Aquaporin (AQP) channels enable regulated transport of water and solutes essential for fluid homeostasis, but they are gaining attention as targets for anticancer therapies. Patterns of AQP expression and survival rates for patients were evaluated by systematic review (PubMed and Embase) and transcriptomic analyses of RNAseq data (Human Protein Atlas database). Meta-analyses confirmed predominantly negative associations between AQP protein and RNA expression levels and patient survival times, most notably for AQP1 in lung, breast and prostate cancers; AQP3 in esophageal, liver and breast cancers; and AQP9 in liver cancer. Patterns of AQP expression were clustered for groups of cancers and associated with risk of death. A quantitative transcriptomic analysis of AQP1-10 in human cancer biopsies similarly showed that increased transcript levels of AQPs 1, 3, 5 and 9 were most frequently associated with poor survival. Unexpectedly, increased AQP7 and AQP8 levels were associated with better survival times in glioma, ovarian and endometrial cancers, and increased AQP11 with better survival in colorectal and breast cancers. Although molecular mechanisms of aquaporins in pathology or protection remain to be fully defined, results here support the

hypothesis that overexpression of selected classes of AQPs differentially augments cancer progression. Beyond fluid homeostasis, potential roles for AQPs in cancers (suggested from an expanding appreciation of their functions in normal tissues) include cell motility, membrane process extension, transport of signaling molecules, control of proliferation and apoptosis, increased mechanical compliance, and gas exchange. AQP expression also has been linked to differences in sensitivity to chemotherapy treatments, suggesting possible roles as biomarkers for personalized treatments. Development of AQP pharmacological modulators, administered in cancer-specific combinations, might inspire new interventions for controlling malignant carcinomas

1.3 Introduction

Membrane channels and transporters have been well characterized as essential for the cellular control of ion and fluid homeostasis and electrical signalling (Dubyak, 2004) and serve key roles in cell proliferation, migration, apoptosis and differentiation that are increasingly being recognized as highly relevant in cancer progression as well as in normal physiological function (Litan and Langhans, 2015, Dubyak, 2004). Several classes of mammalian aquaporin (AQP) channels have been linked to cancer progression, via effects on angiogenesis, proliferation and metastasis (Nico and Ribatti, 2010); pharmacological modulators of aquaporin channels derived from loop diuretics, metal-containing organic compounds, plant natural products, and other small molecules point to therapeutic potential of AQPs as novel targets (De Ieso and Yool, 2018b)

Cancer cells escape normal control mechanisms and invade surrounding tissues or spread to other parts of the body, in a process known as metastasis that makes cancer the world's second greatest cause of mortality based on World Health Organization statistics, accounting for 9.6 million deaths in 2018 (Bray et al., 2018). As life spans increase, the incidence of cancer in the aging population is steadily increasing, with the figures expected to rise by 70% in the next two decades (Ferlay et al., 2015). Treatment options include surgery, chemotherapy and radiotherapy which are targeted primarily at inhibiting cancer proliferation (Siegel et al., 2016). Recurrence of cancers indicates that additional treatments targeting cancer metastasis are greatly needed (Steeg and Theodorescu, 2008).

The migration process underpinning metastasis begins with polarization of a cancer cell to create protrusions at leading edges in the direction of the movement, dependent in part on actin polymerization and extracellular signals (Condeelis, 1993, Devreotes and Horwitz, 2015). In metastasis, cancer cells spread to secondary sites commonly along lymphatics, blood vessels, and coelomic cavities (Spano et al., 2012, Brooks et al., 2010). Cell migration

requires dynamic generation of cytoplasmic protrusions, attachment, and physical displacement through the extracellular matrix (ECM) and stroma. Liotta and colleagues noted the steps involve first the attachment of cancer cells to ECM through cell surface receptors, ECM local degradation by secreted enzymes, and then cancer cell migration (Liotta, 2016). Inhibitors of AQPs are of interest as potential tools for impairing the protrusion and displacement steps in metastatic cell movement (McCoy and Sontheimer, 2007a, McCoy et al., 2010).

Aquaporins

Aquaporins (AQPs) are transmembrane proteins best known as channels that facilitate passive water transport in response to osmotic gradients, which are created by the active transport of solutes between the intracellular and extracellular compartments (Preston et al., 1992, Denker et al., 1988b, Agre et al., 1993). AQPs are highly expressed in the tissues responsible for fluid homeostasis such as kidneys, lung airways and peripheral vascular system (Jenq et al., 1999, Kitchen et al., 2015). Thirteen classes of AQPs (AQP0-AQP12) have been described in higher mammals, and divided into two groups according to general functional properties (Ishibashi et al., 2009, Finn et al., 2014). Classical aquaporins (AQP0,-1,-2,-4,-5,-6,-8) show predominant roles in water transport, though some members of the family also transport gases, urea, hydrogen peroxide, ammonia, and charged particles (Yool, 2007, Yool and Campbell, 2012b). Aquaglyceroporins (AQP3,-7,-9,-10) were defined by permeability to glycerol as well as water. Atypical AQP11 and -12 differ from other mammalian AQPs (Ishibashi, 2009) and their functions remain to be fully defined. AQP channels are tetramers of subunits with signature asparagine-proline-alanine (NPA) motifs in the intrasubunit pore domains, which mediate selective, bidirectional, single-file transport of

water in the classical aquaporins, and water and glycerol in aquaglyceroporins (Sui et al., 2001, Raina et al., 1995).

The first aquaporin, lens major intrinsic protein AQP0, was cloned in 1984, and AQP1 was cloned in 1991 (Gorin et al., 1984, Preston and Agre, 1991b) with other members of the family soon after. The involvement of AQPs in cancer is supported by studies reporting AQP overexpression in at least 12 different tumor cell types (Papadopoulos and Saadoun, 2015). In many tumors, a positive correlation has been observed between histological tumor grade and AQPs expression levels, involving AQPs subtypes not necessarily expressed in the original tissues (Wang et al., 2015b). AQPs are hypothesized to promote cancer metastasis by facilitating tumor cell migration. The purpose of this systematic review was to investigate whether there is a compelling link between AQP subtype expression pattern and cancer survival, based on published observations and cancer transcriptomic databases analyses.

1.4 Materials & methods

Systematic review protocol & Inclusion criteria

Data included in this analysis included all primary research published between 1st January 1990 and 1st January 2019 that was indexed in Pubmed or Embase (OVID version) online databases, which investigated aquaporins and cancers. Papers included work involving use of biopsies, cancer cell lines, animal models and human patient cohort studies. Lists of the search keywords used for querying the online databases are summarized in Table 1.

For positive hits identified using the search keywords, titles and abstracts were screened to determine whether the retrieved studies met the outlined criteria (Table 1). Review articles, conference abstracts and studies that did not have a focus on aquaporins were excluded. For

studies that met selection criteria, the complete published papers were obtained and evaluated (Figure 1).

Forest plot analyses

Hazard ratios for overall survival rates in people with cancer were extracted from a broad literature search. Forest plots using the random effects model were than generated to assess the hazard ratios and 95% confidence interval for each class of AQPs, using Stata software (Stata software, StataCorp, Texas,US).

Overall survival times of cancer patients were obtained from the Human Protein Atlas data base available at https://www.proteinatlas.org/humanproteome/pathology (Ponten et al., 2008, Uhlen et al., 2017). Patients were classified into high expression or low expression groups based on RNAseq data quantified as the fragments per kilobase of exon per million reads mapped (FPKM) values for AQP transcript levels in human biopsy samples. Obtained data were used to calculate hazard ratios for each class AQPs classified by the type of cancer using Graphpad Prism 8 (GraphPad Software, San Diego, CA). Forest plots using the random effects model were generated to determine hazard ratios for each class of AQPs, using Stata software.

Statistical analyses

Box plots were generated using Graphpad Prism 8 software to show transcript levels by AQP class in glioma, colon cancer, lung cancer, breast cancer, ovarian cancer, and endometrial cancer based on Human Protein Atlas transcriptomic data. The median values for the transcript levels for all AQP classes in individual patient samples were used as a point of reference for statistical comparisons. Statistical analyses were done using Graphpad Prism 8.

Statistically significant outcomes determined by the non-parametric Mann-Whitney U test are reported as (*) $p<0.05$. NS is not significant.

1.5 Results

A total of 1546 papers were identified based on the first level of screening of PubMed and Embase databases with search keywords (**Table 1**). After excluding 1185 duplicate search results, 361 records were selected for the second level screening of titles and abstracts. After careful assessment, 76 records were excluded and 285 were retrieved for full review. Papers lacking sufficient focus on AQPs were excluded (with reasons logged), leaving a final set of 224 papers included in this review (**Figure 1**). Within this final set, the largest proportion of studies addressed AQP1, followed by AQP5; AQP4; AQP3; and AQP9 for a diverse array of cancer types, predominantly in brain cancer, followed by lung cancer, breast cancer, and colorectal cancer (**Table 2**).

Forest plots (**Figure 2**) summarizing the survival probabilities of people with cancers reported in the published literature were correlated with levels of expression of different classes of AQPs (based on RNA, protein levels or both), as determined from compiled data from all papers in the final set, which included survival analyses (n = 30). Results indicated strongly negative correlations. AQP1 appeared to be associated with higher risks of death in lung adenocarcinoma patients with a four-fold increase in hazard ratio (HR 4.0) and in pleural mesothelioma (HR 2.7), as well as breast, prostate and some colon cancers (HRs 2.6 to 3.4). Dramatic increases in patient risk were observed for AQP3 in esophageal cancer (HR18.4), and AQP9 in liver cancer (HR 10.8). High hazard ratios for gastric cancer patients correlated with increased AQPs 2, 8 and 10 expression, contrasting with a reduced hazard ratio seen when AQP3 or AQP9 levels were increased. For breast cancer patients, higher hazard ratios were observed with increased AQPs 1 and 3 expression; possible associations

with other AQP classes remain to be evaluated. These data support the idea that AQPs are upregulated in cancers and that the specific classes of AQPs involved and patterns of co-regulation depend on the cancer subtype [10]. It is important to note that, in some cases, such as hepatocellular carcinoma, increased expression of AQP1 is associated with the vasculature, and rarely the cancer cells themselves (Yool et al., 2010).

The finite number of publications in this diverse field precluded an exhaustive comparison of all classes of AQPs across all cancer types. To augment the systematic review data, an independent analysis of AQP expression levels by cancer type was carried out for glioma, colorectal, lung, breast, ovarian and endometrial cancers, with transcriptomic data compiled from the Human Protein Atlase database. Transcriptomic outcomes were compared with the results of the systematic review, as summarized in the sections below, to identify corroborating lines of evidence, inconsistent findings, as well as interesting gaps in knowledge that could merit future research.

Table 1. Search terms used for data collection. (searches combined the left and right sets with AND)

Embase	
Neoplasm/exp OR cancer *: tiab OR neoplasm *: tiab OR Metastas *: tiab OR Tumor *: tiab OR Tumor *: tiab	Aquaporin/de OR "Aquaporin1"/de OR "Aquaporin2"/de OR "Aquaporin3"/de OR "Aquaporin4"/de OR "Aquaporin5"/de OR "Aquaporin6"/de OR "Aquaporin7"/de OR "Aquaporin8"/de OR "Aquaporin9"/de OR "Aquaporin10"/de OR "Aquaporin11"/de OR "Aquaporin12"/de OR "Aquaporin0"/de OR

	Aquaporin *: tiab OR “Water channel *”: tiab OR AQP *: tiab OR CHIP28 *: tiab Aquaporin*:tiab OR "Water channel*":tiab OR AQP*:tiab OR CHIP28*:tiab
Pubmed	
Neoplasms[mh] OR cancer *[tiab] OR neoplasm *[tiab] OR “Neoplasm Metastasis” [mh] OR Metastas *[tiab] OR Tumor *[tiab] OR Tumor *[tiab]	Aquaporins[mh] OR Aquaporin *[tiab] OR “Water channel *”[tiab] OR AQP *[tiab] OR CHIP28 *[tiab] OR MIP *[tiab]

* The asterisk is a wildcard symbol used to broaden search terms for literature database queries.

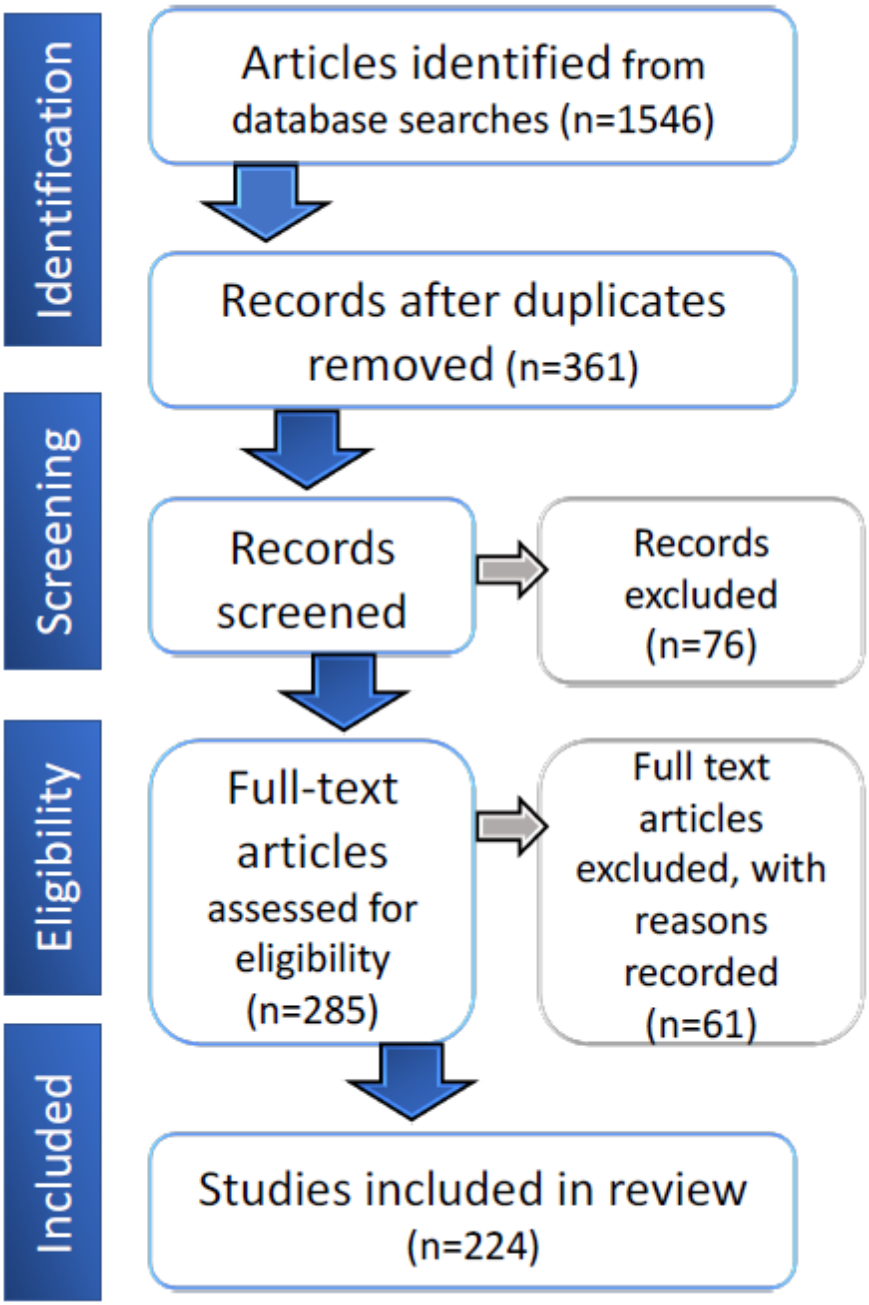
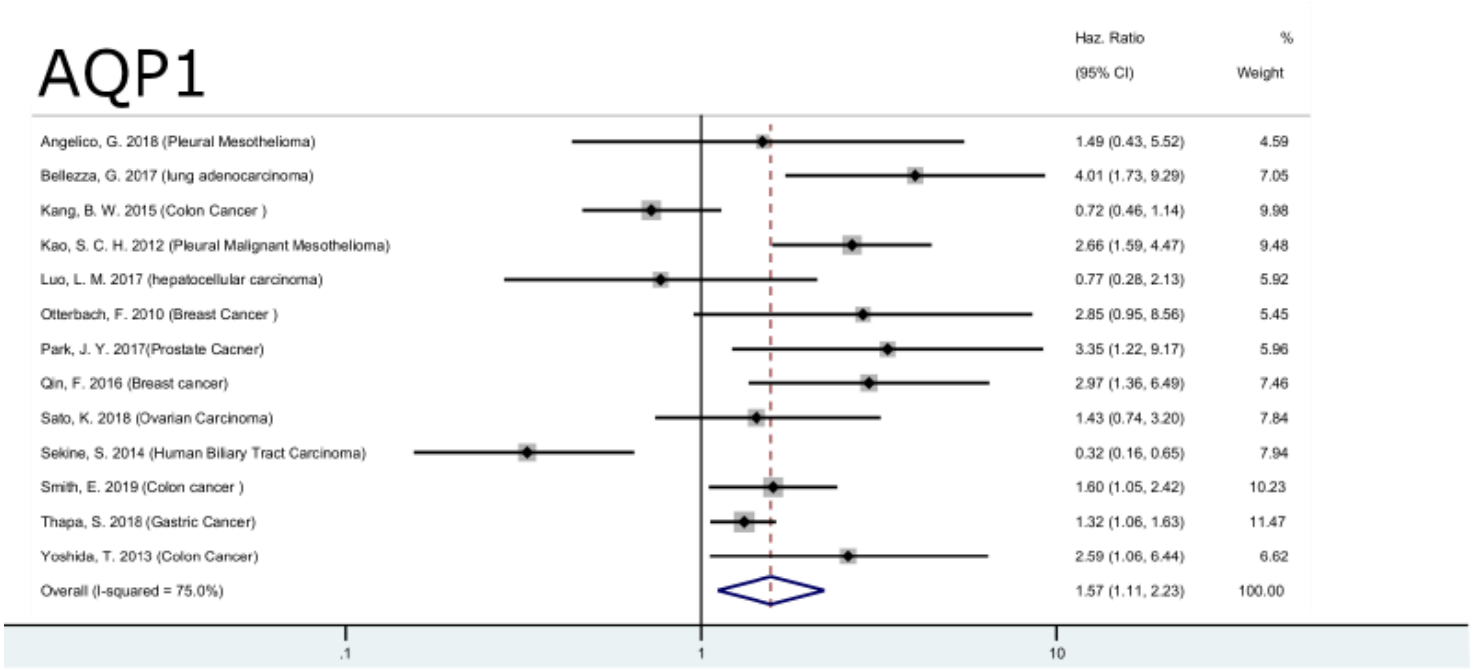
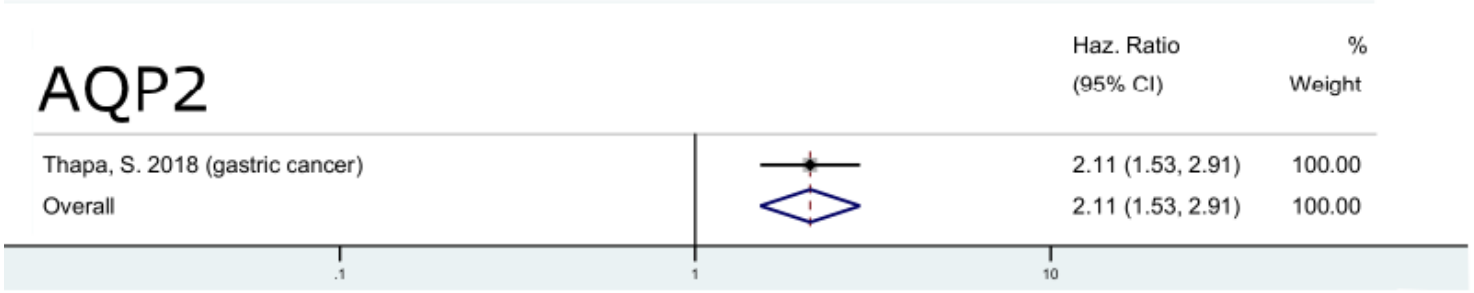
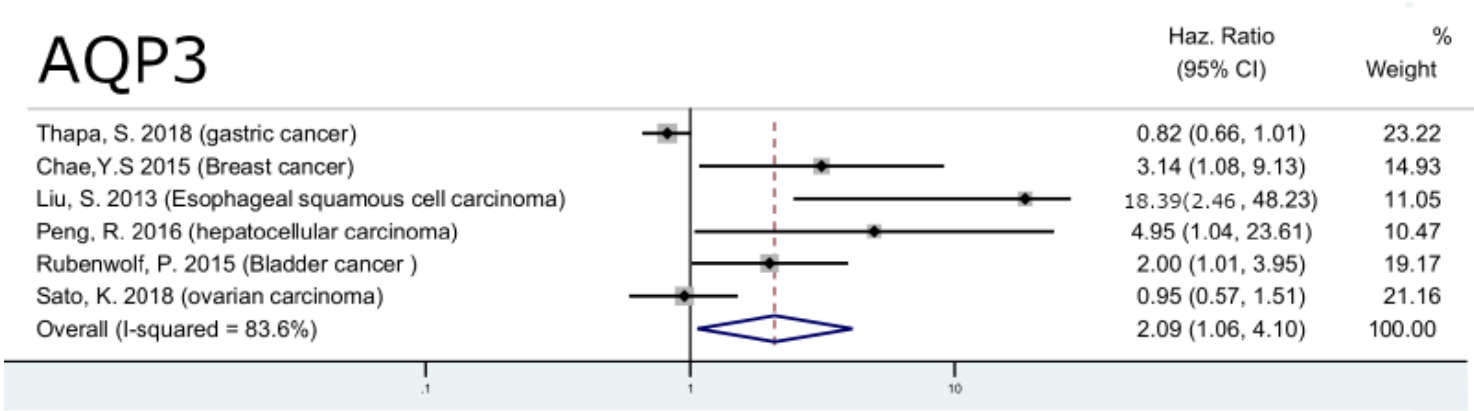
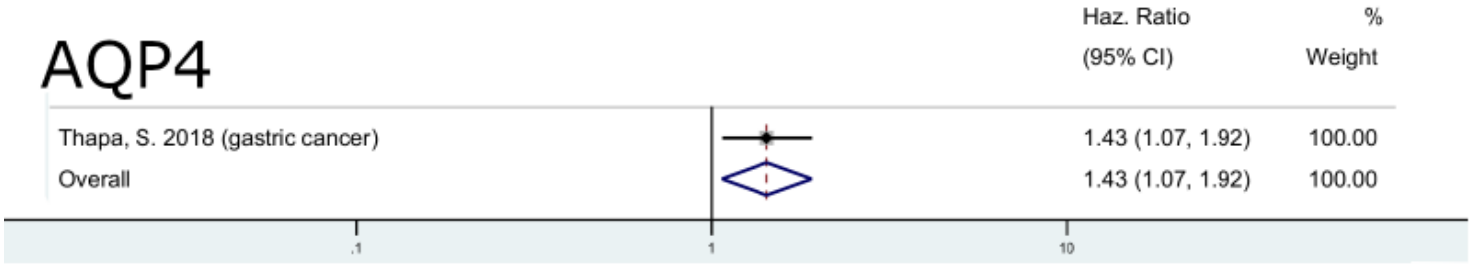


Figure 1. Flow diagram showing the literature evaluation process for the systematic review.


AQP1
Haz. Ratio (95% CI)
% Weight
Angelico, G. 2018 (Pleural Mesothelioma) 1.49 (0.43, 5.52) 4.59
Bellezza, G. 2017 (lung adenocarcinoma) 4.01 (1.73, 9.29) 7.05
Kang, B. W. 2015 (Colon Cancer) 0.72 (0.46, 1.14) 9.98
Kao, S. C. H. 2012 (Pleural Malignant Mesothelioma) 2.66 (1.59, 4.47) 9.48
Luo, L. M. 2017 (hepatocellular carcinoma) 0.77 (0.28, 2.13) 5.92
Otterbach, F. 2010 (Breast Cancer) 2.85 (0.95, 8.56) 5.45
Park, J. Y. 2017(Prostate Cacner) 3.35 (1.22, 9.17) 5.96
Qin, F. 2016 (Breast cancer) 2.97 (1.36, 6.49) 7.46
Sato, K. 2018 (Ovarian Carcinoma) 1.43 (0.74, 3.20) 7.84
Sekine, S. 2014 (Human Biliary Tract Carcinoma) 0.32 (0.16, 0.65) 7.94
Smith, E. 2019 (Colon cancer) 1.60 (1.05, 2.42) 10.23
Thapa, S. 2018 (Gastric Cancer) 1.32 (1.06, 1.63) 11.47
Yoshida, T. 2013 (Colon Cancer) 2.59 (1.06, 6.44) 6.62
Overall (I-squared = 75.0%) 1.57 (1.11, 2.23) 100.00
.1
1
10


AQP2
Haz. Ratio (95% CI)
% Weight
Thapa, S. 2018 (gastric cancer) 2.11 (1.53, 2.91) 100.00
Overall 2.11 (1.53, 2.91) 100.00
.1
1
10


AQP3
Haz. Ratio (95% CI)
% Weight
Thapa, S. 2018 (gastric cancer) 0.82 (0.66, 1.01) 23.22
Chae,Y.S 2015 (Breast cancer) 3.14 (1.08, 9.13) 14.93
Liu, S. 2013 (Esophageal squamous cell carcinoma) 18.39(2.46 , 48.23) 11.05
Peng, R. 2016 (hepatocellular carcinoma) 4.95 (1.04, 23.61) 10.47
Rubenwolf, P. 2015 (Bladder cancer) 2.00 (1.01, 3.95) 19.17
Sato, K. 2018 (ovarian carcinoma) 0.95 (0.57, 1.51) 21.16
Overall (I-squared = 83.6%) 2.09 (1.06, 4.10) 100.00
.1
1
10


AQP4
Haz. Ratio (95% CI)
% Weight
Thapa, S. 2018 (gastric cancer) 1.43 (1.07, 1.92) 100.00
Overall 1.43 (1.07, 1.92) 100.00
.1
1
10

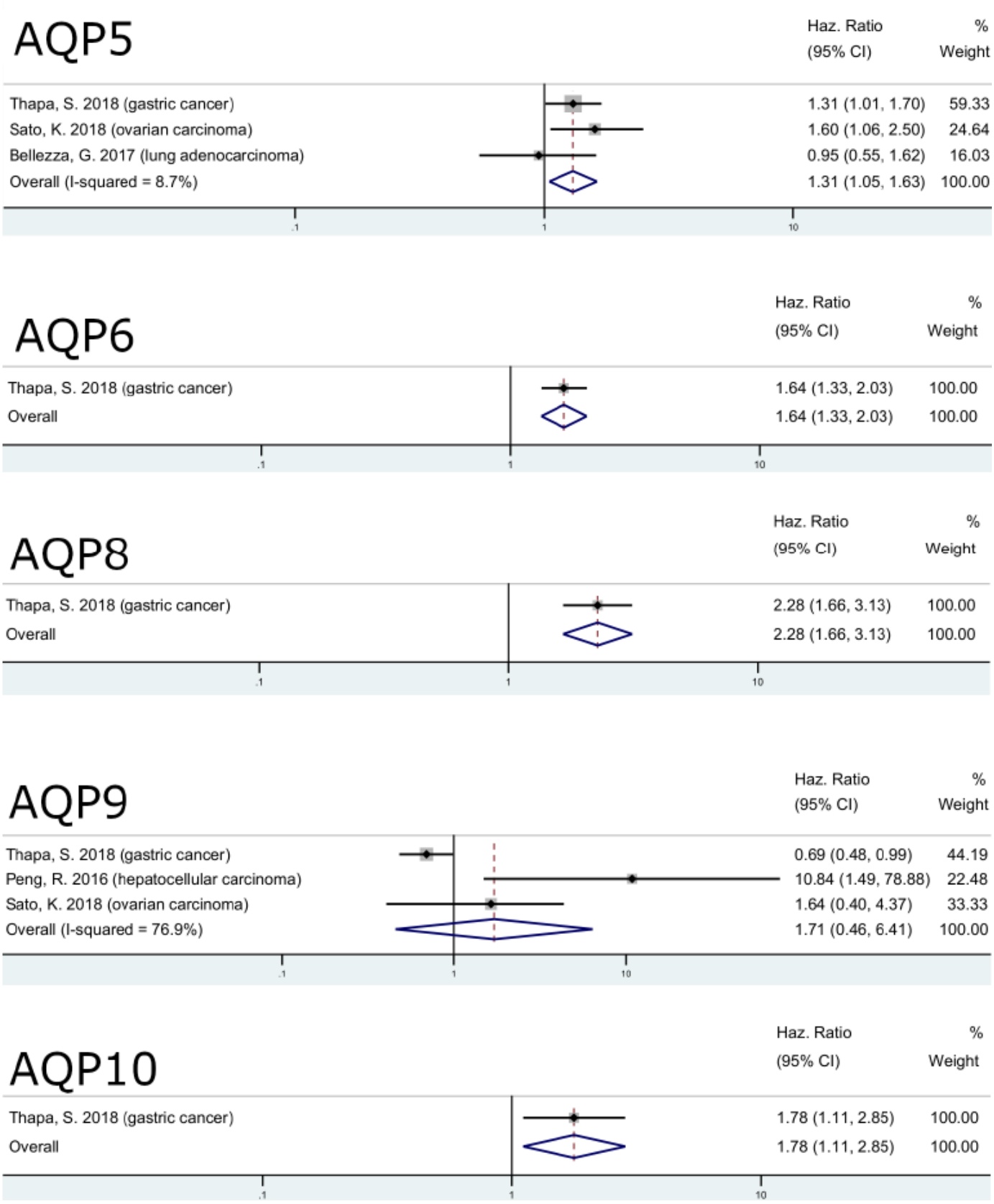


Figure 2. Forest plot from literature compiled for AQP classes, using the hazard ratio and confidence interval (CI) data extracted from published literature, based on analyses of levels of protein, RNA or both.

1.5.1 Aquaporins in gliomas

The human brain consists of 100 billion neurons and 1 trillion glial cells on average (Herculano-Houzel, 2009). Localized in astrocyte end feet throughout the brain and spinal cord, AQP4 enables central nervous system fluid homeostasis and promotes maintenance of the blood brain barrier (Amiry-Moghaddam and Ottersen, 2003). AQP1 normally is expressed in the choroid plexus epithelium where it contributes to cerebral spinal fluid production by mediating water flux from blood to brain (Speake et al., 2001, Boassa et al., 2006), but is otherwise not abundant in brain tissues. AQP9 is permeable to a variety of organic substrates including lactate, glycerol and other solutes, and is expressed at low levels in glia and neurons where it is speculated to play a role in energy metabolism, though details remain to be clarified (Badaut and Regli, 2004).

Upregulation of AQP1 and AQP4 has been the major focus of papers published in the glioma field (**Table 2**), with additional work identifying possible involvement of AQP9. AQP1 overexpression has been observed in diverse types of gliomas, such as glioblastoma, astrocytomas, oligodendroglioma, ependymoma, and glioastrocytoma, and the levels of expression have been reported to correlate with grade of malignancy and the invasiveness of the tumors (Saadoun et al., 2002, Oshio et al., 2005, El Hindy et al., 2013, Deb et al., 2012, Longatti et al., 2006, Endo et al., 1999, Georges et al., 2011). Glioma invasiveness has been linked to AQP1 overexpression (Liao et al., 2016), which is greater in migrating cells than in the tumor core (El Hindy et al., 2013). Dexamethasone, which promotes AQP1 transcription, increased invasiveness of glioma cells (Guan et al., 2018, Hayashi et al., 2007).

AQP4 upregulation and redistribution in glioblastoma (Warth et al., 2007, Hu et al., 2005, Mou et al., 2010, Noell et al., 2015, Schob et al., 2017, Zhao et al., 2012) observed by magnetic resonance imaging has been suggested to contribute to tumor-associated edema (Nico and Ribatti, 2011, Betz et al., 1989, Dua et al., 2010), or enhance clearance of excess

fluid (Yang et al., 2012). Genetic deletion of AQP4 impairs cell migration, actin polymerization and apoptosis (Ding et al., 2013, Ding et al., 2011). Downregulation of AQP4 expression in glioma by pentamidine and temozolomide promoted apoptosis and inhibited cell migration which could be a potential treatment for glioma (Capoccia et al., 2015, Chen et al., 2017). The incidence of epileptiform seizures in glioma patients correlated with increased membrane levels of AQP4 protein, even though transcript levels were not altered (Isoardo et al., 2012), raising the important point that not just expression but also localization of the protein product is essential for understanding functional outcomes. AQP 9 is not highly expressed in normal brain; however, increased levels were observed on human glioma cell membranes and correlated with pathological grades (Fossdal et al., 2012), it was proposed that AQP9 upregulation promoted cell invasion via AKT pathway (Lv et al., 2018).

Independent analysis using transcriptomic data from the Human Protein Atlas pathology database complemented the results of the systematic literature review in terms of AQP expression patterns. Transcript levels in human glioma ranged up to the highest levels for AQP1 and AQP4 (**Figure 3A**); whereas other classes of AQPs generally showed slightly lower levels, or no difference as compared to overall AQP median values. The survival time for glioma patients after diagnosis showed an inverse overall correlation with AQP transcript levels (**Figure 4A**), with risk of death almost doubled for patients with AQP5 transcript levels exceeding the median value (with a hazard ratio (HR) of 1.9) and AQP9 (with HR 1.6). AQP1 showed a modest correlation (HR1.2) but AQP4 showed no effect on hazard ratio (HR 0.9), which is perhaps not surprising given that AQP4 is ubiquitously expressed at high levels in normal glia. A gap in knowledge on possible roles of AQP5 and -9, which have the highest hazard ratios (Figure 3A) but are comparatively underexplored in published work, suggest an area that merits future study in glioma research.

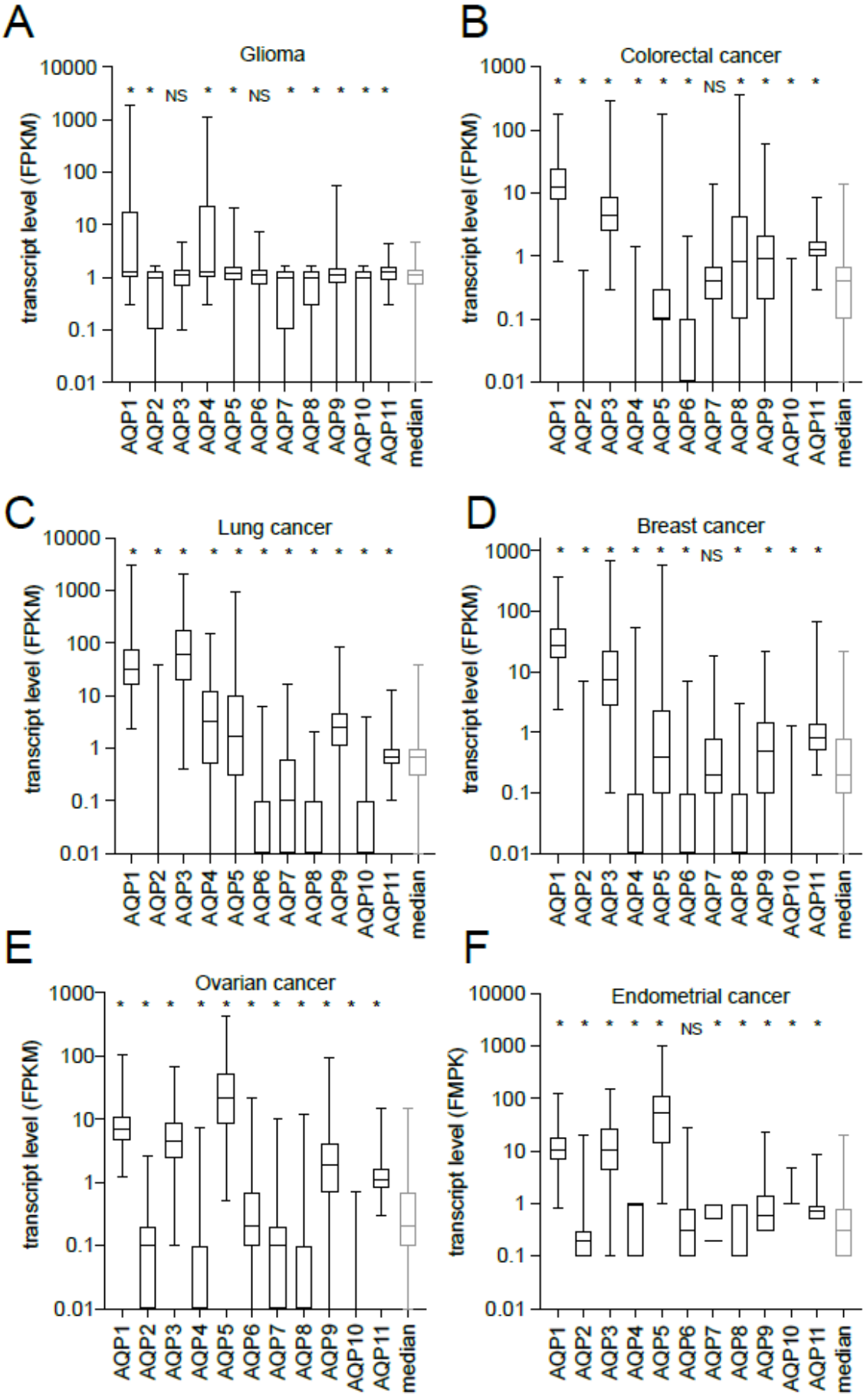
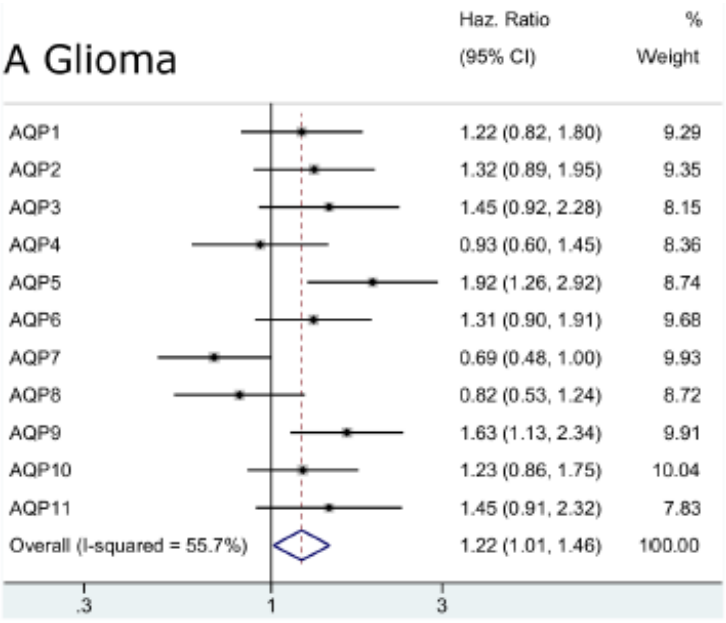
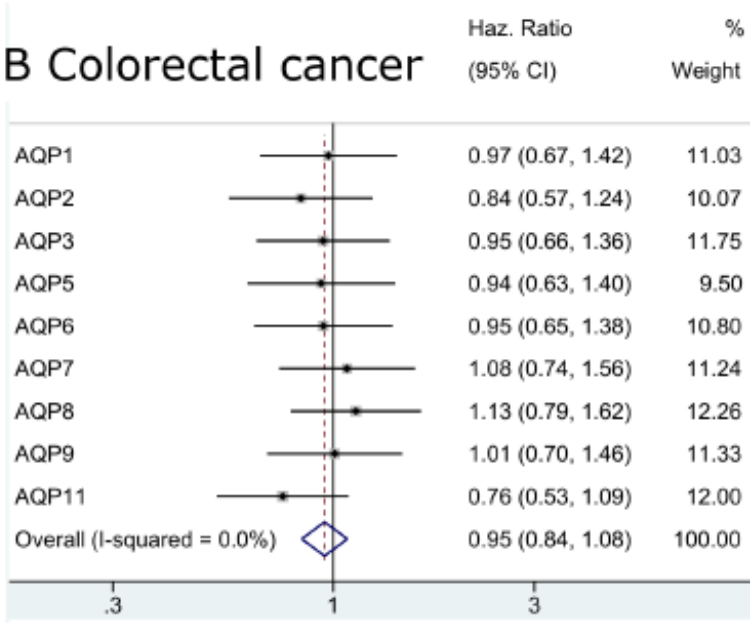
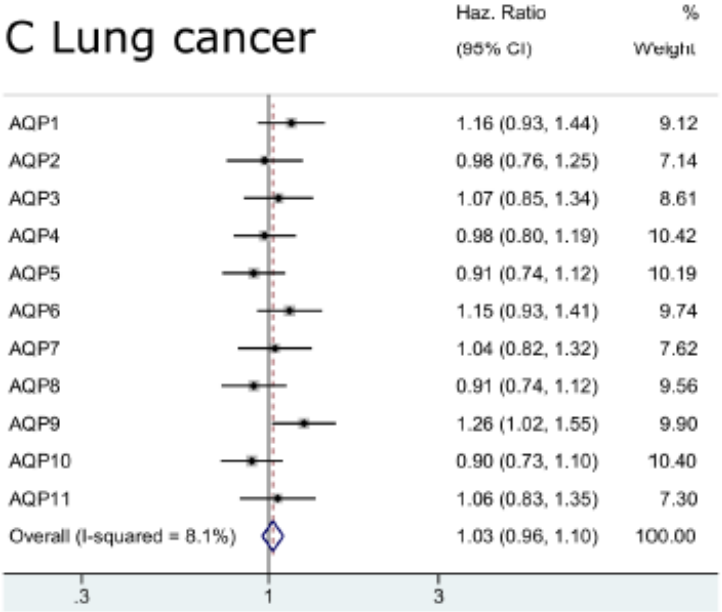
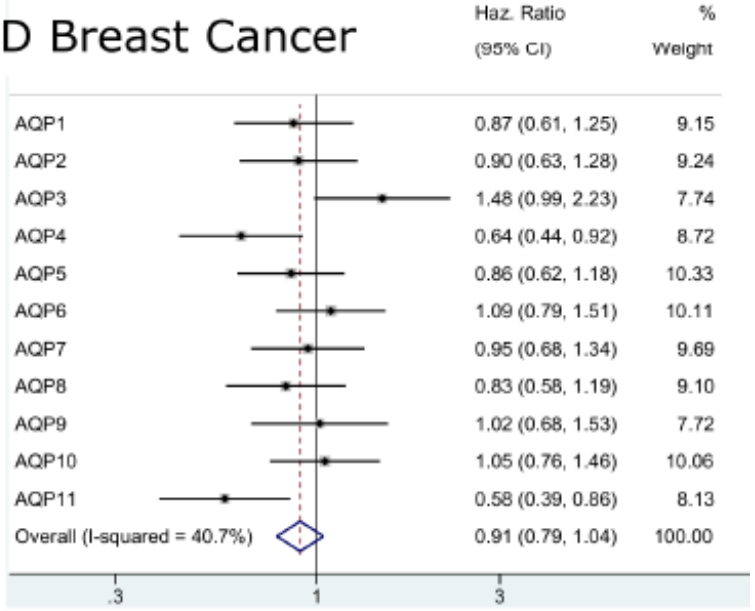

A
Glioma
transcript level (FPKM)
10000
1000
100
10
1
0.1
0.01
NS
NS
AQP1
AQP2
AQP3
AQP4
AQP5
AQP6
AQP7
AQP8
AQP9
AQP10
AQP11
median
B
Colorectal cancer
transcript level (FPKM)
1000
100
10
1
0.1
0.01
NS
C
Lung cancer
transcript level (FPKM)
D
Breast cancer
transcript level (FPKM)
E
Ovarian cancer
transcript level (FPKM)
F
Endometrial cancer
transcript level (FMPK)

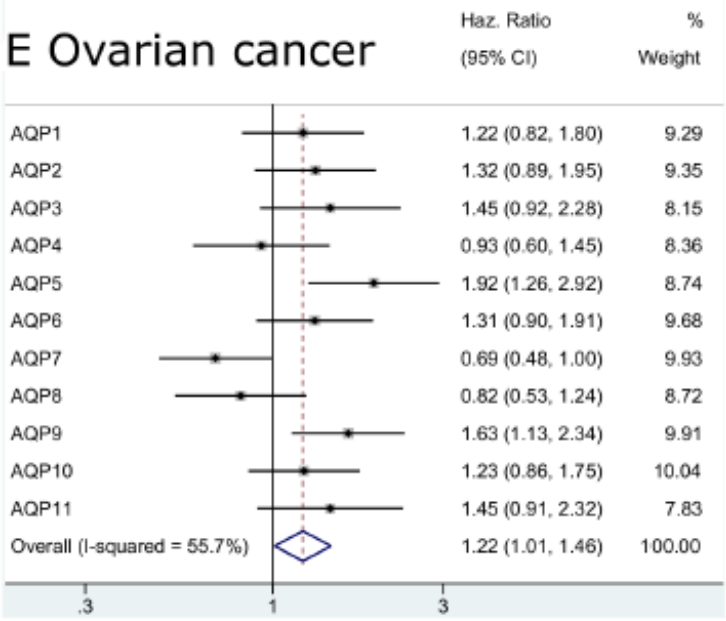
Figure 3. Quantitative transcript levels in human cancer biopsies, calculated as "fragments per kilobase of exon per million reads mapped" (FPKM). Data from the RNAseq transcriptomic database (Human Protein Atlas, https://www.proteinatlas.org), summarized by AQP class, are shown as box plots for six cancer types (A–F, as indicated). Boxes show 50% of data points; error bars show the full range; horizontal bars show median values. Median transcript levels are average FPMK values for all classes of AQPs (1–11) within each cancer type. * $p < 0.05$ as compared with the median values (Mann Whitney U-test); NS is not significant.


A Glioma
Haz. Ratio (95% CI)
%
Weight
AQP1 1.22 (0.82, 1.80) 9.29
AQP2 1.32 (0.89, 1.95) 9.35
AQP3 1.45 (0.92, 2.28) 8.15
AQP4 0.93 (0.60, 1.45) 8.36
AQP5 1.92 (1.26, 2.92) 8.74
AQP6 1.31 (0.90, 1.91) 9.68
AQP7 0.69 (0.48, 1.00) 9.93
AQP8 0.82 (0.53, 1.24) 8.72
AQP9 1.63 (1.13, 2.34) 9.91
AQP10 1.23 (0.86, 1.75) 10.04
AQP11 1.45 (0.91, 2.32) 7.83
Overall (I-squared = 55.7%) 1.22 (1.01, 1.46) 100.00
.3
1
3


B Colorectal cancer
Haz. Ratio (95% CI)
%
Weight
AQP1 0.97 (0.67, 1.42) 11.03
AQP2 0.84 (0.57, 1.24) 10.07
AQP3 0.95 (0.66, 1.36) 11.75
AQP5 0.94 (0.63, 1.40) 9.50
AQP6 0.95 (0.65, 1.38) 10.80
AQP7 1.08 (0.74, 1.56) 11.24
AQP8 1.13 (0.79, 1.62) 12.26
AQP9 1.01 (0.70, 1.46) 11.33
AQP11 0.76 (0.53, 1.09) 12.00
Overall (I-squared = 0.0%) 0.95 (0.84, 1.08) 100.00
.3
1
3


C Lung cancer
Haz. Ratio (95% CI)
%
Weight
AQP1 1.16 (0.93, 1.44) 9.12
AQP2 0.98 (0.76, 1.25) 7.14
AQP3 1.07 (0.85, 1.34) 8.61
AQP4 0.98 (0.80, 1.19) 10.42
AQP5 0.91 (0.74, 1.12) 10.19
AQP6 1.15 (0.93, 1.41) 9.74
AQP7 1.04 (0.82, 1.32) 7.62
AQP8 0.91 (0.74, 1.12) 9.56
AQP9 1.26 (1.02, 1.55) 9.90
AQP10 0.90 (0.73, 1.10) 10.40
AQP11 1.06 (0.83, 1.35) 7.30
Overall (I-squared = 8.1%) 1.03 (0.96, 1.10) 100.00
.3
1
3


D Breast Cancer
Haz. Ratio (95% CI)
%
Weight
AQP1 0.87 (0.61, 1.25) 9.15
AQP2 0.90 (0.63, 1.28) 9.24
AQP3 1.48 (0.99, 2.23) 7.74
AQP4 0.64 (0.44, 0.92) 8.72
AQP5 0.86 (0.62, 1.18) 10.33
AQP6 1.09 (0.79, 1.51) 10.11
AQP7 0.95 (0.68, 1.34) 9.69
AQP8 0.83 (0.58, 1.19) 9.10
AQP9 1.02 (0.68, 1.53) 7.72
AQP10 1.05 (0.76, 1.46) 10.06
AQP11 0.58 (0.39, 0.86) 8.13
Overall (I-squared = 40.7%) 0.91 (0.79, 1.04) 100.00
.3
1
3


E Ovarian cancer
Haz. Ratio (95% CI)
%
Weight
AQP1 1.22 (0.82, 1.80) 9.29
AQP2 1.32 (0.89, 1.95) 9.35
AQP3 1.45 (0.92, 2.28) 8.15
AQP4 0.93 (0.60, 1.45) 8.36
AQP5 1.92 (1.26, 2.92) 8.74
AQP6 1.31 (0.90, 1.91) 9.68
AQP7 0.69 (0.48, 1.00) 9.93
AQP8 0.82 (0.53, 1.24) 8.72
AQP9 1.63 (1.13, 2.34) 9.91
AQP10 1.23 (0.86, 1.75) 10.04
AQP11 1.45 (0.91, 2.32) 7.83
Overall (I-squared = 55.7%) 1.22 (1.01, 1.46) 100.00
.3
1
3

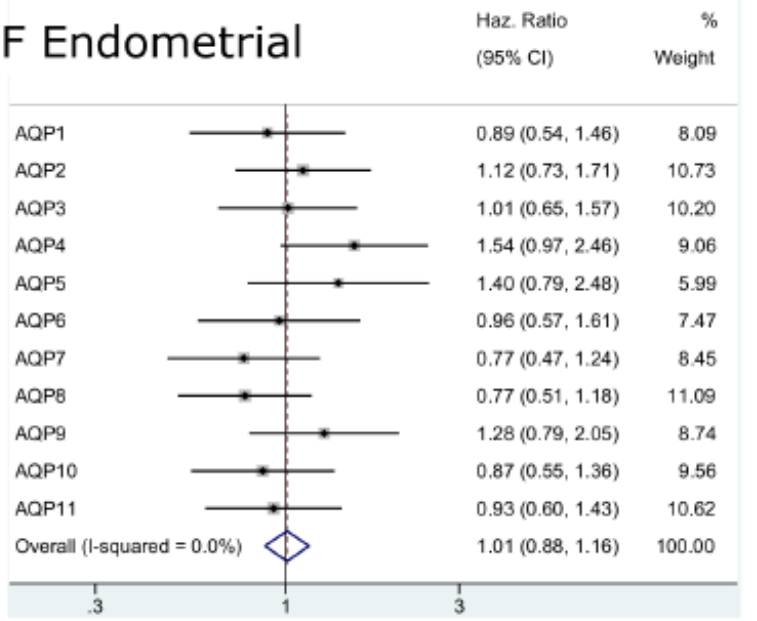

F Endometrial
Haz. Ratio (95% CI)
%
Weight
AQP1 0.89 (0.54, 1.46) 8.09
AQP2 1.12 (0.73, 1.71) 10.73
AQP3 1.01 (0.65, 1.57) 10.20
AQP4 1.54 (0.97, 2.46) 9.06
AQP5 1.40 (0.79, 2.48) 5.99
AQP6 0.96 (0.57, 1.61) 7.47
AQP7 0.77 (0.47, 1.24) 8.45
AQP8 0.77 (0.51, 1.18) 11.09
AQP9 1.28 (0.79, 2.05) 8.74
AQP10 0.87 (0.55, 1.36) 9.56
AQP11 0.93 (0.60, 1.43) 10.62
Overall (I-squared = 0.0%) 1.01 (0.88, 1.16) 100.00
.3
1
3

Figure 4. Forest plot based on survival and median expression data obtained from the Human Protein Atlas database. Hazard ratios were calculated from overall survival times of patients, classified using the default algorithm defined in the Human Protein Atlas to separate populations with optimal significant differences into high and low expression groups according to the median FPKM values. I2 indicates the heterogeneity of the studies.

Table 2. Publications on AQPs in glioma, classified by research approach.

AQP class	# pubs	Research approach (with cited papers in each category)	
		Expression	Function
AQP1	21	35,37,46,47,48, 51, 54, 55,60,62, 78,110,116,117,126,139,142, 143,156,159,191	47,55,60,110,126,156
AQP2	1	78	0
AQP3	2	78,161	161
AQP4	25	5,13,33,38,39,45,51,52,64,78, 111, 126,135,139,140,141,160,164, 165,191,193,196,205,206,221	13,33,38,39,126,196,205
AQP5	2	78, 200	200
AQP6	1	78	0
AQP7	1	78	0
AQP8	2	78,222	0
AQP9	5	53,78,81,121,206	121
AQP10	1	78	0
AQP11	1	78	0

1.5.2 Aquaporins in colon cancer

Colorectal (bowel) cancer initiates from small noncancerous polyps inside the colon, and mainly affects older adults (Bray et al., 2018). In normal colon tissue, AQP1, -3,-4, -7 and -8 are the predominant isoforms, which are responsible for water absorption (Zhu et al., 2016). The expression and function of AQP1 and AQP5 has been the major focus of papers published in the colorectal cancer field (**Table 3**). AQP1 levels are upregulated from early through late stages of colorectal carcinogenesis, and have been suggested to correlate with the invasiveness of the tumors, prompting classification of AQP1 as a negative prognostic

indicator of patient survival (Mobasheri et al., 2005, Yoshida et al., 2013a, Kang et al., 2014). Molecular knockdown and pharmacological inhibition of AQP1 in colon cancer cells significantly impaired migration, providing a possible therapy for colon cancer (Pei et al., 2016b, Dorward et al., 2016, Kourghi et al., 2016a, Smith et al., 2018a, Kang et al., 2014, Yong, 2009). AQP1 was proposed to influence actin organization via RhoA and Rac signalling pathways (Yong, 2009). AQP5 similarly has been suggested as a prognostic biomarker in colorectal cancer, in that AQP5 levels were proportional to the number of circulating tumors cells (Shan et al., 2014, Shan et al., 2015, Kang et al., 2008) and the risk of liver metastasis (Kang et al., 2008). Increased AQP5 induced cell proliferation via Ras-MAPK and signalling pathways (Sung et al., 2008, Kang et al., 2008); conversely, AQP5 knockdown inhibited cell proliferation and induced apoptosis (Shi et al., 2014, Shi et al., 2013). AQP3 modulated tumor differentiation in colon cancer patients via the EGFR pathway, more researches are required to confirm the role of AQP3 (Li et al., 2013).

Interestingly, levels of AQP1, -5 and -9 expression were associated with sensitivity of the cancers to chemotherapy following surgery in patients with stage II and III colorectal carcinoma, suggesting another use for AQPs as biomarkers for personalized medicine (Imaizumi et al., 2018). Genetic knockdown of AQP5 increased sensitivity to chemotherapy and downregulated p38 MAPK signalling in colon cancer cells (Li et al., 2018a, Shi et al., 2014). Non-responsive colon cancer patients to adjuvant chemotherapy were more likely to have low AQP9 expression (Dou et al., 2013, Huang et al., 2017).

In agreement with the systematic review data, AQP transcript data extracted from the Human Protein Atlas database confirmed the elevation of AQP1 and AQP3 levels in colorectal cancers (**Figure 3B**), as well as for AQP5 and AQP9, but unexpectedly also suggested that AQP7, -8, and -11 merit consideration in future work on colorectal cancers. Contrary to predictions, levels of expression of the AQPs showed no correlation with survival time

(**Figure 4B**), suggesting that although classes of AQPs might enable important aspects of cancer progression, they fall short of serving as negative prognostic indicators for survival in colorectal cancer patients.

Table 3. Publications on AQPs in colon cancers, classified by research approach.

AQP class	**# pubs**	**Research approach (with cited papers in each category)**	
		Expression	Function
AQP1	11	41,72,87,95,129,148,183,184,209,210	41,95,96,148,183,184,209
AQP2	0	0	0
AQP3	2	87,101	101
AQP4	0	0	0
AQP5	11	20,49,87,89,105,170,171,173,174,175,187	20,49,89,105,173,174,175,187
AQP6	0	0	0
AQP7	0	0	0
AQP8	1	194	194
AQP9	3	42,65,207	65,207
AQP10	0	0	0
AQP11	0	00	0

1.5.3 Aquaporins in lung cancer

Lung cancers can be divided into two main groups: small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC) which can be further subdivided into adenocarcinoma, squamous cell carcinoma, and large cell carcinoma (Zheng, 2016, Shimasaki et al., 2011, Yun et al., 2016). AQP 1 normally is expressed in microvascular endothelia, and AQP3 and AQP4 are in airway epithelia (Verkman, 2007). In lung cancer, overexpression of AQP1,

AQP3 and AQP5 has been the major focus of papers published in the lung cancer field (**Table 4**). AQP1 was generally up-regulated in lung adenocarcinoma and bronchoalveolar carcinoma, but not in lung squamous cell carcinoma. AQP1 expression was correlated with high risk of postoperative metastasis and low disease-free survival rates in adenocarcinoma, and therefore was proposed be a prognostic factor for stage and histologic differentiation categories of lung cancer (Hoque et al., 2006, Bellezza et al., 2017, Lopez-Campos et al., 2011, Shimasaki et al., 2011, Yun et al., 2016). Interestingly, chemotherapy was associated with a significant upregulation of AQP1 expression, which might cause an unwanted boost in cancer recurrence by enhancing invasiveness (Cagini et al., 2017). Increased expression of AQP1 by transfection of lung cancer cells enhanced cell growth in vitro, suggesting a role for AQP1 in proliferation (Hoque et al., 2006). Overexpression of AQP1 in endothelia of tumor capillaries in lung adenocarcinoma and mesothelioma suggested a role in tumor angiogenesis, in turn facilitatating cancer growth and spread (Lopez-Campos et al., 2011). AQP3 upregulation in lung adenocarcinoma correlated with tumor differentiation and clinical stage (Li et al., 2013). AQP3 promotes angiogenesis in lung cancer through the HIF-2α-VEGF pathway, and invasion through the AKT-MMP pathway (Liu et al., 2007). High levels of AQP4 did not correlate with poorer survival in NSCLC patients (Warth et al., 2011). In contrast, AQP5 overexpression was associated with unfavorable outcomes for patients with NSCLC (Chae et al., 2008, Jo et al., 2016, Song et al., 2015). Genetic knock down of AQP5 in cell lines reduced migration (Chen et al., 2011, Guo and Jin, 2015), whereas upregulation of AQP5 activated epidermal growth factor receptor (EGFR), extracellular receptor kinase (ERK1/2), and p38 mitogen-activated protein kinase (p38 MAPK) pathway to facilitated proliferation and migration (Zhang et al., 2018a). AQP3 coexpression with AQP5 was linked to poor survival, suggesting that combined detection of markers might strengthen prognostic predictions (Liu et al., 2013).

Consistent with the systematic review data, increased transcript levels for AQP1, -3, -4, and -5 were observed (in addition to possible involvement of AQP9) in lung cancer (**Figure 3C**). However, the hazard ratio calculations of overall survival time of patients with lung cancer showed no convincing correlations with the expression level of AQPs 1 to 11 (**Figure 4C**), arguing against value as prognostic indicators for AQPs in lung cancer.

Table 4. Publications on AQPs in lung cancers, classified by research approach.

AQP class	**# pubs**	**Research approach (with cited papers in each category)**	
		Expression	Function
AQP1	12	7,11,22,63,107, 114,118, 122,129,180,195,211	63,107,114,122, 195
AQP2	0	0	0
AQP3	8	6,8,22,59,76,102,115, 180	6,59,76
AQP4	2	22, 192	0
AQP5	13	7,8,11,15,22,36,56,85, 180,185,212,218,220	15,36,56,212,218,220
AQP6	0	0	0
AQP7	0	0	0
AQP8	0	0	0
AQP9	1	127	127
AQP10	0	0	0
AQP11	1	50	0

1.5.4 Aquaporins in breast cancer

Breast cancer is a type of adenocarcinoma, which begins with mutated cells in the milk ducts or the lobules (Sinn and Kreipe, 2013). Upregulation of AQP1, -3 and -5 has been the major focus of papers published in the breast cancer field (**Table 5**). AQP1 upregulation was induced by estrogen in breast cancer (Zou et al., 2013), and overexpression of AQP1 was correlated with poor prognoses for breast cancer patients (Qin et al., 2016a, Yin et al., 2008). Upregulation of AQP3 was observed in the early stages of breast cancer, linked to fibroblast growth factor via FGFR-PI3K or FGFR-ERK signalling pathways, and directly through oestrogen (Cao et al., 2013, Huang et al., 2015). Strong expression of AQP3 was correlated with patient survival rate, therefore was proposed as a potential prognostic marker after curative surgery (Chae et al., 2015, Kang et al., 2015b). Increased invasiveness of cancer cells was observed in cells expressing high levels of AQP3 cell, reported to mediate H_2O_2 transport, which induced CXCL12- cell signalling and migration, indicating the involvement of AQP3 in breast cancer metastasis (Huang et al., 2015, Satooka and Hara-Chikuma, 2016). Similarly, AQP5 expression was upregulated by estrogen in breast cancer patients from early stages, and correlated to survival time, suggesting AQP5 also was a prognostic marker (Lee et al., 2014, Lee et al., 2017). Knockdown of AQP5 activated the MAPK signalling pathway, reducing cell invasiveness and proliferation, and enhanced the chemosensitivity of breast cancer cells, suggesting potential for AQP5 as a pharmacological target (Li et al., 2018b, Jia et al., 2018).

Based on transcriptomic analyses from the Human Protein Atlas database, AQP1, -3, -5, -7, -9 and -11 were upregulated in breast cancer patients (**Figure 3D**). However, apart from AQP3 with a hazard ratio of 1.5, the patterns of upregulated AQP expression were not correlated with survival rates in breast cancer patients (**Figure 4D**). Interestingly, increased

levels of AQP4 and -11 were linked to longer overall survival times (HR 0.6 in both), suggesting several AQPs subtype might have anti-cancer potential.

Table 5. Publications on AQPs in breast cancer, classified by research approach.

AQP class	**# pubs**	**Research approach (with cited papers in each category)**	
		Expression	Function
AQP1	6	129,153,179,208,214	119,153,214
AQP2	1	179	0
AQP3	8	12,16,70,88,161,163,179	6, 12,70,161,163
AQP4	2	108,179	0
AQP5	7	83,86,91,97,98,106,179	83,86,106
AQP6	1	179	0
AQP7	1	179	0
AQP8	1	179	0
AQP9	2	161,179	161
AQP10	1	179	0
AQP11	1	179	0

1.5.5 Aquaporins in ovarian cancer

Expression of AQP1, -2, -3, and -4 was reported in normal ovary (Yang et al., 2005, Thoroddsen et al., 2011). Overexpression of AQP1, AQP3, AQP5 and AQP9 was observed in ovarian tumors (Sato et al., 2018), and has been the major focus of papers published in the ovarian cancer field (**Table 6**). Although AQP1 expression was upregulated in late stage of ovarian tumors than in early stage, the overall survival rate was inversely proportional to the expression, suggesting high expression of AQP1 was not linked to poor prognoses, and in fact might have anti-cancer effect (Sato et al., 2018, Yang et al., 2011, Chen et al., 2014).

AQP3 expression upregulation by EGF promoted cell migration in an ovarian cancer cell line, which was inhibited by curcumin, suggesting a possible pharmacological action on AQP3 (Ji et al., 2008). Multiple studies reported the association of AQP5 expression with tumor grade, lymph node metastasis, and poor prognoses, suggesting AQP5 could be a prognostic factor for ovarian cancer (Abdelrahman et al., 2018, Sato et al., 2018, Yang et al., 2006). AQP5 expression level was associated with the sensitivity of ovarian cancer cells to chemotherapy (Chen et al., 2015) suggesting AQP5 might be one of the targets of the treatment. AQP6 and AQP9 were downregulated and AQP8 was unchanged in ovarian cancer, but their roles remain to be determined (Sato et al., 2018, Ma et al., 2016)

Consistent with systematic review data, transcript levels obtained from the Human Protein Atlas database showed upregulation of AQP1, -3, -5 and -9, and in addition an increase in AQP11 (**Figure 3E**). Increased transcript levels for AQP1, -3 and -9 showed association with poor overall survival time of ovarian cancer patients (HR 1.5, 1.9 and 1.6, respectively), while conversely increased AQP7 expression correlated with longer survival time (**Figure 4E**).

Table 6. Publications on AQPs in ovarian cancer, classified by research approach.

AQP class	# pubs	Research approach (with cited papers in each category)	
		Expression	Function
AQP1	6	30,129,162,201,204,197	30,197
AQP2	3	30,197,204	30,197
AQP3	8	30,82,161,162,197,198,203,204	30,82,161,197
AQP4	3	30,197,204	30,197
AQP5	8	1,30,31,162,197,199,202,204	30,31,197,199
AQP6	4	30,197,204,123	30,197
AQP7	4	30,197,203,204	30,197
AQP8	4	30,197,123,204	30,197
AQP9	6	30,161,162,197,203,204	30,161,197
AQP10	2	30,197	30,197
AQP11	0	0	0

1.5.6 Aquaporins in endometrial cancer

Potential changes in expression levels of AQPs in the endometrial cancer and other less well studied cancers remain a gap in knowledge in the field (**Table 7**). Endometrial cancer is a type of cancer arising from tissue lining the uterus (the endometrium), and accounts for about 95% of uterine cancers. AQP1, AQP2, AQP3, AQP5, AQP7, and AQP9 were expressed in normal endometrium, and involved in fluid exchange and estrogen regulation (Chinigarzadeh et al., 2017, Choi et al., 2019, Cui et al., 2018, Ducza et al., 2019, Ferre-Dolcet et al., 2020, Jiang et al., 2015, Zhou et al., 2019). AQP1 expression was correlated with the histologic

grade, extent of myometrial invasion, and likelihood extrauterine metastasis (Pan et al., 2008), but the functional role of AQP1 remains to be defined.

Data from the Human Protein Atlas database showed that AQP1, AQP3, and AQP5 were upregulated in endometrial cancer (**Figure 3F**). Increased transcript levels for AQP4 and AQP5 were correlated with poor survival (HR 1.5 and 1.4, respectively) (**Figure 4F**). Future research could address this gap in knowledge on the classes of AQPs that might have an impact on overall survival times in endometrial and other cancers.

Table 7. Publications on AQPs in other cancer types

Cancer type	Type of AQP	Cited references
Biliary tract cancer	AQP1	166
	AQP5	167
Bladder cancer	*AQP1*	217
	AQP3	157
Bone cancer	AQP3	154
Cervical cancer	AQP1	28,,29,128,172,176,213
	AQP3	29,172,176
	AQP4	172
	AQP5	34,172,215
	AQP8	17,28,172,176,177,178
Endometrial cancer	AQP1	145
	AQP2	223
Gallbladder cancer	AQP5	168
Gastric cancer	AQP1-11	188
	AQP2	23

	AQP3	25,40,68,84,109
	AQP5	69
Leukemia	AQP5	14
	AQP8	151
	AQP9	9,19,75,100
Liver cancer	AQP1	120,125
	AQP3	21,32,57,149,150
	AQP5	57,61,92,219
	AQP7	32
	AQP9	32,103,144,149,150,216
Melanoma, cutaneous	AQP1	73,74
Mesothelioma	AQP1	3,4,43,4479,80,90,93
Oesophageal cancer	AQP3	113
	AQP4	94
	AQP5	113,181
	AQP8	18
Pancreatic cancer	AQP1	224
	AQP3	66,224
Prostate cancer	AQP1	71,129,146,147
	AQP2	24
	AQP3	27,71,77
	AQP5	104
Renal cancer	AQP1	67,130, 131,132,133,134,155,186,189
	AQP5	2
Skin cancer	AQP1	137,152,182,190
	AQP3	58,59,136,169

Squamous cell carcinoma, oral	AQP3	124
Squamous cell carcinoma, pharyngeal	AQP1,5	99
Squamous cell carcinoma, tongue	AQP3,5	76
Thyroid cancer	AQP3,4	138
Urothelial Carcinoma	AQP1	112

1.6 Conclusion

Overexpression of specific classes of AQPs is consistently observed in clinical and preclinical studies of cancers. Different classes of AQPs, depending on cancer type, are linked to properties of migration, invasion, proliferation, and angiogenesis. Analyses presented in this review provide evidence that upregulation of AQPs in general has a negative correlation with cancer patient survival time, and that AQPs 1,-3,-5, and -9 in particular appear to be relevant to patient survival in cancers including glioma, obarian and endometrial cancers for example. The highest hazard reation were noted for AQP5 in glioma and ovarian cancers, and AQP3 in breast cancer. Not all classes of AQPs are associated with promoting cancer progression; on the contrary, AQPs 7 and -8 merit analysis as components of potentially protective mechanisms. In summary, the current literature provides the foundation for developing potential therapies aimed at specific classes of AQPs. Small molecule inhibitors have been developed, but none have proceeded to clinical trials. Future studies are required to further investigate these pharmacological agents and to fill in the gaps in knowledge on the mechanisms of action of AQPs in cancer growth and progression.

1.7 Thesis Hypothesis and Aims

This thesis aimed to investigate the potential of AQP1-based therapy in cancer and sickle cell disease. The hypothesis is that pharmacological modulation of AQP1 water and ion channels can slow down disease progression. This hypothesis led to the generation of 3 main aims:

1. To discover new pharmacological agent for AQP1 water and ion channel.
2. To test the whether pharmacological block of AQP1 water and ion channel will impede cell migration and invasion in AQP1-expressing cancer cell lines.
3. To identify the role of AQP1 ion channel in sickle cell disease.

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1.9 Supplementary table 1- extracted paper

No.	First author	Study type	Method	Tumour type	Aquaporin type	Expression/ function or both
1.	Abdelrahman, A. E 2018	Biopsy	Immunohistochemistry	Ovarian cancer	AQP5	Expression
2.	Allory, Y. 2008	Biopsy	Immunohistochemistry	Renal cancer	AQP1	Expression
3.	Angelico, G. 2018	Biopsy	Immunohistochemistry	Pleural Mesothelioma	AQP1	Expression
4.	Angelico, G. 2018	Biopsy	Immunohistochemistry	Pleural Mesothelioma	AQP1	Expression
5.	Aras, Y. 2016	In vitro	Immunohistochemistry	Brain cancer	AQP4	Expression
6.	Arif, M. 2018	In vitro	Small interfering RNA RTPCR Fluorescent cell swelling assay Cellular proliferation assay Cellular migration assays Cellular invasion assay Cellular adhesion assay	Breast cancer	AQP3	Expression and function
7.	Bellezza, G. 2017	Biopsy	Immunohistochemistry	Lung cancer	AQP1,5	Expression
8.	Ben, Y. 2008	In vitro	RTPCR Immunohistochemistry	Lung cancer	AQP3,5	Expression
9.	Bhattacharjee, H. 2004	In vitro	RTPCR Up take assay	Leukemia	AQP9	Expression and function
10.	Breyer, J. 2017	Biopsy	Immunohistochemistry	Urothelial cancer	AQP3	Expression

11.	Cagini, L. 2017	Biopsy	RTPCR	Lung cancer	AQP1 AQP5	Expression
12.	Cao, X. C. 2013	In vitro	Wound healing assay Western blot Small interfering RNA	Breast cancer	AQP3	Expression and function
13.	Capoccia, 2015	In vitro	Western blot Wound healing assay DNA fragmentation assay Cell proliferation and survival assays Apoptotic cell staining	Brain cancer	AQP4	Expression and function
14.	Chae, Y. K. 2008	In vitro	Small interfering RNA RT-PCR Immunohistochemistry Immunoblotting Flow cytometry Cytogenetic response	Leukemia	AQP5	Expression and function
15.	Chae, Y. K. 2008	Biopsy In vitro	Tissue microarrays Pull-down assays Invasion assay Immunoprecipitation Immunohistochemistry Fluorescence in situ hybridization	Lung cancer	AQP5	Expression and function
16.	Chae, Y. S. 2015	Biopsy	Tissue array Immunohistochemistry	Breast cancer	AQP3	Expression

17.	Chang, H. 2014	Biopsy	Western blot RT-PCR Immunohistochemistry	Cervical cancer	AQP8	Expression
18.	Chang, H. 2014	In vitro	Wound healing assay Western Blot Immunohistochemistry	Oesophageal cancer	AQP8	Expression and function
19.	Chau, D. 2015	In vitro	Western blot Small interfering RNA RT-PCR Methylation-specific PCR Cytotoxicity assay Apoptosis assay	Leukemia	AQP9	Expression and Function
20.	Chen, C. 2017	In vitro	Wound healing assay Transwell migration Invasion assays Cytotoxicity assay	Colon cancer	AQP5	Expression and function
21.	Chen, G. 2018	In vitro In vivo	Western blot Tumour growth in vivo RT-PCR Invasion assays Immunohistochemistry Cell proliferation	Liver cancer	AQP3	Expression and function
22.	Chen, J. 2004	In vitro	RT PCR	Lung cancer	AQP 1,3,4,5	Expression

23.	Chen, J. 2014	Biopsy In vitro	Western blot Transwell migration Invasion assays Small interfering RNA RT PCR Immunohistochemistry Immunofluorescence assays Cell proliferation assays	Gastric cancer	AQP2	Expression and Function
24.	Chen, J. 2015	In vitro	Wound healing assay Western blot Small interfering RNA RT PCR MMP-3 ELISA assay Invasion assays cDNA microarray	Prostate cancer	AQP2	Expression and function
25.	Chen, L. 2017	Biopsy In vitro	Western blot Small interfering RNA RT PCR Immunohistochemistry Immunofluorescence assays Flow cytometry assay Cellular TAG level detection	Gastric cancer	AQP3	Expression and function

			assay			
26.	Chen, Q. 2016	In vitro	Wound healing assay Western blot Invasion assays Small interfering RNA RT PCR Immunohistochemistry Cell Proliferation Assay Cell apoptosis assay.	Prostate cancer	AQP8	Expression and function
27.	Chen, Q 2018	In vitro	Wound healing assay Western blot Invasion assays Small interfering RNA RT PCR Cell cycle distribution assay Cell apoptosis assay	Prostate cancer	AQP3	Expression and function
28.	Chen, R. 2012	Biopsy	RT PCR Immunohistochemistry	Cervical cancer	AQP 1,3,8	Expression
29.	Chen, R. 2014	Biopsy	RT PCR Immunohistochemistry	Cervical cancer	AQP 1,3	Expression
30.	Chen, X. 2014	In vitro	RT-PCR MTT assay	Ovarian cancer	AQP1 to 10	Expression and function
31.	Chen, X. 2015	In vitro	Western blot	Ovarian cancer	AQP5	Expression and

			Cell proliferation assay Small interfering RNA RT-qPCR			function
32.	Chen, X. F. 2016	Biopsy	Western blot RT-qPCR Immunohistochemistry	Liver cancer	AQP 3,7,9	Expression
33.	Chen, Y. 2017	In vitro	Western blot RT-qPCR Invasion Cell viability assay Cell migration assay Cell cycle analysis	Brain cancer	AQP 4	Expression and function
34.	Chen, Y. 2003	Biopsy	ISH of Tissue Microarrays	Cervical cancer	AQP5	Expression
35.	Chen Y. 2006	In vitro	Western Blots RT-PCR Immunohistochemistry CT scan MRI	Brain cancer	AQP1	Expression
36.	Chen Z. 2011	Biopsy	Western Blot Water permeability assay qRT-PCR Migration assay Invasion assay	Lung cancer	AQP5	Expression and function

			Colony forming test Cellular regulatory volume			
37.	Deb, P. 2012	In vitro	Immunohistochemistry	Brain cancer	AQP1	Expression
38.	Ding, T. 2011	Biopsy In vivo	Western Blot Small interfering RNA Scratch assay Osmotic fragility Invasion assay F-actin measurement Cytotoxicity assay Chemotaxis and chemo kinesis assays Aggregation assay Adhesion assay	Brain cancer	AQP4	Expression and function
39.	Ding, T. 2013	Biopsy In vivo	Western Blot Tumorigenicity Assay Small interfering RNA Osmotic fragility MTT assay Immunohistochemistry Colonies formation assay Apoptosis assay	Brain cancer	AQP4	Expression and function
40.	Dong, X. 2016	Biopsy	Western Blot Small interfering RNA Immunohistochemistry	Gastric cancer	AQP3	Expression and function

			Cell proliferation assays			
41.	Dorward, H. S. 2016	Biopsy In vitro	Wound healing assay Western Blot Immunohistochemistry qPCR Invasion assay Immunofluorescence Cell proliferation assay Angiogenesis assay	Colon cancer	AQP1	Expression and function
42.	Dou, R. 2013	In vitro	MicroRNA data analysis Gene expression data analysis	Colon cancer	AQP9	Expression
43.	Driml, J. 2013	Biopsy In vivo	VEGFA ELISA Small interfering RNA Matrigel VM Assay qRT-PCR Mouse Xenograft MM Model	Malignant mesothelioma	AQP1	Expression and function
44.	Driml, J. 2013	In vitro	Immunohistochemistry	Malignant mesothelioma	AQP1	Expression
45.	Dua, R. K. 2010	In vitro	Immunohistochemistry	Brain cancer	AQP4	Expression
46.	El Hindy, N. 2013	In vitro	Immunohistochemistry	Brain cancer	AQP1	Expression
47.	El Hindy, N. 2013	In vitro	Secreted Alkaline Phosphatase (SEAP) Assay qRT PCR	Brain cancer	AQP1	Expression

			MGMT-Promoter Methylation Analysis Immunohistochemistry			
48.	Endo, M. 1999	In vivo	Immunofluorescence	Brain cancer	AQP1	Expression
49.	Esghaei, M. 2018	Biopsy	MTT assay Immunofluorescence	Colon cancer	AQP5	Expression and function
50.	Evans, J. 2017	Biopsy	Western blot MTT assay Small interfering RNA	Lung cancer	AQP11	Expression
51.	Ewelt, C. 2012	In vitro	Immunofluorescence	Brain cancer	AQP1 AQP4	Expression
52.	Fallier-Becker, P. 2013	Biopsy In vivo	Western blot RT-PCR Immunocytochemistry	Brain cancer	AQP4	Expression
53.	Fossdal, G. 2012	Biopsy In vitro In vivo	Western blot qPCR Immunocytochemistry	Brain cancer	AQP9	Expression
54.	Georges, J. 2011	In vivo In vitro	Western blot RT PCR	Brain cancer	AQP1	Expression

55.	Guan, Y. 2018	In vitro	Western blot Small interfering RNA MTT Test qRT-PCR Migration assay Invasion assay Apoptosis	Brain cancer	AQP1	Expression and function
56.	Guo, K. 2015	Biopsy	Western blot Small interfering RNA MTT assay Migration assay	Lung cancer	AQP5	Expression and function
57.	Guo, X. 2013	In vitro	Immunocytochemistry	Liver cancer	AQP3 AQP5	Expression
58.	Hara-Chikuma, M. 2008	In vivo	Water and glycerol permeability assay RT PCR Cell proliferation assay Cell differentiation assay Cell apoptosis assay ATP production assay	Skin cancer	AQP3	Expression and function
59.	Hara-Chikuma, M. 2016	Biopsy	RT-PCR Immunofluorescence Immunobloting Cellular H2O2 analysis	Skin cancer Lung cancer	AQP3	Expression and function

60.	Hayashi, 2007	In vitro	Western blot Lactate Dehydrogenase Assay Lactate Assay Immunohistochemistry Cathepsin B Enzyme Activity Assay	Brain cancer	AQP1	Expression and function
61.	He, Z. 2017	Biopsy In vivo	Western blot Small interfering RNA qRT-PCR Cell migration assay Cell invasion assay	Liver cancer	AQP5	Expression and function
62.	Hindy, N. E. 2013	In vivo	Immunohistochemistry	Brain cancer	AQP1	Expression
63.	Hoque, M. O. 2006	Biopsy In vitro	Western blot Immunohistochemistry Terminal dUTP Nick-End Labeling (TUNEL) Assay Focus Formation Assay RT-PCR In vitro growth rate ERK Activity assay	Lung cancer	AQP1	Expression and function
64.	Hu, H. 2005	In vivo	Immunohistochemistry	Brain cancer	AQP4	Expression

65.	Huang, D. 2017	Biopsy In vitro In vivo	Western blot Cell growth curve assay QRT-PCR Immunohistochemistry Flow cytometry	Colon cancer	AQP9	Expression and function
66.	Huang, X. 2017	Biopsy	Western blot Small interfering RNA Luciferase reporter assay Cell proliferation and apoptosis assay	Pancreatic cancer	AQP3	Expression and function
67.	Huang, Y. 2009	In vivo	qRT-PCR	Renal cancer	AQP1	Expression
68.	Huang, Y. 2010	Biopsy	Western blot Small interfering RNA Migration assay Cell proliferation assay	Gastric cancer	AQP3	Expression and function
69.	Huang, Y. H.2013	Biopsy In vivo	Western blot RT PCR MTT assay Migration assay Immunohistochemistry Colony formation assay	Gastric cancer	AQP5	Expression and function
70.	Huang, Y. T. 2015	In vivo Biopsy	Western blot Small interfering RNA RT-PCR	Breast cancer	AQP3	Expression and function

			Migration assay Luciferase reporter assay Invasion assay Immunohistochemistry Bioinformation and chromatin immunoprecipitation (ChIP) analyses			
71.	Hwang, I. 2012	In vivo	Western blot Immunofluorescence	Prostate cancer	AQP1,3,9	Expression
72.	Imaizumi, H. 2018	In vivo	Immunohistochemistry	Colon cancer	AQP1	Expression
73.	Imrédi, E. 2018	In vivo	Immunohistochemistry	Skin cancer	AQP1	Expression
74.	Imrédi, E. 2016	In vivo	Immunohistochemistry	Skin cancer	AQP1	Expression
75.	Iriyama, N. 2013	Biopsy In vivo	RT PCR Apoptosis assay	Leukemia	AQP9	Expression and function
76.	Ishimoto, S. 2012	Biopsy In vivo	Immunohistochemistry Western blot Small interfering RNA Cell survival assay Adhesion	Tongue cancer	AQP3 AQP5	Expression and function

77.	Ismail, M. 2009	Biopsy	Western blot Small interfering RNA RT PCR Immunofluorescence Cryoinjury assay	Prostate cancer	AQP3	Expression
78.	Isokpehi, R. D. 2015	Biopsy	Gene analysis	Brain cancer	All AQPs	Expression
79.	Jagirdar, R. 2013	Gene database	BioGRID analysis	Pleural mesothelioma	AQP1	Expression
80.	Jagirdar, R. M. 2016	Biopsy	Western blot Sphere formation assay RT PCR Cell migration assay Cell adhesion	Pleural mesothelioma	AQP1	Expression and function
81.	Jelen, S. 2013	Biopsy In vivo	Immunofluorescence	Brain cancer	AQP9	Expression
82.	Ji, C. 2008	Biopsy	Western Blot Small interfering RNA Phagokinetic track motility assay	Ovarian cancer	AQP3	Expression and function
83.	Jia, B. 2018	Biopsy	Western blot Small interfering RNA RT PCR Invasion assay CCk-8 assay	Breast cancer	AQP5	Expression and function

84.	Jiang, B. 2014	Biopsy In vitro In vivo	Western blot TUNEL assay Tumorigenicity qRT PCR Luciferase assay Immunohistochemistry Cell migration assay Cell invasion assay Cell apoptosis assay Caspase-3 activity assay Colony formation assay 5-ethynyl-2 -deoxyuridine (EdU) assay	Gastric cancer	AQP3	Expression and function
85.	Jo, Y. M. 2016	Biopsy	Immunohistochemistry	Lung cancer	AQP5	Expression
86.	Jung, H. J. 2011	Biopsy In vivo	RT PCR Small interfering RNA Immunohistochemistry BrdU cell proliferation assay Cell migration assay	Breast cancer	AQP5	Expression and function
87.	Kang, B. W. 2015	In vivo	Immunohistochemistry	Colon cancer	AQP1, 3, 5	Expression
88.	Kang, S. 2015	In vivo	Immunohistochemistry	Breast cancer	AQP3	Expression
89.	Kang, S. K. 2008	Biopsy	Small interfering RNA Immunoblotting Cell proliferation assay	Colon cancer	AQP5	Expression and function
90.	Kao, S. C. H. 2012	In vivo	Immunohistochemistry	Pleural Malignant	AQP1	Expression

				Mesothelioma		
91.	Kasimir-Bauer, S. 2009	In vivo	Immunohistochemistry	Breast cancer	AQP5	Expression
92.	Katsurahara, K. 2018	Biopsy	Western blot Small interfering RNA Apoptosis assay RT PCR Migration assay Invasion assay Immunofluorescence Flow cytometer	Liver cancer	AQP5	Expression and function
93.	Klebe, S. 2015	Biopsy In vivo	Small interfering RNA qRT PCR Migration assay Immunohistochemistry Cell proliferation assay Anchorage-Independent Assay	Mesothelioma	AQP1	Expression and function
94.	Kon, T. 2017	In vivo	MRI Immunohistochemistry	Esophageal cancer	AQP4	Expression
95.	Kong, B. 2016	Biopsy	Western blot Small interfering RNA RT PCR MTT assay	Colon cancer	AQP1	Expression and function

96.	Kourghi, M. 2016	Biopsy	Two Electrode Voltage Clamp Recordings Osmotic Swelling Assays Cytotoxicity Cell migration assay	Colon cancer	AQP1	Function
97.	Lee, S. J. 2014	Biopsy	Immunohistochemistry	Breast cancer	AQP5	Expression
98.	Lee, S. J. 2017	Biopsy	Haplotype Identification and Reconstruction	Breast cancer	AQP5	Expression
99.	Lehnerdt, G. F. 2015	Biopsy	Immunohistochemistry	Pharyngeal squamous cell carcinoma	AQP1 AQP5	Expression
100.	Leung, J. 2007	In vivo	Western blot qPCR Arsenic cytotoxicity	Leukemia	AQP9	Expression
101.	Li, A. 2013	Biopsy In vivo	Migration assay Western blot Immunohistochemistry	Colon cancer	AQP3	Expression and function
102.	Li, B. 2012	Biopsy	Immunohistochemistry	Lung cancer	AQP3	Expression
103.	Li, C. F. 2016	Biopsy In vitro In vivo	Western Blot RT-PCR Immunohistochemistry Colony formation assay Cell cycle analysis Apoptosis assay	Liver cancer	AQP9	Expression and function

104.	Li, J. 2014	Biopsy In vivo	Small interfering RNA RT-PCR Immunohistochemistry Immunofluorescence assay FISH staining Cell viability Cell invasion assay	Prostate cancer	AQP5	Expression and function
105.	Li, Q. 2018	In vivo	Western blot Tumor xenograft study Small interfering RNA RT PCR MTT assay Flow cytometry	Colon cancer	AQP5	Expression and function
106.	Li, X. 2018	In vivo	Western blot Small interfering RNA RT PCR MTT assay Flow cytometry Cell migration Cell invasion	Breast cancer	AQP5	Expression and function
107.	Li, X. J. 2007	In vivo	Western blot Immunohistochemistry Carbonic anhydrase activity assay	Lung cancer	AQP1	Expression and function

108.	Li, Y. B. 2016	In vitro	Western blot Small interfering RNA RT PCR Migration assay Invasion assay Cell proliferation assay	Breast cancer	AQP4	Expression and function
109.	Li, Z. 2016	Biopsy In vitro In vivo	Western blot RT PCR Mouse xenograft model Lipid droplet staining Immunohistochemistry G3P assay Colony formation assay Cellular TAG measurement Cellular glycerol detection Cell proliferation assay ATP assay	Gastric cancer	AQP3	Expression and function
110.	Liao, Z. Q. 2016	Biopsy In vitro	Small interfering RNA Western blot RT PCR MTT assay Matrigel invasion assay Dual-Luciferase reporter gene	Brain cancer	AQP1	Expression and function

			assay ChIP assay Cell migration assays			
111.	Lim, B. C. 2014	Biopsy	Immunosorbent assay	Brain cancer	AQP4	Expression
112.	Liu, J. 2015	Biopsy	Immunohistochemistry	Uroepithelium cancer	AQP1	Expression
113.	Liu, S. 2013	Biopsy	Immunohistochemistry	Esophageal cancer	AQP3,5	Expression
114.	Liu, Y. H. 2015	In vitro	Western blot RT PCR MTT assay Invasion assay Flow cytometry	Lung Cancer	AQP1	Expression and function
115.	Liu, Y. L. 2007	Biopsy In vitro	Western blot RT- PCR Immunohistochemistry	Lung cancer	AQP3	Expression
116.	Longatti, P. 2006	Biopsy	Immunohistochemistry	Brain cancer	AQP1	Expression
117.	Longatti, P. 2006	Biopsy	Immunohistochemistry	Brain cancer	AQP1	Expression
118.	Lopez-Campos, J. L. 2011	Biopsy	Immunohistochemistry	Lung cancer	AQP1	Expression

119.	Luo, L. 2018	Biopsy In vitro	qTT-PCR Migration assays Luciferase assays Invasion assays Immunohistochemistry Cell proliferation assays	Breast cancer	AQP1	Expression and function
120.	Luo, L. M. 2017	Biopsy	Immunohistochemistry	Liver cancer	AQP1	Expression
121.	Lv, Y. 2018	In vitro	Western blot RT-qPCR Invasion assay Cell migration assay	Brain cancer	AQP9	Expression and function
122.	Ma, B. 2004	In vivo	Western blot Metastases assays Immunohistochemistry Carbonic anhydrase activity assay	Lung cancer	AQP1	Expression Function
123.	Ma, J. 2016	Biopsy	Western Blot Immunohistochemistry	Ovarian cancer	AQP6,8	Expression
124.	Matsuo, K. 2014	Biopsy	Immunohistochemistry	Oral cancer	AQP3	Expression
125.	Mazal, P. R. 2005	Biopsy	Immunohistochemistry	Liver cancer	AQP1	Expression

126.	McCoy, E. S. 2010	In vitro In vivo	Western blot Site directed mutagenesis Migration assay Immunocytochemistry Cell volume measurement Cell adhesion assay	Brain cancer	AQP1,4	Expression and function
127.	Miao, Z. F.2009	In vitro	MTT cytotoxicity assay Measurement of intracellular glutathione concentration Mass spectrometry Immunoblotting Graphite furnace atomic absorption spectrometry Cell proliferation assay	Lung cancer	AQP9	Expression and function
128.	Ming, L. 2008	Biopsy	Immunohistochemistry	Cervical cancer	AQP1	Expression
129.	Mobasheri, A. 2005	Biopsy	Immunohistochemistry	Prostate Cancer Breast Cancer Ovary Cancer Colon cancer Lung cancer	AQP1	Expression
130.	Mobley, J. 2013	Biopsy	Western blot	Renal cancer	AQP1	Expression
131.	Mobley, J. 2013	Biopsy	Western blot	Renal cancer	AQP1	Expression

132.	Morrissey, J. J. 2013	Biopsy	Western blot	Renal cancer	AQP1	Expression
133.	Morrissey, J. J. 2015	Biopsy	Western blot	Renal cancer	AQP1	Expression
134.	Morrissey, J. J. 2014	Biopsy	Western blot	Renal cancer	AQP1	Expression
135.	Mou, K. 2010	Biopsy	Western blot Immunofluorescence Cranial MRI	Brain cancer	AQP4	Expression
136.	Nakakoshi, M. 2006	In vitro In vivo	RT-PCR Northern blot Immunoblotting	Skin cancer	AQP3	Expression
137.	Nicchia, G. P. 2013	In vitro	Western blot Small interfering RNA Proliferation assay Immunofluorescence	Skin cancer	AQP1	Expression
138.	Niu, D. 2012	Biopsy In vitro	Western blot RT PCR Immunohistofluorescence Immunohistochemistry	Thyroid cancer	AQP3,4	Expression
139.	Noell, S. 2015	Biopsy	RT PCR Electron microscopy Immunohistochemistry	Brain cancer	AQP1,4	Expression

140.	Noell, S. 2012	Biopsy In vitro In vivo	Western blot RT PCR Immunohistochemistry Freeze Fracture	Brain cancer	AQP4	Expression
141.	Noell, S. 2012	Biopsy	Immunohistochemistry Electron microscopy Immunogold labeling Immunoblotting Electrophoresis	Brain cancer	AQP4	Expression
142.	Oshio, K. 2005	Biopsy	Western blot RT PCR Immunohistochemistry Gene Array	Brain cancer	AQP1	Expression
143.	Otterbach, F. 2010	Biopsy	Immunohistochemistry	Breast cancer	AQP1	Expression
144.	Padma, S. 2009	Biopsy	Immunohistochemistry Immunofluorescent	Liver cancer	AQP9	Expression
145.	Pan, H. 2008	Biopsy	Immunohistochemistry	Skin cancer	AQP1	Expression
146.	Pan, X. Y. 2012	In vitro	Western blot Small interfering RNA Migration assay	Prostate cancer	AQP1	Expression and function

			Invasion assay Dual-luciferase reporter assay Cell proliferation assay			
147.	Park, J. Y. 2017	Biopsy	Immunohistochemistry	Prostate cancer	AQP1	Expression
148.	Pei, J. V. 2016	In vitro	Quantitative Oocyte Swelling Assays QRTPCR Western blot Immunocytochemistry Molecular Docking Migration assay Live cell imaging Electrophysiology Cytotoxicity assay	Colon cancer	AQP1	Expression and function
149.	Peng, R. 2016	In vitro	Western blot RT PCR Immunofluorescence Immunocytochemistry Cell proliferation assay	Liver cancer	AQP3,9	Expression and function

150.	Peng, R. 2016	Biopsy In vivo	Xenograft tumor model Western blot RT PCR ELISA Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling assay	Liver cancer	AQP3,9	Expression
151.	Prata, C. 2018	In vitro	Western blot RT-PCR Measurement of Intracellular ROS Level Immunofluorescence Electrophoresis Cell Viability	Leukemia	AQP8	Expression and function
152.	Pulford, E. 2017	In vitro	Xenograft MM Model VEGFA ELISA Small interfering RNA qRT PCR Matrigel VM assay	Skin cancer	AQP1	Expression and function
153.	Qin, F. 2016	Biopsy	Western blot Proliferation assay Immunofluorescence Immunocytochemistry Colony formation Matrigel invasion	Breast cancer	AQP1	Expression and function

154.	Qiu, J. 2018	Biopsy In vitro	Western blot RT PCR Luciferase reporter assay Invasion Assay Cell proliferation	Bone cancer	AQP3	Expression and function
155.	Rentsch, C. A. 2009	Biopsy	qRT PCR	Renal cancer	AQP1	Expression
156.	Rouzaire-Dubois, B. 2009	In vitro	RT PCR Small interfering RNA Sodium imaging	Brain cancer	AQP1	Expression and function
157.	Rubenwolf, P. 2015	Biopsy	Immunohistochemistry	Bladder cancer	AQP3	Expression
158.	Rubenwolf, P. C. 2013	Biopsy In vitro	RT PCR Immunohistochemistry Immunofluorescence	Urothelial cancer	AQP3	Expression
159.	Saadoun, S. 2002	Biopsy	Immunohistochemistry	Brain cancer	AQP1	Expression
160.	Saadoun, S. 2003	Biopsy	Immunohistochemistry	Brain cancer	AQP4	Expression
161.	Saito, Y. 2013	In vitro In vivo	Western blot Glycerol Uptake assay Immunohistochemistry	Ovarian cancer Brain cancer Breast cancer	AQP3,9	Expression and function
162.	Sato, K. 2018	Biopsy	Immunohistochemistry	Ovarian cancer	AQP1,3,5,9	Expression
163.	Satooka, H. 2016	In vitro	Time-lapse imaging of chemotaxis and H2O2 uptake.	Breast cancer	AQP3	Expression and function

			Spontaneous metastasis RT PCR PTP1B oxidation PTEN oxidation Osmotic water permeability assay Migration assay Immunofluorescence Invasion assay Immunoblotting			
164.	Sawada, T. 2007	Biopsy	Immunohistochemistry	Brain cancer	AQP4	Expression
165.	Schob, S.2017	Biopsy	Immunohistochemistry	Brain cancer	AQP4	Expression
166.	Sekine, S. 2014	Biopsy	Immunohistochemistry	Biliary tract cancer	AQP1	Expression
167.	Sekine, S. 2012	Biopsy	Immunohistochemistry	Biliary tract cancer	AQP5	Expression
168.	Sekine, S. 2014	In vitro	Small interfering RNA RT PCR Migration assay Invasion assay Immunohistochemistry Cell proliferation assay	Gallbladder cancer	AQP5	Expression and function

169.	Seleit, I. 2015	Biopsy	Immunohistochemistry	Skin cancer	AQP3	Expression
170.	Shan, T. 2014	Biopsy	Western blot RT PCR Immunohistochemistry Immunofluorescence FISH staining(Saito et al., 2013)	Colon cancer	AQP5	Expression
171.	Shan, T. 2014	Biopsy	Immunohistochemistry	Colon cancer	AQP5	Expression
172.	Shen, Q. 2016	Biopsy	Immunohistochemistry	Cervical cancer	AQP1,3,4,5,8	Expression
173.	Shi, X. 2013	In vitro	Western Blot Small interfering RNA RT PCR Proliferation assay Flow cytometry	Colon cancer	AQP5	Expression and function
174.	Shi, X. 2014	Biopsy In vitro	Western blot Sulforhodamine B staining Small interfering RNA qRT PCR Immunohistochemistry	Colon cancer	AQP5	Expression and function

175.	Shi, X. M. 2015	In vitro	Western Blot Small interfering RNA MTT assay Flow cytometry	Colon cancer	AQP5	Expression and function
176.	Shi, Y. H. 2012	Biopsy	RT PCR Immunohistochemistry Immunofluorescence	Cervical cancer	AQP1,3,8	Expression
177.	Shi, Y. H. 2013	In vitro	Xenografts model Migration assay Invasion assay Cell proliferation Adherence assay	Cervical cancer	AQP8	Function
178.	Shi, Y. H. 2014	In vitro	Western blot Migration assay Immunofluorescence	Cervical cancer	AQP8	Expression and function
179.	Shi, Z. 2012	Biopsy	Western Blot RT PCR Immunohistochemistry	Breast cancer	AQP0-12	Expression
180.	Shimasaki, M. 2011	Biopsy	Western Blot qRT-PCR Immunostaining	Lung cancer	AQP1,3,5	Expression
181.	Shimizu, H. 2014	In vitro Biopsy	Western blot Small interfering RNA RT-PCR cell proliferation assay	Esophageal cancer	AQP5	Expression and function

			Cell cycle analysis Apoptosis assay			
182.	Simone, L. 2018	In vitro In vivo	Xenograft model Western blot Small interfering RNA Immunofluorescence	Skin cancer	AQP1	Expression and function
183.	Smith, E. 2019	In vitro	Western blot qRT-PCR Cell growth assay Cell cycle analysis Apoptosis Acridine Orange Staining	Colon cancer	AQP1	Expression and function
184.	Smith, E. 2019	Biopsy In vitro	qRT PCR 5-aza-2′-deoxycytidine assay	Colon cancer	AQP1	Expression and function
185.	Song, T. 2015	Biopsy	Immunohistochemistry	Lung cancer	AQP5	Expression
186.	Sreedharan, S. 2014	Biopsy	Western blot	Renal cancer	AQP1	Expression
187.	Sung, K. K. 2008	In vitro	Small interfering RNA Immunohistochemistry Immunoblotting Cell proliferation assay	Colon cancer	AQP5	Expression and function

188.	Thapa, S. 2018	Biopsy	Immunohistochemistry	Gastric cancer	AQP1-11	Expression
189.	Ticozzi-Valerio, D. 2007	Biopsy	Immunoblotting Electrophoresis	Renal cancer	AQP1	Expression
190.	Vacca, A. 2001	Biopsy	Western blot Immunoblotting Bone marrow angiogenesis staining	Skin cancer	AQP1	Expression
191.	Wang, D. 2011	Biopsy	Western blot RT PCR Immunohistochemistry	Brain cancer	AQP1,4	Expression
192.	Warth, A. 2011	Biopsy	Western blot qRT PCR Immunohistochemistry	Lung cancer	AQP4	Expression
193.	Warth A. 2007	Biopsy	Immunohistochemistry	Brain cancer	AQP4	Expression

194.	Wu, D. Q. 2018	Biopsy In vitro	Xenograft model Western blot qPCR Migration assay Invasion assay Immunofluorescence Colony formation assay Cell proliferation	Colon cancer	AQP8	Expression and function
195.	Xia, H. 2013	In vitro	Western blot RT PCR MTT Invasion assay Flow cytometry	Lung cancer	AQP1	Expression and function
196.	Xiong, W. 2018	Biopsy In vivo	Western blot Small interfering RNA qRT PCR Migration assay Invasion assay Immunohistochemistry Immunofluorescence H&E staining Dual-luciferase reporter assay	Brain cancer	AQP4	Expression and function
197.	Xuejun, C. 2014	In vitro	PCR MTT assay	Ovarian cancer	AQP1-10	Expression and function
198.	Yang, C. 2016	Biopsy	qRT PCR Immunohistochemistry	Ovarian cancer	AQP3	Expression

199.	Yang, J. 2012	In vitro	Western blot RT PCR Growth rate assay	Ovarian cancer	AQP5	Expression and function
200.	Yang, J. 2017	Biopsy In vitro	Western blot qRT PCR MTT assay Migration assay Immunohistochemistry Flow cytometry	Brain cancer	AQP5	Expression and function
201.	Yang, J. H. 2006	Biopsy	Immunohistochemistry	Ovarian cancer	AQP1	Expression
202.	Yang, J. H. 2006	Biopsy	Immunohistochemistry Western blot RT PCR	Ovarian cancer	AQP5	Expression
203.	Yang, J. H. 2011	EX-vivo	Western blot Immunohistochemistry	Ovarian cancer	AQP3,7,9	Expression
204.	Yang, J. H. 2011	Biopsy	Immunohistochemistry	Ovarian cancer	AQP1-9	Expression
205.	Yang, L. 2012	In vitro In vivo	Xenograft glioma model VEGF activity assay Tumor vessel permeability assay The water contents of tumor tissue assays qPCR Immunohistochemistry ELISA	Brain cancer	AQP4	Expression and function

206.	Yang, W. C. 2015	Biopsy	Western blot Immunohistochemistry	Brain cancer	AQP4,9	Expression
207.	Yang, Z. H. 2015	In vitro In vivo	Xenograft model Western blot Immunostaining Flow cytometry Cell proliferation assay Cell apoptosis	Colon cancer	AQP9	Expression and function
208.	Yin, T. 2008	Biopsy	Immunohistochemistry	Breast cancer	AQP1	Expression
209.	Yong, J. 2009(Yong, 2009)(Yong, 2009)(Yong, 2009)	In vitro In vivo	Xenograft model Water Permeability Measurements RT PCR Migration assay Measurement of RhoA and Rac Activities Invasion assay Immunofluorescence Immunoblotting	Colon cancer	AQP1	Expression and function
210.	Yoshida, T. 2013	Biopsy	Immunostaining H&E staining	Colon cancer	AQP1	Expression
211.	Yun, S. 2016	Biopsy	Immunohistochemistry	Lung cancer	AQP1	Expression

212.	Zhang, L. 2018	In vitro In vivo	Xenograft model Western blot TUNEL assay RT-qPCR MTT assay Flow cytometry Colony formation assay.	Lung cancer	AQP5	Expression and function
213.	Zhang, L. 2018	Biopsy	Pet/CT scan Immunohistochemistry	Cervical cancer	AQP1	Expression
214.	Zhang, T. 2013	Biopsy	Western blot qRT PCR Migration assay Luciferase assays Invasion assay Immunohistochemistry Cell proliferation assay	Breast cancer	AQP1	Expression and function
215.	Zhang, T. 2012	Biopsy	Western Blot qRT PCR Immunohistochemistry	Cervical cancer	AQP5	Expression
216.	Zhang, W. G. 2016	Biopsy In vitro In vivo	Western Blot Small interfering RNA qRT-PCR Invasion assay Immunohistochemistry Cell migration assay	Liver cancer	AQP9	Expression and function

217.	Zhang, X. 2018	In vitro	Western Blot qRT PCR MTT assay Flow cytometry	Bladder Cancer	AQP1	Expression and function
218.	Zhang, Z. 2010	In vitro In vivo	Xeongrafted model Western blot Small interfering RNA RT PCR Osmotic water permeability measurement Migration assay Invasion assay Immunohistochemistry Immunofluorescence	Lung Cancer	AQP5	Expression and function
219.	Zhang, Z. 2019	Biopsy In vivo	Western blot qRT PCR Luciferase reporter assay Flow cytometry assay Detection of HBsAg and HBeAg Cell viability assay Cell proliferation assay Caspase-3 activity assay Apoptosis	Liver cancer	AQP5	Expression and function
220.	Zhang, Z. Q. 2011	In vitro In vivo	Xenograft model Western Blot RT PCR Immunohistochemistry Immunofluorescence	Lung cancer	AQP5	Expression and function

221.	Zhao, W. J. 2012	Biopsy In vitro	Immunohistochemistry Immunofluorescence	Brain cancer	AQP4	Expression
222.	Zhu, S. J. 2013	Biopsy	Immunohistochemistry Immunoblotting RT-PCR	Brain cancer	AQP8	Expression
223.	Zou, L. B. 2011	Biopsy In vitro	Western blot siRNA qRT PCR Migration assay Luciferase reporter assay Invasion assay Immunohistochemistry Immunofluorescence Electron microscopic Cell proliferation Adhesion assay	Endometrial cancer	AQP2	Expression and function
224.	Zou, W. 2019	Biopsy	Western blot Immunohistochemistry	Pancreatic cancer	AQP1,3	Expression

1.9.2 Supplementary table 2 – excluded paper

Systematic review - paper excluded

No.	Study	Aquaporin type	Reason for exclusion
1	Aboulenein-Djamshidian, F. 2015	AQP4	Not related to cancer
2	Aikman, B. 2018		Review article
3	Amiry-Moghaddam, M. 2010		Review article
4	Angelico, G. 2017		Conference abstract
5	Angelico, G., 2017		Conference Abstract
6	Annus, A, 2018	AQP5	Only use as a marker for cell type
7	Arcangeli, A. 2010		Review article

8	Armağan, H. 2012		Not directly link to cancer
9	Belkacemi, L. 2008	AQP1	Not directly link to cancer
10.	Carlson, M. R. J. 2007	AQP3	Not directly link to cancer, focusing in oedema
11.	Chan, K. H. 2010		Conference Abstract
12.	Chan, K. H. 2010		Not directly link to cancer
13.	Chen Z. 2006	AQP5	Not directly link to cancer
14.	Clapp,C. 2006		Mini- review
15.	Collinson,K 2009		Not directly link to cancer
16.	Dajani, S. 2018		Not directly link to cancer

17.	Ding, T. 2010	AQP4	Review
18.	Direito, I. 2016	AQP5	Review
19.	Echevarría, M. 2018	AQP1 and AQp3	Conference abstract
20.	Engelhorn, T. 2009		Not focusing on AQP
21.	Erguven, M. 2012		Not focusing on AQP
22.	Fennell, D. A. 2014		Not focusing on AQP
23.	Fischer, H. 2001	AQP8	Not directly link to cancer
24.	Fu, C. 2015		Not focusing on AQP
25.	Fujita-Yoshigaki, J. 2008		Not focusing on AQP
26.	Gawlitza, M. 2017		Not directly link to cancer
27.	Hanada, S. 2008		Not directly link to AQP

28.	Hua, Y. 2017		Not directly lint to AQP
29.	Ikarashi, N. 2011		Not directly link to cancer
30.	Isoardo, G. 2012		Not directly link to cancer
31.	Jarius, S. 2010		Letter
32.	Jensen, H. H. 2016		Review
33.	Kourghi, M. 2017		Not directly link to cancer
34.	Lan, Y. L. 2017		Review
35.	Liang, L. 2017		Not focusing on AQP
36.	Liao, W. 2019		Not directly link to cancer
37.	Login, F. H. 2019		Not directly link to cancer
38.	Longatti, P. L. 2004		Not directly link to cancer
39.	Magouliotis, D. 2018		Not a full paper

40.	Mitsuda, M. 2018		Not the right author
41.	Monzani, E. 2007		Review
42.	Moon, C. 1997		Not directly link to cancer
43.	Nico, B. 2010		Mini review
44.	Nico, B. 2011		Review
45.	Otterbach, F. 2008		In German
46.	Papadopoulos, M. C. 2015		review
47.	Pei, J. V. 2019		Not directly link to cancer
48.	Pollo, B. 2017		Abstract
49.	Rodrigues, C. 2013		Conference paper

50.	Rossel, T. 2015		Case report
51.	Rubenwolf, P. 2013		Abstract
52.	Rubenwolf, P. C. 2012		Letter
53.	Saadoun, S. 2002		Not directly link to cancer
54.	Saadoun, S. 2005		Not directly link to cancer
55.	Shepard, M. J. 2017		Not directly link to cancer
56.	Stigliano, C. 2013		Not directly link to cancer
57.	Suero Molina, E. J. 2013		Not directly link to cancer
58.	Ye, H. J. 2016		Not directly lint to AQP
59.	Yool, A. J. 2010		Review

60.	Zhang, H. 2016		Not directly link to cancer
61.	Zhang, H. 2014		Not directly link to cancer

Chapter 2: 5-hydroxymethyl-furfural and structurally related compounds block the ion conductance in human aquaporin-1 channels, and slow cancer cell migration and invasion.

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Chapter 2 has been submitted to Molecular Pharmacology.

2.1 Abstract

Aquaporin-1 (AQP1), a dual water and ion channel, enhances migration and invasion when upregulated in leading edges of certain classes of cancer cells. Work here identified structurally-related furfural compounds as novel inhibitors of AQP1 ion channels and tested their effects on migration in AQP1-expressing cancer cell lines. Effects of 5-Hydroxymethyl-2-furfural (5HMF), a component of natural medicinal honeys, and three structurally related furan compounds nitrofuroic acid (5NFA), acetoxymethylfuraldehyde (5AMF), and methylnitrofuroate (M5NF), were determined for water and ion channel activities of human AQP1 expressed in Xenopus oocytes. Twoelectrode voltage clamp demonstrated dose-dependent block of the AQP1 ion current by 5HMF>5NFA>5AMF (IC_{50} 0.43 mM, 1.2 mM and ~3 mM, respectively), but no inhibition by M5NF. In silico docking predicted the same order for favorable energies of interaction, locating putative binding sites in the intracellular central pore vestibule. Water fluxes were unaltered by the furans (up to 5mM). Effects on in vitro cell migration, invasion and cytoskeletal organization were tested in high AQP1-expressing cancer lines, HT29 and MDA-MB-231, and SW480 with low AQP1. The same order of potency for AQP1 ion channel block was observed for impairment of motility, except

M5NF which immobilized all cancer lines tested by disrupting actin cytoskeleton, an effect not seen for the other compounds. No cytotoxicity occurred at doses effective for blocking motility. These results define a new class of AQP1 ion channel inhibitors, and confirm the AQP1 ion conductance influences cell motility. Furans hold promise as novel lead compounds for the development of aquaporin channel inhibitors.

2.2 Introduction

Aquaporin (AQP) channels found in all kingdoms of life serve essential roles in transmembrane fluid and solute fluxes, enabling regulation of volume and osmotic gradients across cell membranes (Agre et al., 1993; Gomes et al., 2009; Hohmann et al., 2000; Reizer et al., 1993). Aquaporin channels are tetramers of subunits, with monomeric pores that facilitate osmotic water flux (Jung et al., 1994; Sui et al., 2001). In AQP1, the central pore is a cGMPgated nonselective cation channel, permeable to monovalent cations such as Na+, K+ and Cs+, and pharmacologically distinct from the intra-subunit water pores (Boassa and Yool, 2003; Campbell et al., 2012; Kourghi et al., 2018; Saparov et al., 2001; Yool et al., 1996; Yu et al., 2006). Ion channel activity has been reported for other aquaporins expressed in mammals (AQP0, AQP6), as well as in insects (Drosophila Big Brain) and plants (soybean nodulin 26 and rockcress AtPIP2;1) (Anthony et al., 2000; Boassa et al., 2006; Byrt et al., 2017; Hazama et al., 2002; Weaver et al., 1994; Yanochko and Yool, 2002).

Chemical modulators of AQP water and glycerol channel activities have been characterized by research teams around the world (Huber et al., 2012; Martins et al., 2013; Pei et al., 2016a; Seeliger et al., 2013; Yool et al., 2009). Inhibitors of the human AQP1 ion conductance identified thus far include bacopaside I from the Ayurvedic medicinal plant Bacopa monnieri and derivatives of bumetanide, AqB007 and AqB011, which also act to slow cell migration and decrease invasiveness in classes of cancer cell lines which express high levels of AQP1 (Kourghi et al., 2016; Pei et al., 2016b). Divalent cations such as Cd2+ and Ca2+ also inhibit AQP1 ion conductance (Boassa et al., 2006; Kourghi et al., 2017). The ongoing search for modulators is important for developing a globally accessible pharmacological armementarium for AQP research, for identifying drug candidates aimed at potential future clinical translations,

and for building understanding of ligand structure-activity relationships for diverse classes of aquaporins.

Traditional medicines have been used by humans for more than 4000 years as valuable sources of agents with therapeutic effects, including anti-cancer activities (He et al., 2019; Khan, 2014). Natural remedies based on Manuka and Tualang honeys include antioxidant (Khalil et al., 2012), anti-inflammatory (Gasparrini et al., 2018), antibacterial (Girma et al., 2019), and antidiabetic (Lori et al., 2019) effects, with benefits in gastrointestinal disorders (Ghosh and Playford, 2003), wound infections (Shan, 2019), cancers (Abel et al., 2018; Afrin et al., 2018; Aryappalli et al., 2017; Attia et al., 2008), and other conditions. Honey components are of interest as possible therapeutic agents (Afrin et al., 2019). Given the role of AQP1 as one of the pathways influencing cancer cell motility, we hypothesized that one (or more) of the compounds in honey extract might act by inhibiting AQP1 water or ion channel activity.

Traditional medicines have been used by humans for more than 4000 years as valuable mixtures of agents with likely therapeutic effects, including anticancer activities (Khan, 2014; He et al., 2019). Natural remedies based on manuka and tualang honeys are reported to have antioxidant (Khalil et al., 2012), anti-inflammatory (Gasparrini et al., 2018), antibacterial (Girma et al., 2019), and antidiabetic (Lori et al., 2019) effects, with benefits in gastrointestinal disorders (Ghosh and Playford, 2003), wound infections (Shan, 2019), and cancers (Attia et al., 2008; Aryappalli et al., 2017; Abel et al., 2018; Afrin et al., 2018). Medicinal extracts have been useful as sources of new pharmacological agents to modulate proteins involved in diverse physiologic functions, including for example ion channels, receptors, and transporters (Sucher and Carles, 2015). One of the compounds naturally occurring in both manuka and tualang honeys is 5-hydroxymethyl-2-furfural (5HMF), which shows differences in concentration

levels that have been suggested to correlate with therapeutic effectiveness (Ahmed and Othman, 2013). 5HMF confers protective effects in brain and cardiac ischemic injury models. Intraperitoneally injected 5HMF (12 mg/kg) reduced neurologic deficits and brain edema in mice after transient global cerebral ischemia (Ya et al., 2017). In perfused isolated rat hearts, 5 mM 5HMF reduced damage during 20-minute no-flow ischemia, enhanced coronary artery relaxation, and accelerated recovery to normal sinus rhythm during reperfusion (Wölkartet al., 2017).

Work here tested for effects of 5HMF and related compounds on the ion conductance and the water channel activity of human AQP1 channels expressed in Xenopus oocytes and evaluated the effects of the same agents on the rates of migration and invasiveness of AQP1-expressing breast cancer (MDA) and colon cancer (HT29) cell lines and a colon cancer line with low levels of AQP1 (SW480). Results showed that 5HMF blocked the ion conductance but not the osmotic water flux mediated by AQP1, and provided evidence for a structureactivity relationship for furan compounds, which was supported by results from in silico docking modeling. Block of cell migration in AQP1-positive lines correlated with effectiveness in inhibiting AQP1 for three (5HMF, 5ANF, 5NFA) of the four agents tested. The fourth compound, M5NF, blocked motility in all cell lines by an AQP1-independent mechanism. These results expand the panel of known AQP1 modulatory agents and identify new low-cost pharmacological antagonists that are available from commercial suppliers internationally. Possible therapeutic activities of improved furan derivatives in animal models of cancer metastasis could be of interest in future research. Materials and M

2.3 Material and Methods

2.3.1 Oocyte preparation and cRNA injection

Unfertilized oocytes were harvested from anesthetized female Xenopus laevis frogs in accord with national guidelines (Australian Code of Practice for the Care and Use of Animals for Scientific Purposes), using protocols approved by the University of Adelaide Animal Ethics Committee (M2018-016). Harvested oocytes were defolliculated in collagenase type 1A (2 mg/ml) in isotonic saline (100mM NaCl, 2mM KCl, 5mM $MgCl_2$, and 5mM HEPES; pH 7.6) for 1.5 hours at approximately 18°C. Oocyte were washed three times with isotonic saline, and transferred into frog ringers saline (isotonic saline supplemented with 0.6 mM $CaCl_2$, 5% horse serum (v/v), 100 units/ml penicillin, 0.1 mg/ml streptomycin, and 0.5 mg/ml tetracycline). Healthy oocytes were injected with 50 nl of sterile water (non-AQP1 control oocytes), or 50 nl wild type hAQP1 cRNA (approximately 1 ng in sterile water). Injected oocytes were incubated in frog ringers saline at 18°C for 48 h or more to allow time for protein expression. Prior to experimental assays, control and AQP1-expressing oocytes were rinsed in isotonic saline (without serum, antibiotic free) for at least 1 hour.

Human AQP1 cDNA (National Center for Biotechnology Information GenBank NM_198098) (Preston et al., 1992) subcloned in a Xenopus ß-globin expression plasmid was linearized with BamHI and transcribed using T3 polymerase (T3 mMessage mMachine; Ambion, Austin, TX, USA). The cRNA was resuspended in sterile water and stored at -80°C. All chemicals are from Sigma Aldrich Chemicals (St Louis, MO, USA) unless otherwise indicated.

2.3.2 Furan compounds

5-hydroxymethyl-2-furfural (5HMF) and three structurally related compounds: 5-nitro-2-furoic acid (5NFA); 5-acetoxymethyl-2-furaldehyde 9 (5AMF), and methyl 5-nitro-2-furoate (M5NF), were purchased from Sigma Aldrich Chemicals. 5-HMF was dissolved in water while other compounds were dissolved in dimethylsulfoxide (DMSO) to create 1000x stock solutions, diluted 1 µl/ml into experimental salines to final concentrations. The equivalent amount of DMSO alone (0.1%) in saline or cell culture medium was used as the vehicle control.

2.3.3 Quantitative swelling assay

For the swelling assays, each oocyte served as its own control, as described previously (Migliati et al., 2009). Each oocyte was tested first without drug treatment, and then incubated for 2 hours in isotonic saline with vehicle or with one of the furfural-related compounds, and then reassessed in a second swelling assay. Swelling rates were measured in 50% hypotonic saline (isotonic saline diluted with an equal volume of water, without test compounds present). Oocytes were imaged with a grayscale camera (Cohu, San Diego, CA) on a dissecting microscope (Olympus SZ-PT; Olympus, Macquarie Park, Australia) at 1 frame per second for 30s using NIH ImageJ software. Oocytes were then incubated for 2 hrs in isotonic saline alone, with vehicle, or with the indicated compound, and reassessed in a second swelling assay. The swelling rates were calculated from slope values of linear regression fits of cross-sectional areas as a function of time using GraphPad Prism.

2.3.4 Electrophysiology

Two-electrode voltage clamp recordings of control and AQP1-expressing oocytes in isotonic

Na^+ saline were done with capillary glass electrodes (1–3 MΩ) filled with 1 M KCl, using a GeneClamp amplifier. Bath application of membrane permeable 8CPT-cGMP (8-(4chlorophenylthio)-guanosine 3′,5′-cyclic monophosphate) activated the ionic conductance in AQP1-expressing oocytes, as described previously (Campbell et al., 2012). The ionic conductance in AQP1-expressing oocytes was activated by application of a bolus of 8CPTcGMP to achieve a final bath concentration of 10 μM. Changes in current over time were monitored by brief repeated voltage step protocols to +40 mV from a holding potential of -40 every 6 s. Conductance values were measured using voltage steps from +60 to -110 mV. Recordings were filtered at 2 kHz, and stored to hard disk for offline analysis. Data were analyzed with Clampex 9.0 software (pClamp 9.0, Molecular Devices, Sunnyvale CA USA) and Prism software (GraphPad, San Diego CA USA).

2.3.5 Molecular modeling

In silico modeling was conducted as reported previously (Pei et al., 2016b). The protein crystal structures of human AQP1 were obtained from the protein data bank(PDB ID:1FQY). Structures for 5-HMF and related compounds were downloaded from PubChem and converted into software-compatible 3D structures in .pdb format using the online SMILES Translator and Structure File Generator (National Cancer Institute, U.S.Department Health and Human Services, Washington, DC). Both AQP1 and ligand coordinates were prepared for docking using MGLtools (Version 1.5.4; Scripps Institute, San Diego, CA). The docking was carried using Autodock Vina (Trott and Olson, 2010), with a docking grid covering the intracellular face of the tetrameric pore.

2.3.6 Cancer Cell Culture and Migration Assays

HT29 and SW480 colon cancer cell lines and MDA-MB-231 (referred to here as 'MDA') breast cancer cell lines (from American Type Culture Collection, Manassas, VA) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 1× glutaMAX (Life Technologies, Mulgrave, Australia), penicillin and streptomycin (100 U/ml each), and 10% fetal bovine serum (v/v) at 37°C in a 5% CO_2 humidified environment. For wound healing assays of two-dimensional migration over flat surfaces, confluent cultures of cancer cell lines were tested using the circular wound closure method (De Ieso and Pei, 2018) to measure the effect of 5HMF and related compounds on the rates of cell migration. Cells were plated in flat-bottomed 96- well plates at 1.25 x 10^5 cells/well for HT29, or 1x10^5 cells/well for MDA and SW480 lines, in Dulbecco's modified Eagle's medium (DMEM) supplemented with 1× glutaMAX (Life Technologies, Mulgrave, Australia), penicillin and streptomycin (100 U/ml each), and 10% fetal bovine serum. Cultures were incubated at 37°C in 5% CO_2 for 18-24 hours. Once cells achieved 80-90% confluence, the culture medium was replaced with reduced serum (2% FBS) DMEM medium with 400 nM of the mitotic inhibitor 5-fluoro-2'deoxyuridine (FUDR) to minimize cell proliferation, and cells were incubated overnight to achieve a confluent monolayer. Circular wounds were created with a sterile p10 pipette tip; wells were washed two times with phosphate-buffered saline (PBS) to remove cell debris. Reduced serum DMEM culture medium (containing either vehicle or furfural derivatives) was applied into the wells. Images of wounds were taken at 0 h and 24 h with a Canon 6D camera on an Olympus CK2 microscope (10x objective). Wound areas were quantified using NIH ImageJ software (U.S. National Institutes of Health) as described previously (De Ieso and Pei, 2018).

Transwell invasion assays were used to measure three-dimensional migration through an extracellular matrix. Cells were grown to approximately 40% confluence under normal culture conditions and transferred into reduced serum (2% FBS) DMEM medium for 32-34 hours

before seeding. Corning Transwell polycarbonate membrane cell culture inserts (8 μm pore size; product #3422; Sigma-Aldrich, St. Louis, MO) were prepared by coating the upper surface of the filter with 40 μL of extracellular matrix-like gel from Engelbreth-Holm-Swarm murine sarcoma (diluted to 25 μg/mL in sterile water; Sigma-Aldrich, St. Louis, MO), allowed to dry overnight in a sterile hood, then rehydrated with 50 μL of serum-free DMEM 2 h before cell seeding. In the lower chamber, 600 μL of DMEM with 10% serum (chemoattractant) was added with or without the vehicle or furan agents. Cells were seeded into the upper chamber at 2.5×10^5 per well for HT29, 1.5×10^5 per well for SW480, and 1×10^5 per well for MDA-MB-231 in serum-free DMEM, with DMSO or furan agents matching the lower chamber. Durations of incubation for transwell assays were optimized empirically in previous work (Nourmohammadi et al., 2019); durations used were 6 h for MDA, and 24 h for HT29 and SW480, at 37°C in 5% CO_2. For quantitation of invasion, non-migrated cells were wiped from the upper surface of the membrane with a cotton swab; migrated cells on the trans (lower) surface were counted after staining with crystal violet (Sigma-Aldrich, St Louis, MO, USA). Numbers of migrated cells were determined for three fields per replicate, with two replicates per experiment, and normalized to the mean number of migrated cells in the vehicle control treatment for the same cell line.

2.3.7 Cytotoxicity Assay

HT29, MDA and SW480 cell viabilities were assessed using the AlamarBlue assay (Molecular Probes, Eugene, OR). Cells were plated at 10^4 cells/well in 96 well plates, and fluorescence signals were measured with a FLUOstar Optima microplate reader (BMG Labtech, Ortenberg, Germany) after 24 hours of incubation with vehicle, 5HMF or related furan compounds.

2.3.8 Actin staining

HT29, SW480 and MDA cells were cultured in μ-Plate 8 Well dishes (Ibidi, Munich, Germany), in 2% FBS with FUDR (400 nM) and incubated at 37°C in 5% CO2. After12-18 hours incubation, cells were treated with DMSO (vehicle) or furfural derivatives at 1mM, and incubated for another 24 h. Cells were then washed with PBS, and fixed with 4% paraformaldehyde at room temperature for 10–30 min. Fixed cells were rinsed 2–3 times with PBS, and permeabilized with 200 μl of 0.1% Triton X-100 in PBS for 3–5 min at room temperature. Phalloidin-iFluor 488 Reagent CytoPainter (ab176753; Abcam, MA, USA) at 1:1000 dilution was used to stain F-actin cytoskeleton at room temperature in the dark for 1–2 hours. 1:1000 diluted Hoechst stain (cat # 861405; Sigma-Aldrich) was used to label cell nuclei. Cells were visualized using a SP5 laser scanning confocal microscope (Leica, Germany) at Adelaide Microscopy core facilities.

2.3.9 Quantitative PCR analysis of AQP1 expression

HT29, SW480 and MDA cells were seeded in triplicate at 4×10^5 cells per well in 6-well plates, and incubated at 37°C in humidified 5% CO_2 environment overnight. The PureLink™ RNA Mini Kit (Invitrogen; Carlsbad CA, USA) was used for total RNA extraction for all cancer cell lines. cDNA was synthesized from 1μg of extracted RNA using QuantiTect Reverse Transcription Kit (Qiagen; Hilden, Germany). Synthesized cDNA was quantified using a NanoDrop (Life Technologies; Carlsbad, CA USA). A final concentration of 50ng cDNA was used to perform Real-time qRT-PCR analyses using SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA USA). StepOne Plus Real-Time PCR software was used for data analysis. The primer sequences used for AQP1 were: forward 5′-CGCAGAGTGTGGGCCACATCA- 3′, and reverse 5′ -CCCGAGTTCACACCATCAGCC -3′, amplifying a product of 217 bp. RPS13 was used as a standard and target mRNA levels relative to RPS13 were calculated using the formula 2-ΔCT.

2.3.10 Data analysis and statistics

Results compiled from replicate experiments are presented as box plots to show the full range of data points. The box represents 50% of the data, the error bars indicate the full range, and the horizontal bar is the median value. Statistical differences were evaluated using one-way ANOVA with post-hoc Bonferroni or paired Students T-tests, and reported as $p<0.00001$ (****); $p<0.0001$ (***); $p< 0.0020$ (**); $p<0.05$ (*); or not significant (NS; $p\geq0.05$).

2.4 Results

2.4.1 Effects of furfural compounds on the AQP1 ion channel conductance

Effects of 5-hydroxymethyl-2-furfural (5HMF) and three structurally related compounds: 5nitro-2-furoic acid (5NFA); 5-acetoxymethyl-2-furaldehyde 9 (5AMF), and methyl 5-nitro-2furoate (M5NF) were assessed using two-electrode voltage clamp electrophysiology to record ion conductance levels in AQP1-expressing and non-AQP control oocytes (**Figure 1**).

Currents were measured before ('initial') and 30 min after activation by 8CPT-cGMP ('cGMP 1st'). Oocytes were then incubated 2 hours in isotonic saline with vehicle or the indicated agents. During the incubation period (without 8CPT-cGMP present), the ionic conductance recovered to initial levels, as described previously (Kourghi et al., 2016). Incubation with the vehicle (DMSO) did not impair the second conductance response to 8CPT-cGMP ('cGMP 2nd'). Responses were inhibited following incubation in 1 mM 5HMF, 3 mM 5NFA, and partially in 5 mM 5AMF. No appreciable block was observed after incubation with M5NF (5 mM).

Compiled data for conductance responses in a box plot (**Figure 2**) summarize amplitudes of cGMP-activated conductances in AQP1-expressing oocytes before and after incubation with vehicle or furfural derivatives, as indicated. Conductance responses to cGMP were significantly reduced after treatment with 5HMF, 5NFA or 5AMF as compared with the initial cGMP-activated responses in the same oocytes (Fig 2A). M5NF was not effective. Structures of the furfural derivatives are illustrated in Fig 2B. An intact carboxylic acid group on a furan ring is a feature seen in all three active agents; methylation of the carboxylic acid group in M5NF correlated with absence of apparent inhibition of AQP1 ion channels. Trend plots (**Figure 3**) summarize conductance responses measured by two-electrode voltage clamp for individual oocytes expressing AQP1 and non-AQP-expressing controls. Initial conductances in

AQP1-expressing and control oocytes were similarly low before addition of 8CPT-cGMP. After application of the agonist ('1st cGMP'), currents in AQP1-expressing oocytes increased to a maximum amplitude by 30 min; whereas control oocytes showed no substantial effect of cGMP in the same time. Oocytes were then transferred into incubation salines containing vehicle or the indicated furfural derivatives for 2 hours. Initial ionic conductances recorded after the incubation period ('post incub') were comparable to those in the starting initial condition, confirming that ion channel activation was reversible. Second applications of CPT-cGMP in normal saline were used to assess levels of inhibition established during the incubation period. The AQP1 conductance was fully reactivated by 8CPT-cGMP ('2nd cGMP') after incubation in saline with vehicle, showing that repeated recordings were well tolerated. The impairment of AQP1 reactivation by cGMP after incubation in 1 mM 5HMF, 3 mM 5NFA, or 5 mM 5AMF indicated inhibition of the ionic conductance. M5NF showed no appreciable blocking effect. Non-AQP1 expressing control oocytes showed little or no effects of cGMP, vehicle, or furfural derivatives.

The blocking effects of the active furfural derivatives on the AQP1 ionic conductance were dose-dependent and required time to establish (**Figure 4**). The dose-response relationships (Fig 4A) show percent block as a function of concentration, yielding estimated IC_{50} values for 5HMF (0.43 mM), 5NFA (1.2 mM), and 5AMF (approximately 3 mM or higher). The onset of block of the AQP1 conductance by 5HMF was not immediate, and required time for establishment (Fig 4B). Ion conductances were measured for AQP1-expressing oocytes after activation by 8CPT-cGMP. Oocytes were then transferred into incubation saline with 1 mM 5HMF, where they remained for 15, 30 or 60 mins before being rinsed in standard saline and tested for reactivation by a second application of 8CPT-cGMP. A non-significant decline was observed after 15 minutes. The magnitude of block significantly increased with longer times of incubation in 1 mM 5HMF, reaching essentially complete block after 1 hour incubation (Fig

4B), at which time the ion conductance was not significantly different from that in non-AQPexpressing control oocytes (data not shown). The time needed to establish block was consistent with a predicted intracellular site of action of 5HMF at the AQP1 channel. Similar latencies for onset of block (1-2 h) have been described for agents such as AqB013, AqB011, and bacopasides that act at the intracellular side of the AQP1 channel (Kourghi et al., 2016; Migliati et al., 2009; Pei et al., 2016b), and require time to cross the plasma membrane.

In silico docking analyses were used to investigate candidate sites for interaction of the ligand 5-hydroxymethyl-2-furfural with the human AQP1 channel (**Figure 5**). Views from the intracellular side of the channel show the sites with the most favorable energies of interaction are located at loop D interface in the central pore of the tetramer (Fig 5A). These cytoplasmic loops between the 4th and 5th transmembrane domains of each subunit modulate AQP channel gating (Kourghi et al., 2018; Nyblom et al., 2009; Yu et al., 2006). In silico models of predicted binding sites for 5HMF and 5NFA suggest formation of hydrogen bond interactions between the ligand carboxylic acid and glycine 165 (Fig 5b), a residue conserved in AQP1 amino acid loop D sequences across species (Kourghi et al., 2018). Calculated predicted energies of interaction (kcal/mol) are: 5HMF (-4.9) > 5NFA (-4.3) > 5AMF (-4.0) > M5NF (-3.9), matching the order of potency for inhibition of the ion conductance (Fig 1).

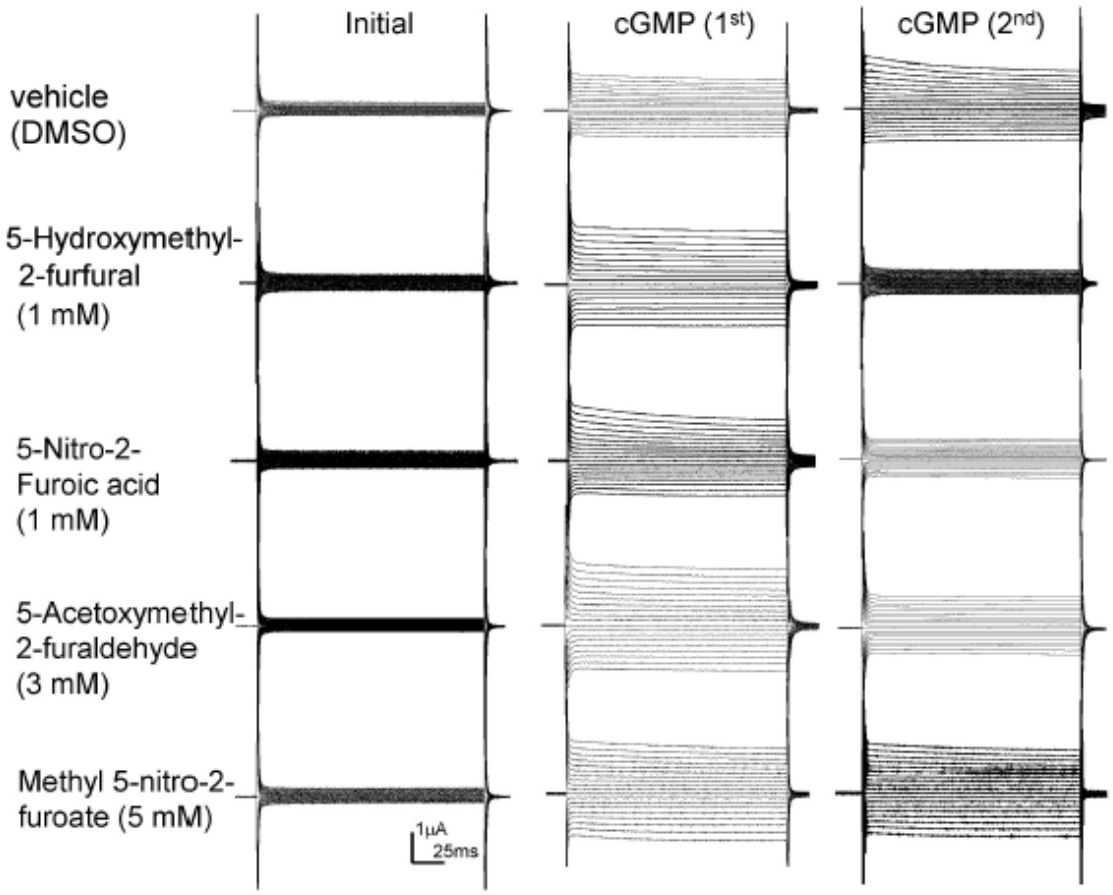

Figure 1. Electrophysiological recordings illustrating the effects of 5HMF and related compounds on the cGMP-activated AQP1 ion conductance. 5-Hydroxymethyl-2-furfural, 5-nitro-2-furoic acid, 5-acetoxymethyl-2-furaldehyde and methyl 5-nitro-2-furoate were tested for effects on the ionic conductance of AQP1-expressing oocytes. Each row shows responses recorded during the sequential treatment of a single oocyte: first prior to application of 8CPTcGMP (initial; left); second at 30 min after the first application of 8CPT-cGMP (cGMP 1st; middle); and third after 2 h incubation in the indicated treatment followed by 30 min reapplication of 8CPT-cGMP (cGMP 2nd; right).

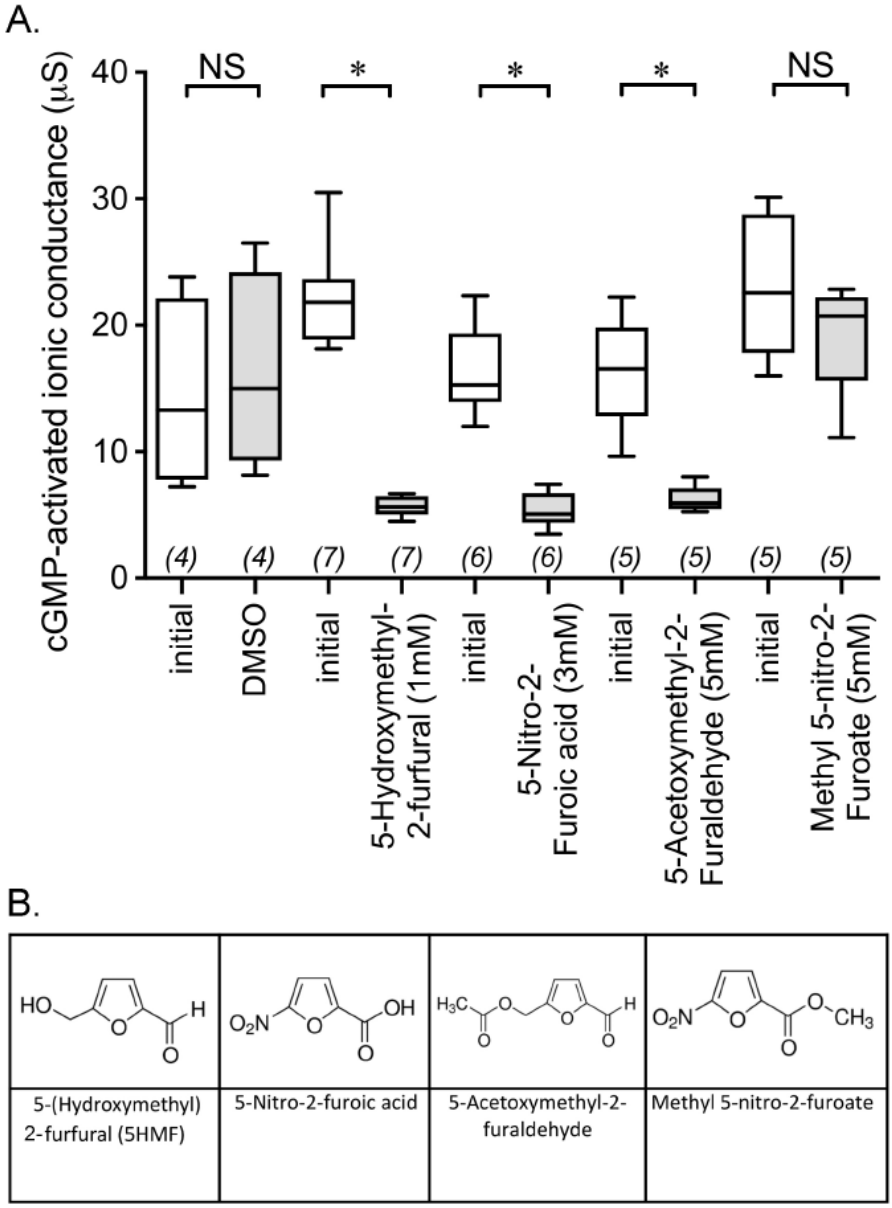

Figure 2. Differential inhibition of the cGMP-activated AQP1 conductance by 5HMF and related compounds. (A) Box plot showing statistically significant inhibition of the cGMPactivated AQP1 ionic conductance with 5-hydroxymethyl-2-furfural, 5-nitro-2-furoic acid and 5-acetoxymethyl-2-furaldehyde but not with vehicle (DMSO) or methyl 5-nitro-2-furoate. (* $p < 0.05$; NS not significant). Boxes contain 50% of the data points; bars show the full range of data values; horizontal bars show the median value. n values are above the x-axis. (B) Structures of the compounds tested.

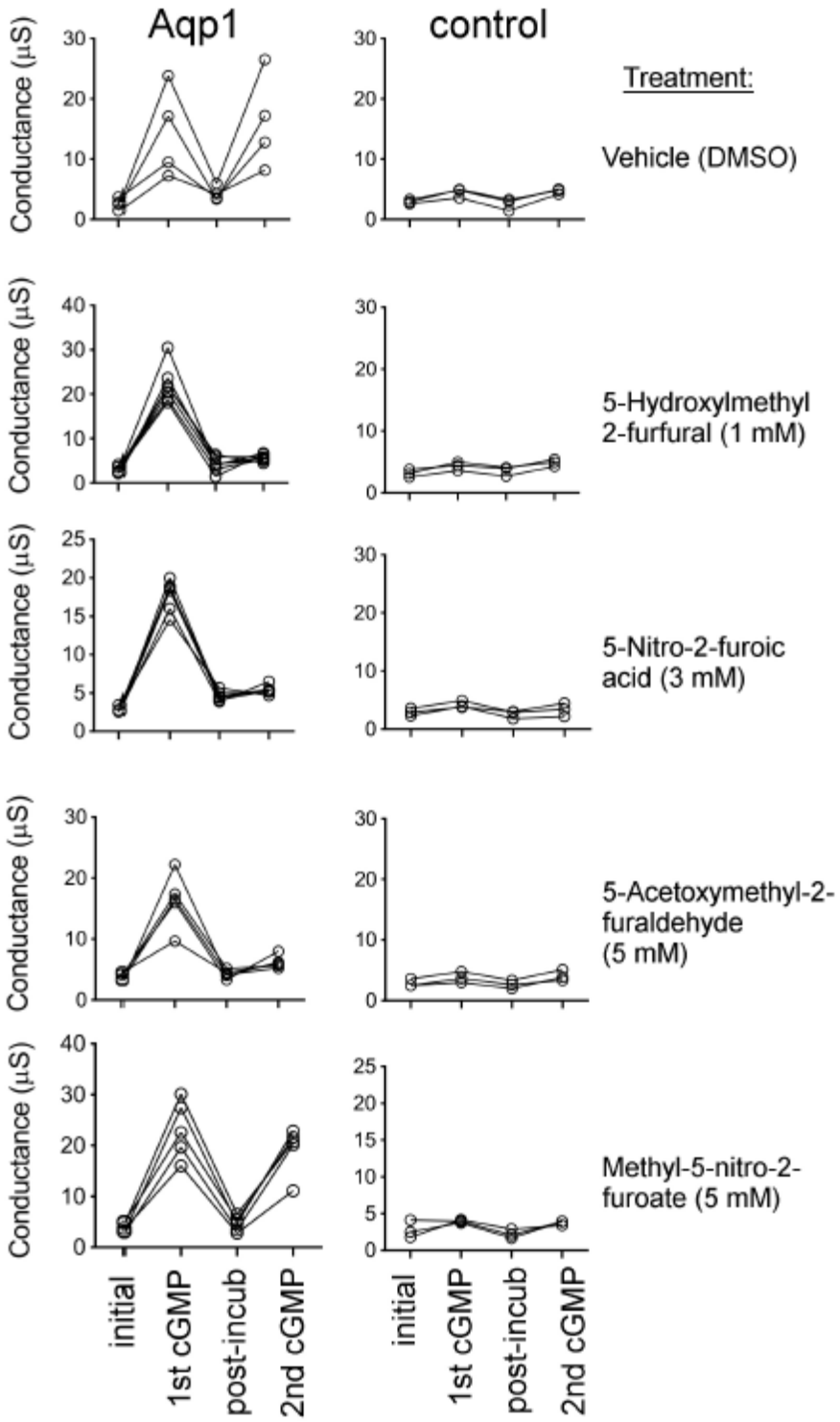

Figure 3. Trend plots showing the effects of 5HMF and related compounds on ion conductances. Conductance values were measured for AQP1- expressing (left) and non–AQP-expressing (right) oocytes. Lines link the values for single oocytes recorded before 8CPT-cGMP (initial), 30 minutes after 8CPT-cGMP ("1st cGMP"), after 2-hour incubation in saline

with vehicle or furan compounds without cGMP (“post-incub”), and after reapplication of 8CPT-cGMP (“2nd cGMP”). Treatments (listed right) were applied during the 2-hour recovery interlude between “1st cGMP” and “post-incub” steps. n values are shown in italics above the x-axis.

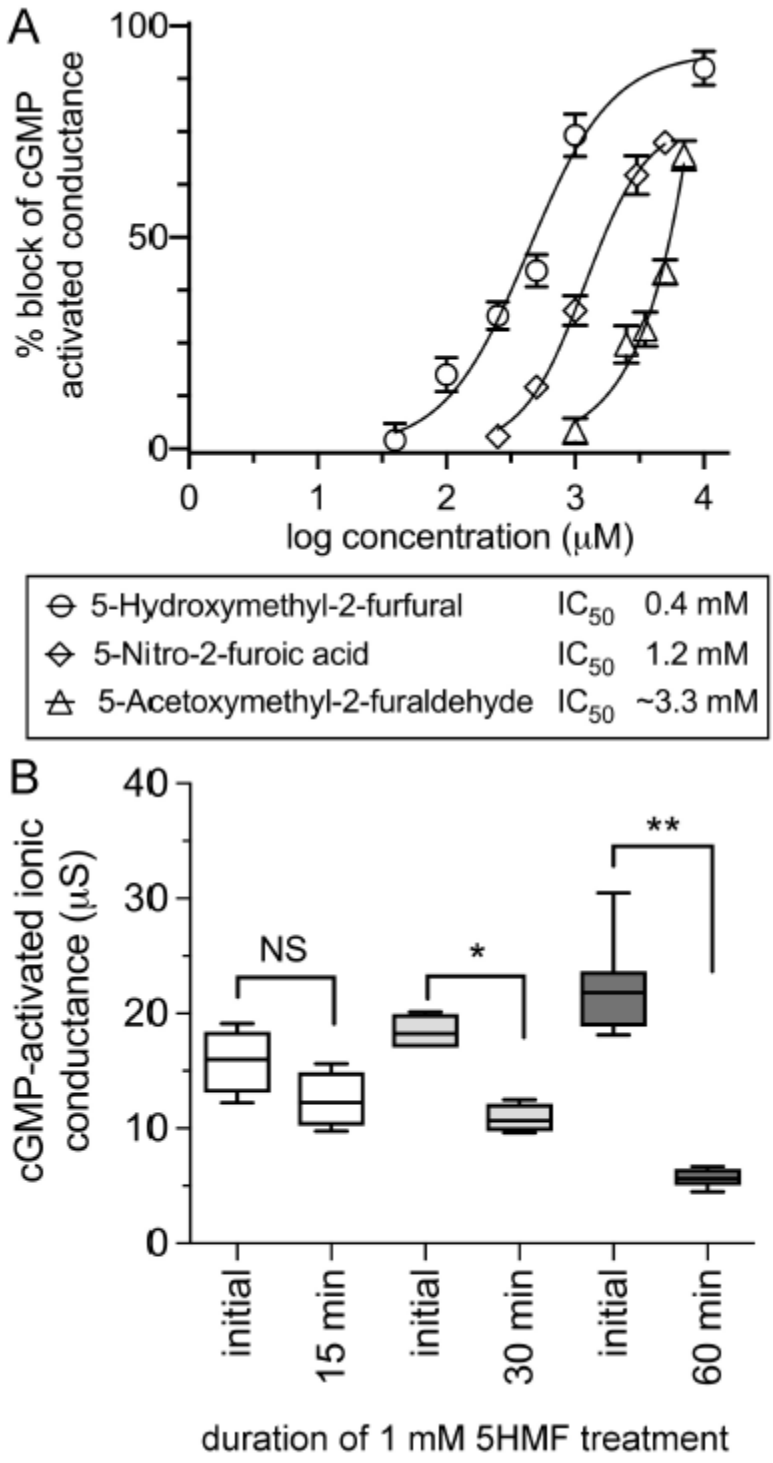

Figure 4. Dose-dependence and rate of onset of block of the cGMP-activated AQP1 ionic conductance. (A) Dose-response curves showing mean percent block (± SEM) as a function of concentration of the furan compounds. Estimated IC_{50} values are listed in the legend. n values were 3 per dose for 5-hydroxymethyl-2-furfural (except 1mM which was n=7), 3 per

dose for 5-nitro-2-furoic acid, and 3 per dose for 5-acetoxymethyl-2-furaldehyde (except 5 mM which was n=4). (B) Time of onset of block of AQP1 ionic conductance during incubation in 1mM 5HMF. The mean level of inhibition of the AQP1 ionic conductance (± SD) was 21 ± 3% at 15 min (n=4), 41 ± 6% at 30 min (n=4), and 75 ± 5% at 60 min (n=7) of incubation time.

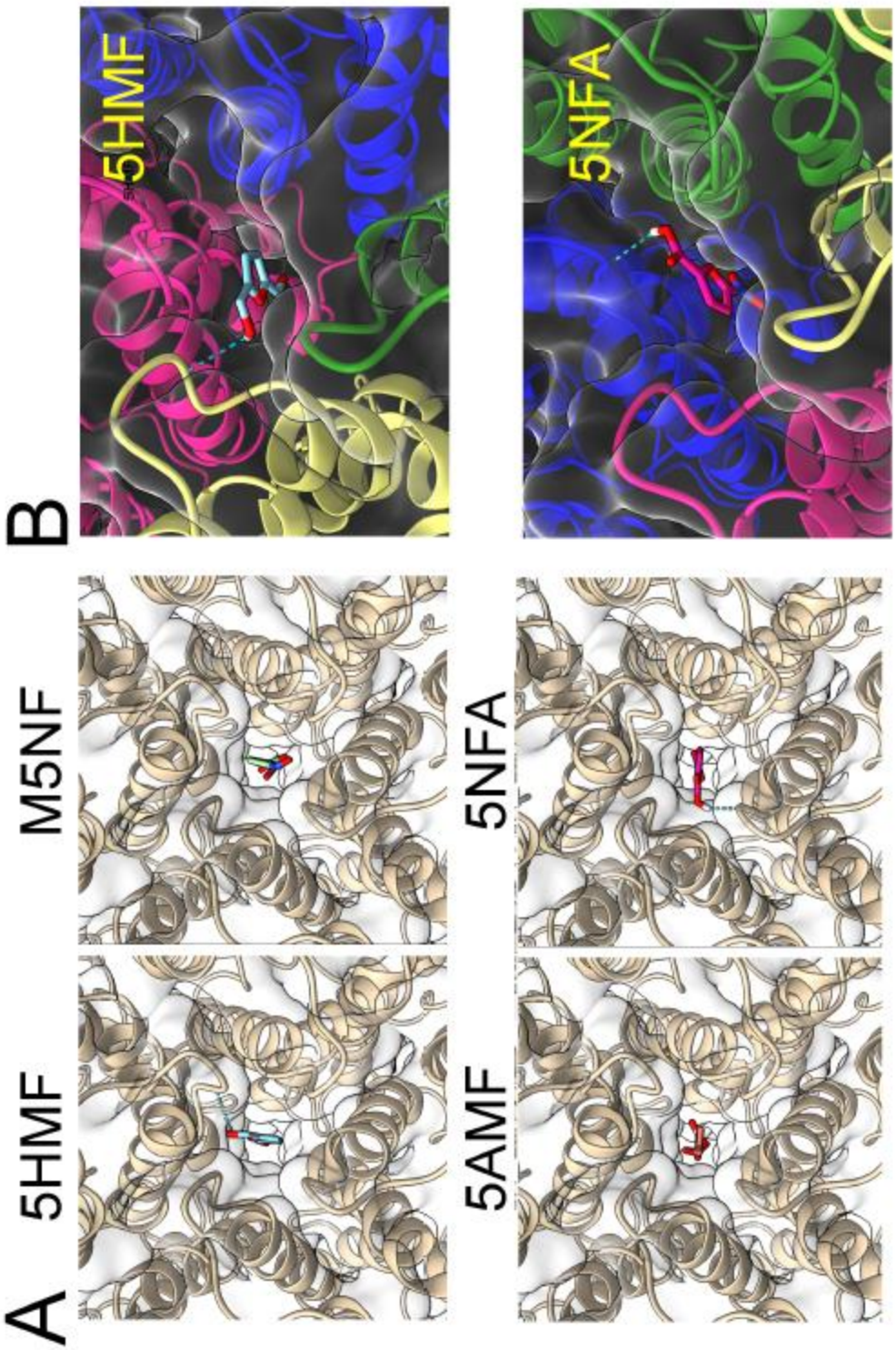

Figure 5. In silico modeling of predicted binding sites for 5HMF and related compounds. (A) Docking modelling results indicated the most favorable energy of interaction for the ligands was in the loop D domains, which surround the tetrameric central pore. Glycine 165 in

human AQP1 is predicted to interact by hydrogen bonding (blue dotted line) with the carboxylic acid moieties of 5HMF and 5NFA, correlating with their higher inhibitory efficacy. 5AMF and M5NF appeared to fit into the central pore domain, but specific amino acid interactions were not identified by the model. (B) Magnified views of hydrogen bonding interactions for 5HMF and 5NFA with Gly165 as predicted by in silico modelling.

2.4.2 Effects of furfural compounds on AQP1 osmotic water fluxes

The furfural-related derivatives did not inhibit AQP1 osmotic water permeability (**Figure 6**). Oocytes expressing AQP1 and non-AQP1 control oocytes were assessed for osmotic water permeability as quantified by swelling rates in 50% hypotonic saline (Fig 6A) as per published methods (Migliati et al., 2009). Swelling rates were measured for AQP1-expressing oocytes before treatment. Oocytes were then transferred into isotonic saline with DMSO vehicle or with furfural derivatives (5 mM) for 1 hour. After incubation, oocytes were rinsed briefly in saline, and tested again. There were no significant differences between the first and second swelling rates in any of the treatment groups (Fig 6B), indicating that none of the furan compounds affected AQP1 water permeability.

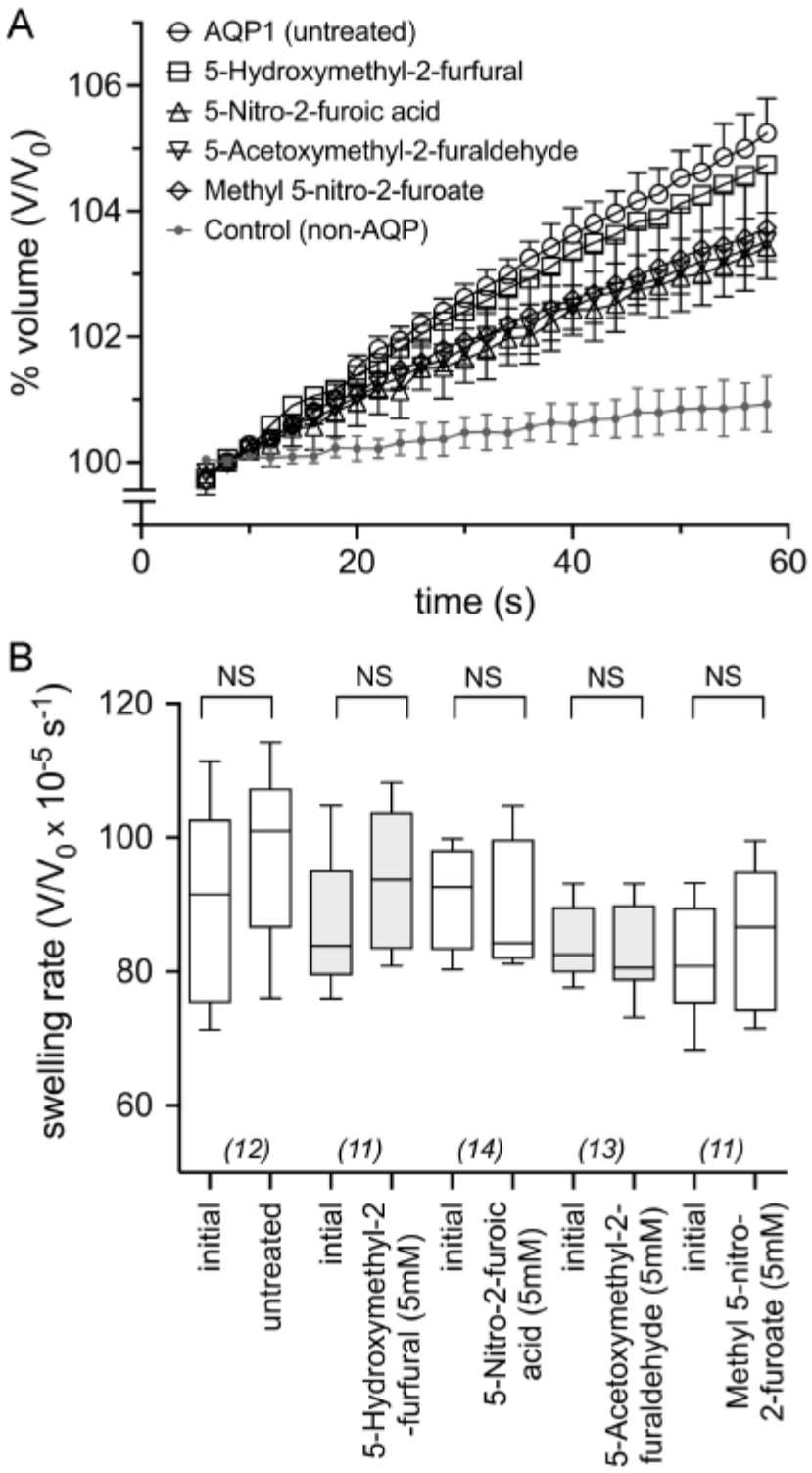

Figure 6. Osmotic water permeability of AQP1-expressing oocytes is not altered by treatment with 5HMF or related compounds. (A) Relative volumes of oocytes, standardized to initial volumes, were measured as a function of time after introduction of oocytes into 50% hypotonic saline at time zero. Data are mean ± SEM. The control n value was 10; other n values were as

shown in panel B. (B) Box plot of compiled data for swelling responses (from panel A) of AQP1-expressing oocytes before (initial) and after 2 h incubation in saline with vehicle or 5 mM furan compounds as indicated. Boxes show 50% of the data; whiskers show the full range; horizontal bars are median values; n values are in italics above the x-axis. None of the treatment groups showed responses that were significantly different than untreated.

2.4.3 Effects of furfural derivatives on cancer cell migration rates

Effects of the furfural derivatives on the migration of breast and colon cancer cell lines were tested using a circular wound closure assay. Results in **Figure 7** show that HT29 cell migration was reduced by furfural derivatives as compared to vehicle control. Cell migration was reduced 85% by 5HMF, 68% by 5NFA, and 37% by 5AMF (each at 1 mM) as, illustrating an order of potency comparable to that seen for block of the AQP1 ion conductance. A similar pattern was observed for MDA cell migration, which was reduced 43% by 5HMF, 37% by 5NFA, and 27% by 5AMF. In contrast, SW480 cells showed no sensitivity to 5HMF, 5NFA or 5AMF, with migration rates not significantly different from that of vehicle control, consistent with the low level of membrane AQP1 protein in this cell line. However, M5NF (which did not affect AQP1 water or ion channel activities), unexpectedly inhibited migration in all three cell lines, reducing HT29 cell migration by 87%, SW480 by 77%, and MDA by 60%. These results suggest M5NF targets a widespread process which is required for cell motility, and not mediated by AQP1.

Cell viability measured with the AlamarBlue assay was not impaired by 5HMF or related compounds (**Figure 8**) at doses found to be effective in cell motility assays, suggesting that the inhibitory effects of the agents on cancer cell motility did not result indirectly from toxicity or reduced cell viability. Results were normalized to untreated controls in each of the cell lines (Fig 8A,B,C). Furfural derivatives did show cytotoxicity at higher concentrations (2 mM in the mammalian cell lines), indicating an upper limit for the potential therapeutic window of doses that might be considered in translational work.

Levels of AQP1 transcript measured using quantitative PCR were confirmed to be high in MDA and HT29 cell lines, and low in SW480 cells (Fig 8D), consistent with results reported previously (Nakhjavani et al., 2019; Pei et al., 2019; Pei et al., 2016b). The low level of AQP1

protein that is expressed in SW480 cells has been shown to be mainly intracellular, explaining the insenstivity of these cells to effects of AQP1 channel blockers (De Ieso et al., 2019).

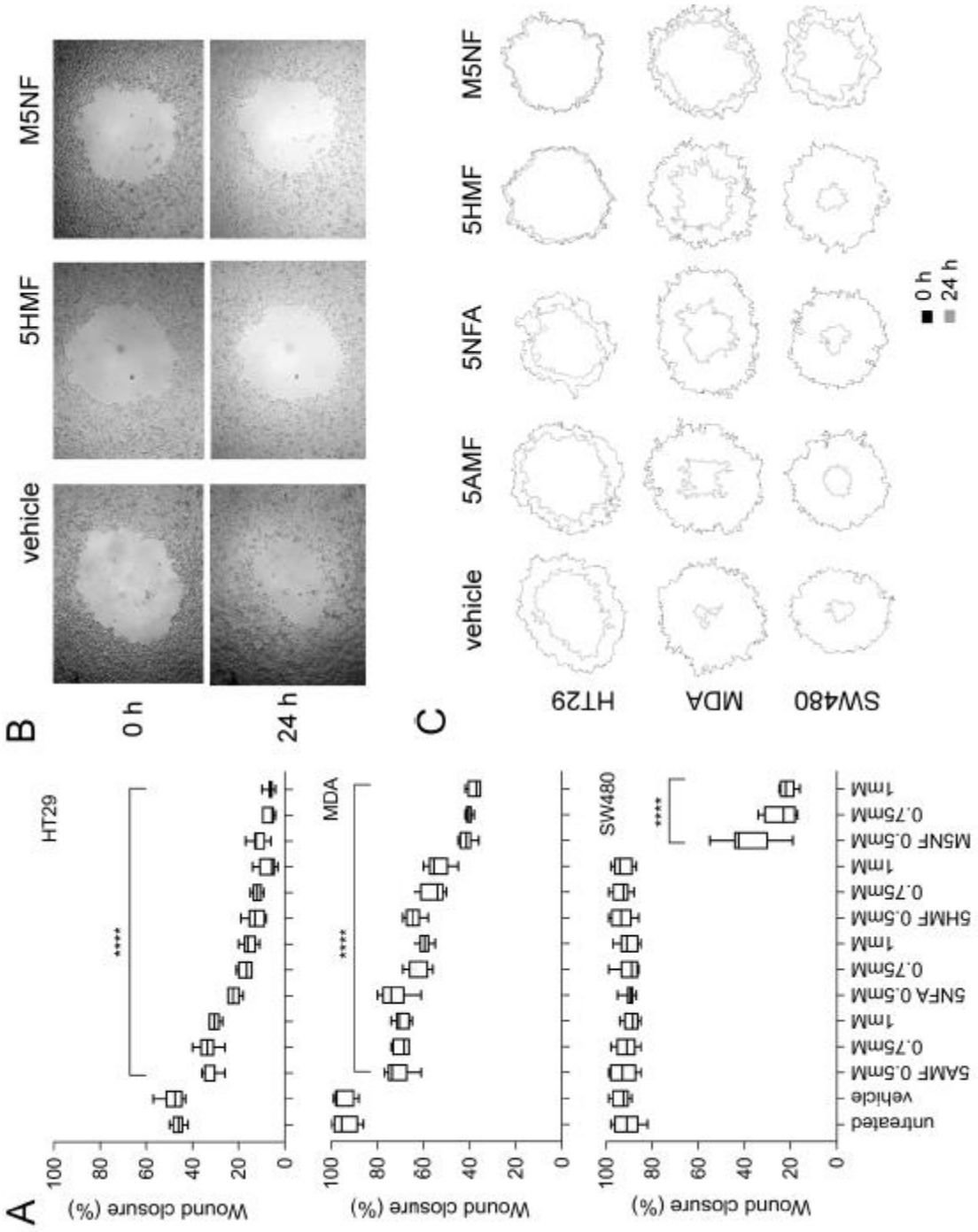

Figure 7. Circular wound closure in HT29, MDA, and SW480 cells is differentially impaired by 5HMF and related compounds. (A) Box plots depicting results for percent wound closure of HT29, MDA, and SW480 cells following treatment with or without vehicle or furan derivatives. HT29 and MDA showed significant block by all compounds; SW480 was sensitive

only to M5NF. n=8 for all groups except HT29 5NFA (0.75mM) and HT29 M5NF (0.75 mM) which were n=7 each. Statistically significant differences for each column were assessed by comparison to vehicle control. See Methods for details. (B) Representative images showing circular wounds at 0 hours (upper row) and 24 hours (bottom row) in cultured HT29 cells treated with vehicle, 5HMF, or M5NF. (C) Superimposed outlines of circular wound perimeters at 0 (black) and 24 (grey) hours for representative examples from each treatment group were generated by ImageJ software.

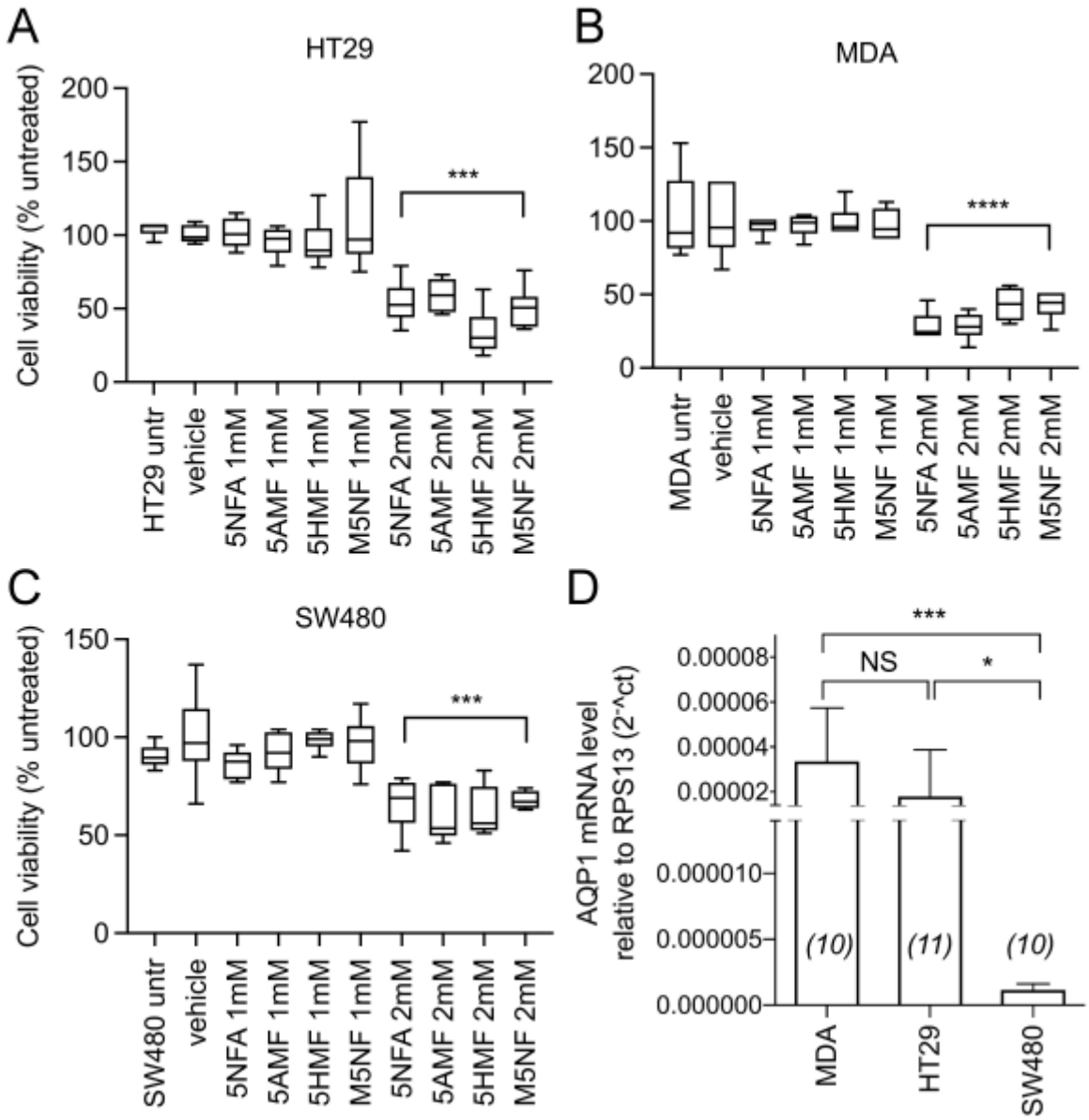

Figure 8. Analysis of dose-dependent cytotoxic effects of treatments, and confirmation of AQP1 mRNA expression levels in the cancer cell lines. Cell viability shown in box plot summaries was measured by alamarBlue assay for (A) HT29, (B) MDA, and (C) SW480 cells. Data were standardized to results for untreated cells. Statistically significant differences for each column were assessed by comparison to vehicle controls; see Methods for details. n=6 in all treament groups. (D) AQP1 transcript levels, standardized to transcript levels of ribosomal

protein S13 (RPS13), were higher in HT29 and MDA than in SW480, as determined by quantitative reverse transcription PCR. Histogram bars show mean ± SD; n values are indicated in italics.

2.4.4 Effects of furfural derivatives on cancer cell invasiveness

The effect of furfural derivatives on cancer cell invasiveness was tested using a transwell invasion assay, in which cells migrated through an extracellular matrix-like material layered on a semi-permeable membrane towards a chemoattractant (fetal bovine serum), then were stained with crystal violet and counted on the trans side of the filter (**Figure 9**). HT29 cell invasiveness was impaired 30% by 5HMF and 25% by 5NFA as compared to vehicle control; 5AMF was not effective. In MDA cells, invasiveness was blocked 49% by 5HMF; NFA and

5AMF were not effective. SW480 cells showed no significant block of invasiveness by 5HMF, 5NFA or 5AMF. However, M5NF intriguingly caused almost complete block of invasiveness in all three cell lines, suggesting it targets a general mechanism of cell motility.

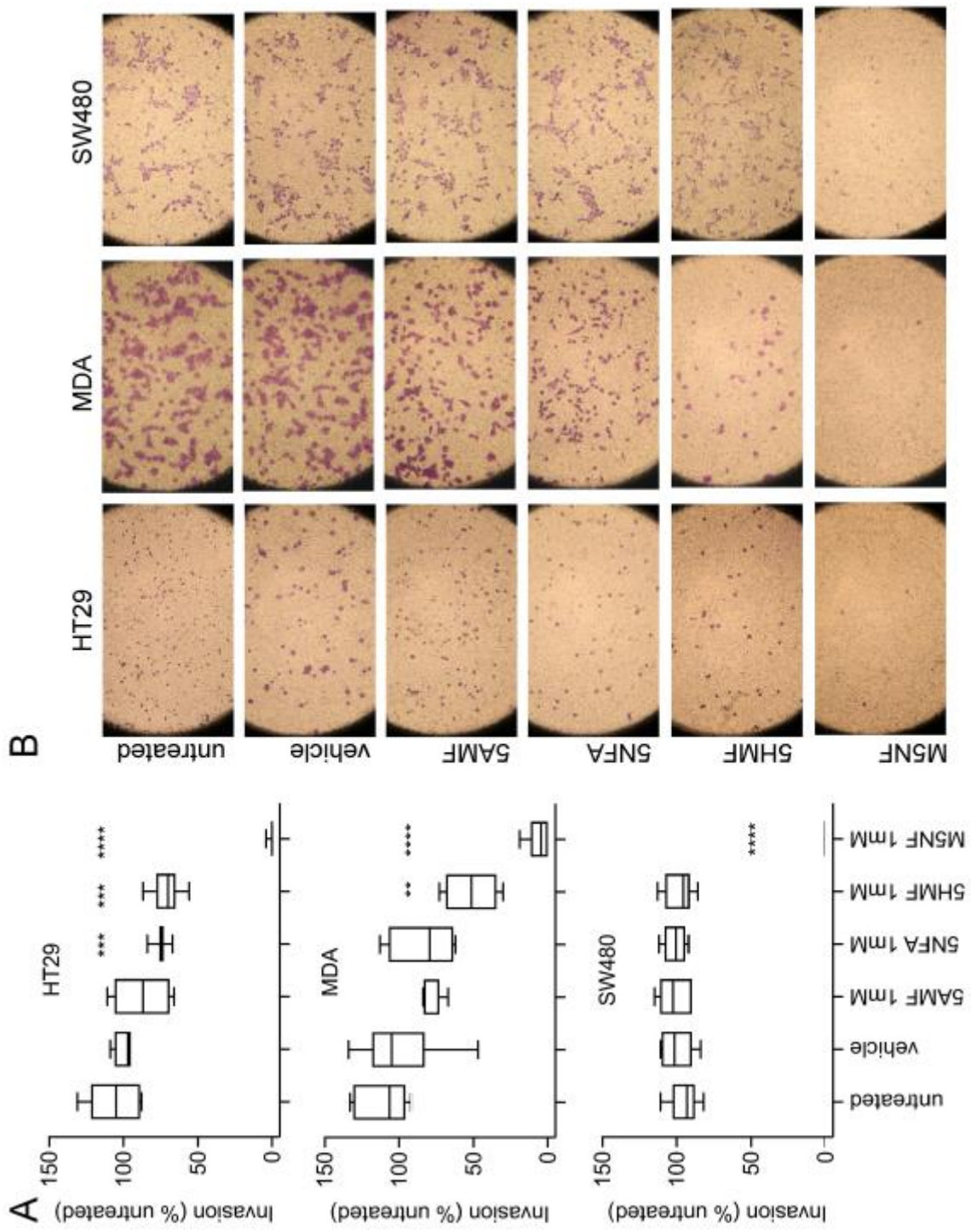

Figure 9. Effects of 5HMF and related compounds on transwell cell invasiveness of HT29, MDA, and SW480 cells. (A) Box plots depicting HT29, MDA, and SW480 cell migration across transwell filters with extracellular matrix, with or without treatment with vehicle or furan derivatives at 1mM as indicated. Statistically significant differences were assessed as

compared to vehicle controls; see Methods for details. (B) Images illustrating differences in the abundance of successfully migrated cells (stained purple) on the trans sides of filters for the three cell lines untreated, and in the different treatment conditions (at 1 mM, or equivalent DMSO for vehicle).

2.4.5 Effect of furfural derivatives on actin polymerization.

Pathways involved in cancer cell motility and metastasis are frequently found to include kinase and GTPase signaling cascades that converge on the regulation of actin cytoskeletal organization (Foxall et al., 2016). To examine the effects of M5NF on a common endpoint of F-actin polymerization (Hinz and Jucker, 2019), HT29, MDA and SW480 cells were treated with furfural derivatives and then labelled with fluorescent phallodin, which binds F-actin polymers with high affinity and reveals actin parallel fiber and network structures (Wulf et al., 1979).

Confocal imaging results showed the fluorescent signal intensities were reduced in all three cell lines after treatment with M5NF, which also disrupted the transverse parallel fiber tracts of actin, leaving trace residual stainining against cell boundaries (**Figure 10)**. In contrast, treatments of HT29, MDA and SW480 cells with the other furfural derivatives had no discernable effects on actin signal intensity or structural organization as compared to vehicle controls. Methyl 5-nitro-2-furoate effects on cell motility are distinct from those of the other furfural derivatives, and appear to involve pathways controlling actin assembly.

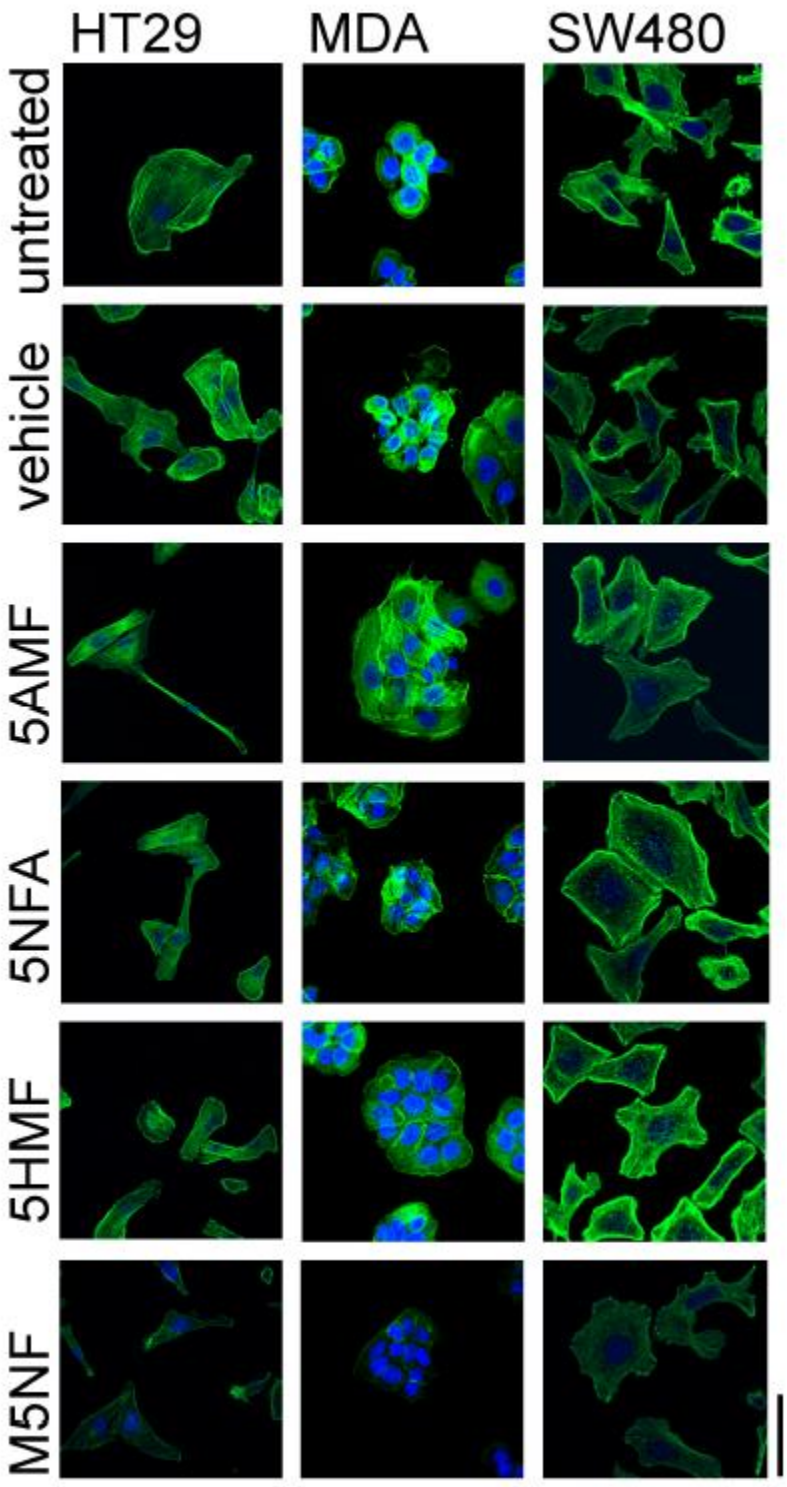

Figure 10. Abundance and patterns of distribution of polymerized F-actin seen in HT29, MDA, and SW480 cells after treatment with or without vehicle or furan derivatives. Factin was labeled with fluorescently-tagged phalloidin (green). Nuclei were visualized by

Hoechst stain (blue). Fluorescence signal intensities and the apparent organization of filaments into parallel strands were visibly reduced in cells treated with M5NF, but not those treated with other furan compounds or vehicle. Scale bar 25 μm (lower right).

2.5 Discussion

AQP1 is upregulated in classes of breast, glioblastoma, colorectal and other cancers and has been correlated with poor prognoses (El Hindy et al., 2013; Luo et al., 2018; Shimasaki et al., 2018; Wang et al., 2017; Yoshida et al., 2013). The dual water and ion channel function of AQP1 has been shown to facilitate cancer cell migration and invasiveness in vitro (De Ieso et al., 2019; Kourghi et al., 2016; Pei et al., 2016b; Tomita et al., 2019), suggesting this channel is a target of interest for new approaches to control cancer metastasis (De Ieso and Yool, 2018). Fifteen classes of aquaporin (AQP0-14) have been identified in mammals (Denker et al., 1988; Finn et al., 2014; Ishibashi et al., 2009; Preston and Agre, 1991); furans remain to be tested on other classes of AQPs.

AQP1 expression has been linked to migration in certain aggressive cancer subtypes, and proposed as a target for novel treatments to control cancer metastasis (De Ieso and Yool, 2018; Dorward et al., 2016; Hu and Verkman, 2006; Jiang et al., 2009; McCoy and Sontheimer, 2007; Pei et al., 2016a). In these subytpes, AQP1 has been found to be localized at the leading edges of migrating cells (McCoy and Sontheimer, 2007; Pei et al., 2019), where it is proposed to facilitate rapid cell volume changes by mediating water and ion fluxes (Yool and Campbell, 2012). Previous studies have been shown that pharmacological targeting of the AQP1 water flux, ion channel activity, and both have inhibitory effects in cancer cell migration and invasion in vitro (De Ieso et al., 2019; Kourghi et al., 2016; Pei et al., 2016b). This study hypothesized that one of the compounds found in natural medicinal honey treatments, 5HMF, might impair cancer cell migration and invasion by exerting an inhibitory effect on AQP1 water or ion channel activities.

The results showed that 5HMF significantly blocked the cationic conductance of AQP1 with an IC_{50} value of approximately 400 µM. The order of potency of block of the AQP1 ion current was 5HMF > 5-NFA > 5-AMF; no AQP1 inhibition was observed with M5NF. All

the AQP1-active compounds had a furfural core, consisting of a furan ring and carboxylic acid. Excluding M5NF, the order of potency was inversely related to the size of the moiety at position 5 on the furan ring; the most potent agent 5HMF has the smallest group (hydroxymethyl), and the least effective agent 5AMF has the largest group (acetoxy-methyl). Electrophysiology results were consistent with molecular docking studies, indicating the most potent agent had the most favorable binding energy. The AQP loop D domain has been suggested to be a gating region regulating channel activity (Kourghi et al., 2018; TornrothHorsefield et al., 2006; Yu et al., 2006). In AQP1, pharmacological interaction or mutagenesis in this region impairs activation of the ion conductance by cGMP. Structural differences between ligands have been shown previously to correlate with differences in predicted interactions between the ligands and loop D, and with corresponding potency of inhibition of the AQP1 ion channel (Kourghi et al., 2016; Pei et al., 2016b). Results here are consistent with these previous findings. The predicted interaction at loop D had no effect on osmotic swelling through parallel intrasubunit pores, but inhibited the ion conductance thought to occur via the central pore (Campbell et al., 2012; Yu et al., 2006).

Pharmacological targeting of the AQP1 ion channel with the furfural derivatives resulted in slowing of migration and invasion in AQP1-expressing cancer cell lines, as tested with two- and three-dimensional migration assays. The high AQP1-expressing cell lines HT29 and MDA were sensitive, whereas the low AQP1-expressing cell line SW480 was not sensitive to furfural-based AQP1 ion channel blockers. The order of potency of inhibition of motility by the different furfural compounds in AQP1-expressing cancer cells (but not SW480) followed the order of potency determined by electrophysiological analyses, consistent with the idea that observed impairments of cell migration and invasion were mediated in large part by direct block of AQP1 ion channel. The insensitivity of SW480 cell motility to the furfural agents showed 5HMF, 5NFA and 5AMF did not act via indirect toxic effects or disruption of

cytoskeletal actin organization. The selectivity of the AQP1 active compounds only for AQP1expressing cell lines argues for a specific AQP1-dependent mechanism of action. In contrast, M5FA impaired migration through a more widespread mechanism that appears to involve actin disorganization, which might limit usefulness as a therapeutic agent. The furan compounds at 5 mM did not affect osmotic water fluxes, demonstrating that the pharmacological treatments did not alter the levels of AQP1 protein in the plasma membrane, nor did they disrupt AQP1 subunit tetrameric organization, which is prerequisite for water channel activity (Jung et al., 1994).

In summary, results here are the first to show that 5HMF and the related furfural compounds 5NFA and 5AMF can serve as AQP1 ion channel blockers. The pharmacological effects are dose-dependent and provide a first step towards understanding structure-activity relationships for this class of compounds. Cancer cell line wound closure and invasion assays are the first to demonstrate inhibitory effects of furfural-based derivatives on cell motility, and provide another line of evidence for the importance of AQP1 ion channel function in aggressive cancers that upregulate this target protein. While the concentrations needed for biological activity are comparatively high, these concentrations have been shown to be tolerated in vivo, suggesting this line of agents might have promise for the development of new AQP1-based therapeutics using 5 hydroxymethyl furfural as a lead compound.

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Chapter 3 Dose-dependent inhibition of colon cancer cell migration by a natural medical plant extract, KeenMind, targeting human AQP1 water channel activity

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3.1 Abstract

Aqpauporin-1 (AQP1) is a water channel that mediates flux of water and small solutes across the cell membrane. Upregulation of AQP1 in colon cancer cells facilitates volume change of cancer cell that are thought to promote cell migration; hence, inhibiting AQP1 channel function could be a potential therapy for slowing cancer metastasis. Evidence suggests there is pharmacological value for traditional medicinal plants *Bacopa monnieri* in different diseases; however, their effects on cancer remain uncertain. One of the active ingredient in *Bacopa monnieri*, bacopad side II, has reported to have an inhibitory effect in colorectal cancer by targeting AQP1 water channel activity. KeenMind is a commercial supplement extracted from medicinal plant b*acopa monnieri,* therefore it also contains bacopdaside II. Thus we hypothesized that KeenMind extracts inhibits cell migration on HT29 colorectal cancer cells by blocking the AQP1 water channel. Using the circular migration assay develop in our lab, results here showed that KeenMind extracts significantly impaired HT 29 colon cancer cell migration starting at 60μg/ml. Effect of KeenMind extracts were then determined for water activity in AQP1 expressed oocytes. Results from oocyte swelling assay showed that KeenMind extracts significantly inhibited AQP1 water permeability in a dose dependent manner, with a half-maximal block at 290 µg/ml. Data here suggests that KeenMind is a

potential AQP1 water channel blocker and has an inhibitory effect in cell migration. With further investigation, KeenMind extracts could be used for developing a potential therapy for colon cancer.

3.2 Introduction

Aquaporins are a family of integral membrane proteins that serve mainly as water channels (Nielsen et al., 1993). Upregulation of aquaporin 1 (AQP1) in colorectal cancer has been reported in a number of studies, in which the expression level was correlated to the tumor grade (Yoshida et al., 2013a, Kang et al., 2015a). Genetic knockdown of AQP1 in cancer cell lines with small-interfering RNAs led to a significant reduction in cell migration rate in vitro, whereas increasing AQP1 levels by transfection into low-AQP1 expressing lines facilitated cell migration and increased the chance of metastases in vivo (Saadoun et al., 2005, Hu et al., 2006), suggesting AQP1 plays an important role in cancer migration. Pharmacological inhibition of AQP1 ion or water channel significantly reduced cancer migration and invasiveness (De Ieso et al., 2019, Pei et al., 2016, Kourghi et al., 2016); therefore AQP1 inhibitors could be a potential therapy for cancer.

Traditional medicines have been used for over a thousand years in China and India, and often uses as complementary medicine (He et al., 2019, Khan, 2014). The medicinal plant *Bacopa monnieri*, also known as Brahmi, has been used in Ayurveda (traditional indian medicine) for improving memory, reducing stress, counteracting inflammation, relieving pain, and strengthening the immune system (Gohil and Patel, 2010). Intensive studies have been conducted to investigate the cognitive enhancing effects of the plant extracts and its isolated primary active ingredients, bacosides (Aguiar and Borowski, 2013, Russo and Borrelli, 2005, Chaudhari et al., 2017). Possible mechanisms of action include neuroprotection through antioxidant effect (Simpson et al., 2015, Uddin et al., 2016), promotion of cerebral blood flow (Kamkaew et al., 2013), neurotransmitter modulation (Rauf et al., 2012, Swathi et al., 2013), and β-amyloid reduction (Chaudhari et al., 2017). Plant extracts of *Bacopa monnieri* have been reported to have anti-tumorigenic and anti-proliferative activity by interrupting cell cycle at the M phase in multiple cell lines including colon, lung, and breast (Mallick et al.,

2015). However, there are only limited studies that have investigated the effect of the plant extract in cancer metastasis. Previous work from our lab and others had demonstrated that bacopaside II can selectively block the AQP1 water channel and inhibit cell migration of the high AQP1-expressing colon cancer cell line HT29, at dose that were not cytotoxic; however, bacopaside II had minimal effect on the migration of the low AQP1-expressing SW480 colon cancer cell line (Pei et al., 2016b, De Ieso et al., 2019a, Palethorpe et al., 2018). Keen Mind is a commercial dietary supplement produces from *Bacopa monnieri*, and clinical trial has showed the beneficial effect of KeenMind in cognitive function and memory (Benson et al., 2014, Downey et al., 2013, Stough et al., 2008). However, the effect of KeenMind in cancer remains unknown. Work here tested the hypothesis that this traditional medicinal plant extract would have an inhibitory effect on cancer cell migration through targeting AQP1 water channel. Data here show that KeenMind extract significantly inhibited HT29 colon cancer cell migration and the AQP1 water channel function in quantitative swelling assays, suggesting its potential pharmacological value in cancer.

3.3 Materials and Methods

3.3.1 Chemical compounds

KeenMind (lot K0078702, exp 02-2021) was purchased at local pharmacy (TerryWhite Chemist, Adelaide Australia). Powder extracted from KeenMind tablets was used for preparing a stock solution. Total 3.03g powder was dissolved into 25 ml of 70% methanol (17.5 ml HPLC-grade methanol with 7.5 ml reverse-osmosis MilliQ-filtered water), then sonicated for 30 minutes, and centrifuged at 12 k at 18^{o}C for 15 min. The stock methanol extract of KeenMind (120mg/ml) was stored in sterile microfuge tubes at 4^{o}C. The prepared stock solution was serially diluted to 110 to 450μg/mL in isotonic Na^{+} saline for oocyte swelling assays. BacopasideII was purchased from Sigma-Aldrich (NSW Australia), and

dissolved in 70% methanol to yield a 100x stock solution (5mM). The experimental solution was diluted with isotonic saline to create across a concentration range of 10 to 200μM. Vehicle control saline was made using the same volume of methanol alone in isotonic saline.

3.3.2 Cancer cell culture and migration assay

Human colorectal adenocarcinoma cell lines HT29 (ATCC® HTB-38TM) was grown in T-75 flasks in complete medium composed of 15ml Dulbecco's Modified Eagle Medium with 1× glutaMAX (Life Technologies, Mulgrave, Australia), penicillin and streptomycin (100 U/ml each), and 10% fetal bovine serum. Cultures were kept in 5% CO2 at 37^{o}C. The colorectal cancer cell lines HT29 tested here is AQP1 positive, as previously shown (De Ieso et al., 2019, Chow et al.,2019 under review). To prepare cell migration assay, colon cancer cells were seeded at 1.25 × 106 cells/ml in a 96 well plate to produce a confluent monolayer. When cells reached 80% to 90% confluence, cells were serum-starved in 2% fetal bovine serum (FBS), in the presence of 400 nM of the mitotic inhibitor 5-fluoro-2′-deoxyuridine (FUDR) (Parsels et al., 2004). Circular wounds were created with a sterile p10 pipette tip; drug-treated media (with 2% FBS and FUDR) was applied following wounding (De Ieso and Pei, 2018). Created wounds were then imaged at 10x magnification with a Canon 6D camera on a Nikon inverted microscope. Xn converter (XnSoft, Reims, France) was used to standardise the dimensions of each image. Standardised wound images were quantified by generating an outline to caculate wound area using imageJ. Migration rate was calculated by wound closure as a percentage of the initial wound area as a function of time.

3.3.3 Cell viability assay

HT29 viability was quantified using an alamarBlue assay, following the manufacturer's protocol (Life Technologies). HT29 cells were cultured in media with FUDR and 2%serum at

10^5 cells/ml in a 96-well plate. Treatments were applied 12- 18 hours after seeding. Cells were incubated for 24 hours. 10% alamar blue was added to cancer cells for 1-2 hours, and the fluorescence signal levels were measured with a FLUOstar Optima microplate reader (BMG Labtech, Ortenberg, Germany) to obtain quantitative measures of cell viability

3.3.4 Oocyte preparation and quantitative swelling assay

Female Xenopus laevis frogs were anesthetized and ovarian lobes was surgically removed using protocols approved by the University of Adelaide Animal Ethics Committee (M2018-016) in accord with Australian National Guidelines for animal use. Oocytes were defolliculated with collagenase type 1A (2 mg/ml) in OR-2 saline (82mM NaCl, 2.5mM KCl, 1mM $MgCl_2$, and 5mM HEPES; pH 7.6) for 1.5 hours. Collagenase were removed by washing oocytes with in OR-2 saline, and oocytes were then stored in Frog Ringers saline (96 mM NaCl, 2 mM KCl, 0.6 mM $CaCl_2$, 5 mM $MgCl_2$, and 5 mM HEPES supplemented with 10% horse serum, 100 U/ml penicillin, 100 mg/ml streptomycin, and 50 mg/ml tetracycline, pH 7.6) at 16 to 18°C. For the double swelling assays, healthly looking oocytes were selected for injection. 1 ng of AQP1 cRNA or sterile water was injected into each oocyte, and cells were incubated for 2 to 3 days at 16–18°C to allow AQP1 expression. To perform the swelling assay, each oocyte was first tested for swelling in 50% hypotonic saline to achieve a baseline swelling rate, as described previously (Migliati et al., 2009a). Oocytes were then incubated for 2 hrs in isotonic saline with or without drug treatment, and reassessed in a second swelling assay. During the swelling assay, oocytes were imaged by a grayscale camera (Cohu, San Diego, CA) fixed on a dissecting microscope (Olympus SZ-PT; Olympus, Macquarie Park, Australia) at 1 frames per second for 30s using NIH ImageJ software. The swelling rates were calculated from slope values of linear regression fits of the cross-sectional area as a function of time using GraphPad Prism (Calif, USA). Human AQP1

cDNA (NCBI GenBank: BC022486.1)(Preston et al., 1992) sub cloned Xenopus ß-globin expression plasmid was linearized with BamHI and transcribed with T3 polymerase (T3 mMessage mMachine; Ambion, Austin, TX).

3.3.5 Data analysis and statistics

Statistical analyses performed with GraphPad Prism software involved one-way ANOVA. Statistically significant outcomes are represented as $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***), or $p<0.0001$ (****); NS is not significant. All data are presented as mean ± standard error of the mean (SEM); n values for independent samples are indicated in italics above the x-axes in histogram figures.

3.4 Results

3.4.1 Effects of KeenMind extracts on cancer migration rates

To investigate whether KeenMind extracts have any effect of cancer metastasis, we tested KeenMind extracts in colorectal cancer cell line HT29 . KeenMind extracts treated colon cancer cells were compared to the untreated cells and vehicle treated cells after 24 hours incubation (Fig.1). Two independent migration assays was performed (Fig 1A&B). Results from two separate experiments showed that cell migration was significantly reduced in KeenMind extracts treated cells (Figure 1 A&B), for 60 to 150 µg/ml. To calculate the percentage bock , data were normalized to the vehicle control; KeenMind caused 55% inhibition at 60µg/ml, 62% inhibition at 110 µg/ml, and 75% at 150 µg/ml (Figure 1C). These results demonstrated the KeenMind blocked wound closure in HT29 cell lines and suggested that it could have an inhibitory effect on AQP1 channel function.

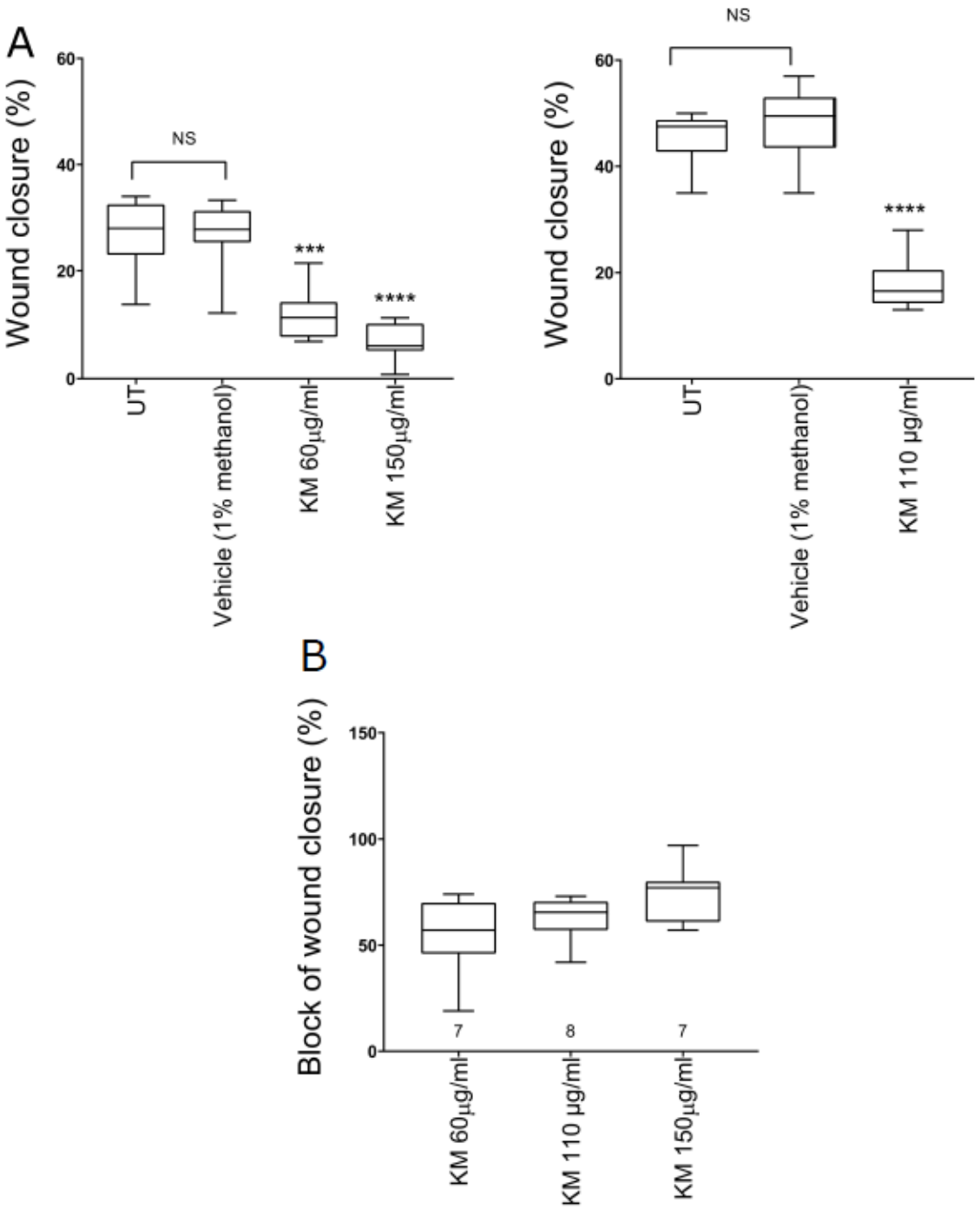

Figure 1. Effect of KeenMind extracts on wound closure in HT29 cancer cell line.

(A) Results from two independent experiments. Box plots depicting percentage of wound closure of cancer cells following treatments with vehicle and KeenMind extracts. Each group had 8 replicates. (B) Block of wound closure of KeenMind was calculated by normalizing data to vehicle control.

3.4.2 *Effects of KeenMind extracts on cancer cell viability*

To ensure KeenMind didnt induce any toxicity, HT29 cell viability was measured with the AlamarBlue assay. Data were standardized to the vehicle (Figure 2). Results showed that the Keenmind extracts did not impair cell viability at doses tested in circular wound assay, suggesting that the inhibitory effects of the agents on cancer cell motility did not result indirectly from toxicity or reduced cell viability.

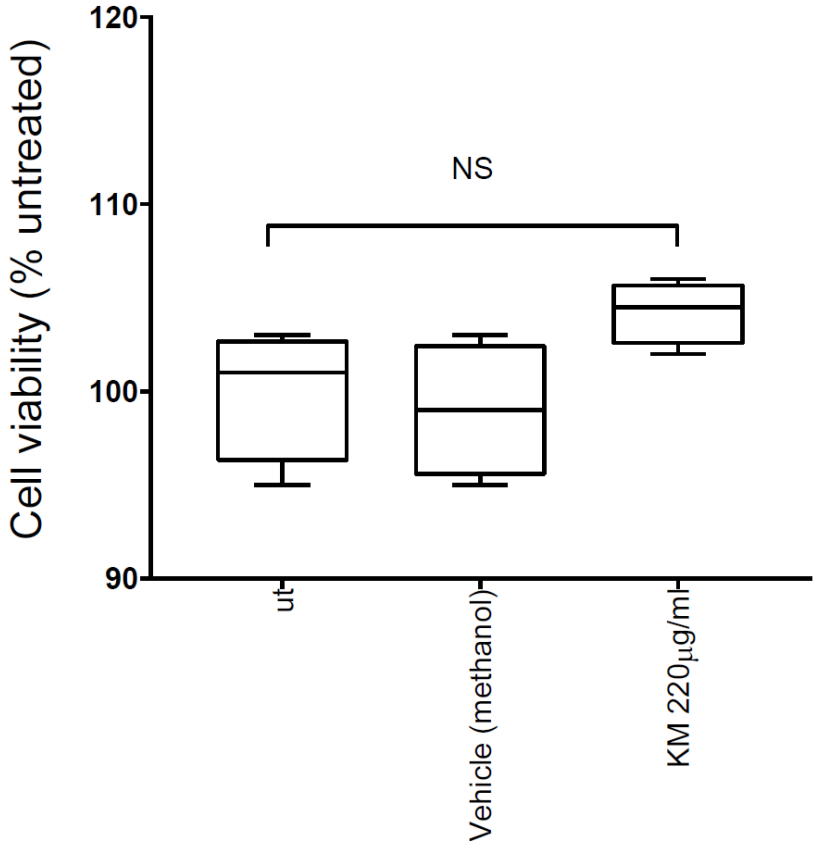

Figure 2. Analysis of cytotoxic effects of treatments in HT29 cells. HT29 Cells viability shown in box plot summaries was measured by alamarblue assay (n=4). Data were standardized to results for untreated cells. Statistically significant differences for each column were assessed by comparison to vehicle controls.

3.4.3 Block of osmotic water permeability in AQP1 expressing oocytes by KeenMind extracts

To further investigate the mechanism of KeenMind extracts, oocyte swelling assays were performed. Prior to the swelling assay, the control and AQP1-expressing oocytes were preincubated in isotonic saline (serum and antibiotic-free) at 16-18^{o}C for 1 hour. The osmotic water permeabilities of oocytes expressing AQP1 and non-AQP1 control oocytes were assessed by measuring the swelling rates in 50% hypotonic saline. After two hours incubation in the vehicle (Methanol 1%) or KeenMind extracts, the volume of oocytes were standardized as a percentage of the initial volume at the beginning (Figure 3). The volume of AQP1 expressing oocytes treated with KeenMind extracts at 110µg/ml, 220µg/ml, and 450µg/ml was significantly reduced when compared to the vehicle control, suggesting KeenMind extracts inhibited AQP1 water channel.

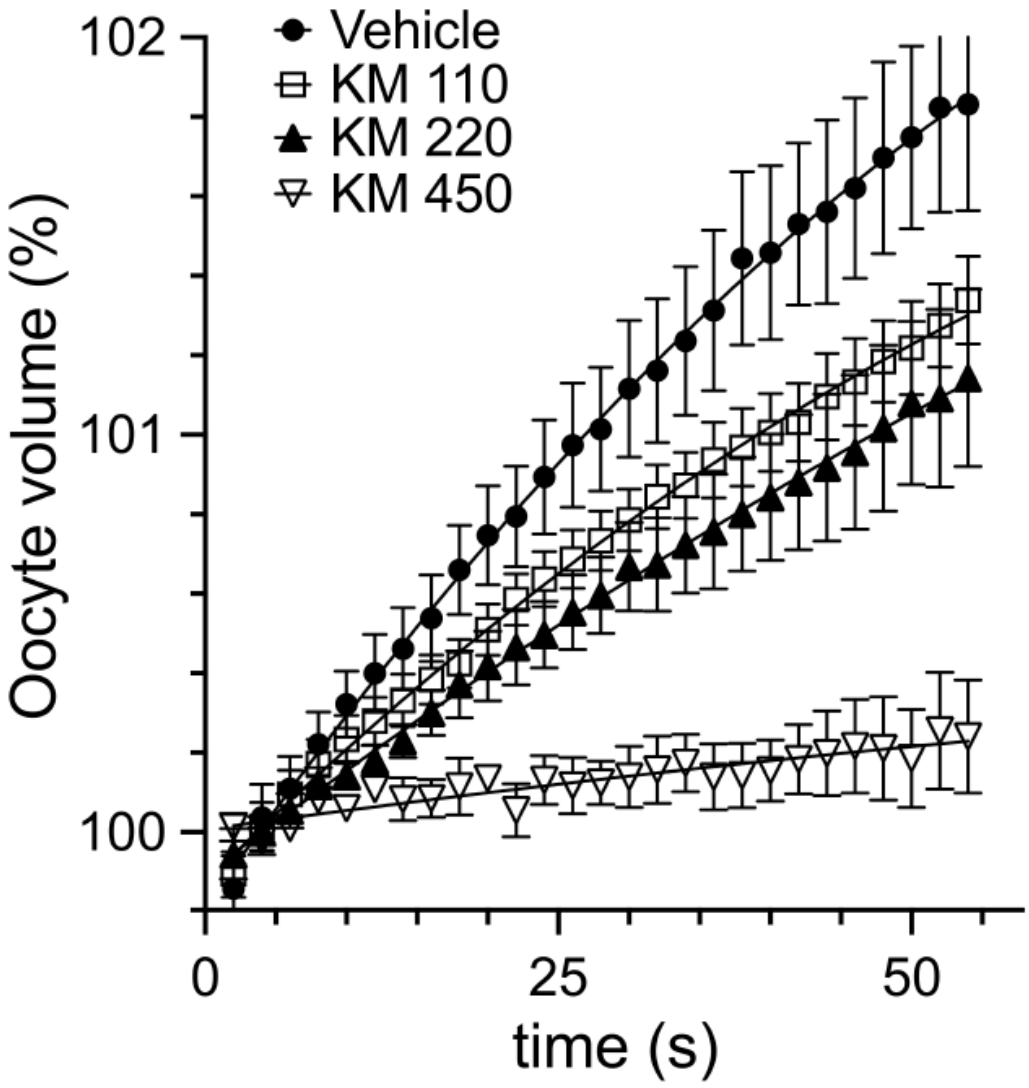

Figure 3. KeenMind extracts reduced the water permeability of AQP1 expressing oocyte. Volumes of AQP1-expressing oocytes was standardized as a percentage of the initial volume after introduction into 50% hypotonic saline at time zero,. Oocytes were treated with vehicle or KeenMind extracts at 110µg/ml, 220µg/ml, and 450µg/ml (n=4).

3.4.4 KeenMind extracts reduced water permeability of AQP1 expressing oocytes in double swelling assay

Trend plots illustrated the swelling rates for each AQP1-expressing oocyte during first and second swelling assays (Figure 4). The swelling rate of AQP1 expressing oocytes was first tested before incubation in the vehicle control or KeenMind extracts. After 2 hours of incubation with the vehicle control or KeenMind extracts, the swelling rate was tested for the second time. Results showed that there was no significant difference between first and second respond in vehicle control, but a significant reduction in second response after incubation with KeenMind extracts. Inhibitory effects were seen at 110μg/ml, and almost completely blocked the AQP1 water channel activity at 450μg/ml. In summary, KeenMind extracts significantly inhibited the water permeability of AQP1, and the inhibitory effect was dose-dependent.

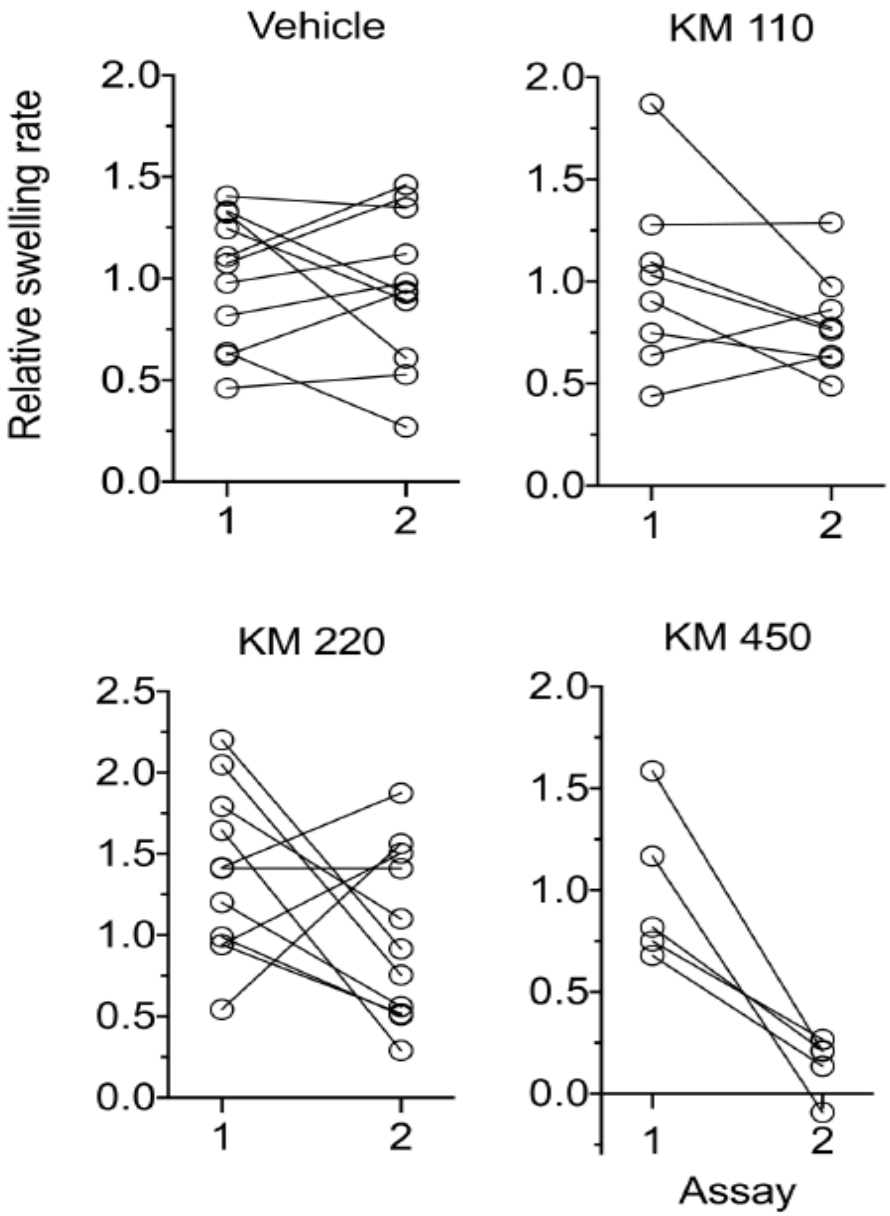

Figure 4. KeenMind extracts reduced the swelling response in the second swell assay. Trend plots illustrate the swelling rates measured for each oocyte (empty circle) in the initial untreated condition (1) and post-incubation in vehicle or KeenMind extracts (2). The swelling rate was calculated by comparing the surface area of the oocyte before and after osmotic challenge.

3.4.5 Dose-dependent inhibition of AQP1 mediated osmotic swelling by KeenMind and bacopasidesII

KeenMind extracts and bacopaside II inhibited the water permeability of AQP1-expressing oocytes in a dose-dependent manner. Each KeenMind capsule consists of about 3% bacopaside II (Dowell et al., 2015), therefore, the inhibitory effect of KeenMind extracts could be compared to that of the pure bacopaside II compound. Bacopaside II significantly inhibited the second swelling response of AQP1 expressing oocyte at 20μM and 50μM (Fig 5A), consistent with previous observations (Pei et al., 2016). KeenMind extracts significantly blocked the AQP1 water channel at 290 μg/ml and 450μg/ml (Fig 5 B). The half-maximal block of Bacopaside II was 18μM, whereas the half-maximal block of KeenMind was 290 μg/ml (Fig 5C), which approximately contains 15μM of bacoadaside II. Data here suggested that KeenMind extracts blocked the AQP1 water channel mainly by bacopaside II, although other bacopaside compounds might have a small contribution to the inhibitory effect.

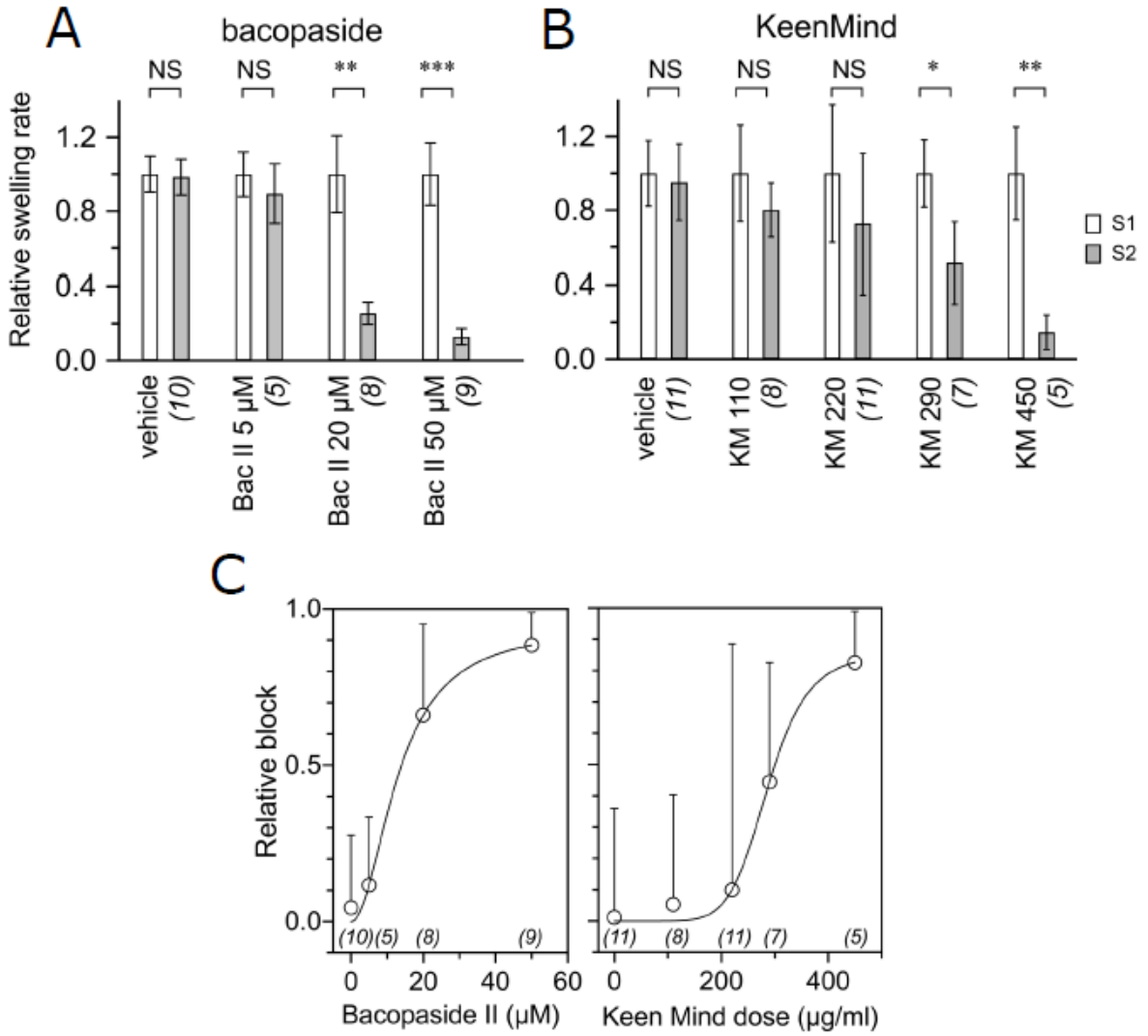

Figure 5. Dose-dependent inhibition by KeenMind and bacopaside II of osmotic water permeability in oocytes expressing human AQP1 channels. (A & B) Histogram summary of swelling rates in the first untreated assay (S1) and the second post incubation assay (S2) conditions. (C) Relative block by bacopaside II and KeenMind was calculated per oocyte from the difference in swelling rates between the first and second assays, divided by the rate in the first assay.

3.5 Discussion

Cancer is the second leading cause of death worldwide, accounting for 9.6 million deaths in 2018 (Bray et al., 2018). Among all types of cancer, colorectal cancer is the second most freguently diagnosed cancer in women and the third in men (Bray et al., 2018). Current treatments for cancer such as surgery, chemotherapy, and radiotherapy which are targeted primarily at inhibiting cancer proliferation are available; however, high recurrence of cancers after treatment suggests additional treatments are required (Miller et al., 2019, Koeller et al., 2002). Cell migration is the key determinant of cancer metastasis, which is the major cause of death in cancer patients (Koeller et al., 2002, Cramer, 1999). AQP1 expression has been reported to facilitates cell migration and proposed as a prognostic factor (Yoshida et al., 2013b, Park and Yoon, 2017, Qin et al., 2016b). AQP1 was up regulated in colorectal cancer and found to be localized at the leading edges of migrating cell (McCoy and Sontheimer, 2007b). Previous studies from our group have shown that pharmacological targeting AQP1 can impair cancer cell migration and invasion in vitro (De Ieso et al., 2019a, Pei et al., 2016b, Kourghi et al., 2016b). Therefore, pharmacological inhibitors for AQP1 could be a potential treatment for cancer.

A field of great interest was the identification of pharmacological modulators of aquaporins (Papadopoulos et al., 2008, Devuyst and Yool, 2010). Inhibitors for AQP1 such as mercury (Preston et al., 1993), silver and gold (Niemietz and Tyerman, 2002), acetazolamide (Gao et al., 2006), and tetraethylammonium ion (Brooks et al., 2000, Yool et al., 2002) were discovered early in work. However, high toxicity and varies in efficacy between experiments limited the usefulness of these inhibitors. More recently, small pharmacological agents such as the bumetanide derivative, AqB013, which targets AQP 1 and AQP4 water channels; AqB011, which targeting gated AQP1 ion channel; furosemide derivative AqF026, also

targeting AQP1 water channels and act as an agonist of the AQP1 water channel; and Phloretin, which is targeting AQP3 water channel (Müller-Lucks et al., 2013). Compounds found in traditional medicine such as bacopaside II from *Bacopa monnieri*, which is targeting AQP1 water channel (Pei et al., 2016); 5 hydroxymethyl furfural from honey which is targeting AQP1 ion channel (Chow et al., 2019 under review); and curcumin from curcuma longa plants, which is targeting AQP4 water channel (Wang et al., 2015a). These AQP inhibitors identified could be used therapeutically.

This study aims to broaden the panel of AQP inhibitors by evaluating natural medicinal plants as sources of active compounds. Data here supported the hypothesized that the commercial supplement KeenMind impairs cancer cell migration by exerting an inhibitory effect on AQP1 water channel activities. Results here showed that cell migration of HT29 colon cancer cells was impaired by KeenMind extract in a dose-dependent manner. KeenMind extracts showed no effect on cell viability, suggesting the inhibitory effect observed in cell migration was not due to drug toxicity. The effect of KeenMind extracts on AQP1 water channel was confirmed by oocyte swelling assay. AQP1-expressing oocytes incubated in vehicle saline showed a robust swelling response as a function of time in 50% hypotonic media, whereas oocytes incubated in KeenMind extract showed significant reductions in the swelling rates.

KeenMind is a commercial supplement from *Bacopa monnieri,* and bacopaside II is one of the active ingredients that has been shown to have an inhibitory effect in cancer proliferation, angiogenesis, and migration (Smith et al., 2018b, Palethorpe et al., 2018, Pei et al., 2016b). Our group previously reported that Bacopaside II significantly reduced HT29 colon cancer cell migration with an IC_{50} at 15 μM, by pharmacologically inhibiting the AQP1 water

channel. Works here showed that KeenMind extracts achieved 75% inhibition of HT29 cell migration at 150µg/ml, which contains approximately 5 µM of bacopadise II. KeenMind extracts had a stronger inhibitory effect in cancer migration than bacopaside II alone. Similar results were observed in oocyte swelling assays. The IC_{50} of KeenMind extracts was 290 µg/ml, which approximately contains 15µM of bacoadaside II, while the IC_{50} of pure bacopaside II was 18 µM. Bacopaside I and II has be shown to have synergistic effect in blocking breast cancer cell migration and invasion (Palethorpe et al., 2019), thus the additional inhibitory effect from KeenMind extracts might be a synergistic effect of bacopaside compounds. Human clinical studies have tested the chronic effect of KeenMind in health human subjects (Stough et al., 2001, Downey et al., 2013, Stough et al., 2008, Roodenrys et al., 2002); volunteers administrated 300 to 640mg/ day for several days showed no serious adverse events, thus the dosages tested here is within the safely tolerated range.

In summary, data here supported the hypothesis that KeenMind extracts inhibited HT29 cell migration through targeting AQP1. KeenMind extracts appeared to have a stronger inhibitory effect in cancer cell migration and AQP1 water permeability than bacopaside II alone, due to the present of different bacopaside compounds. Future work should test the effects of KeenMind extracts in invasion assay. Considering the cost and the inhibitory effect in cancer, KeenMind could be a potential treatment for colon cancer.

3.6 Acknowledgements

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Chapter 4: Furan compounds that are effective in preventing red blood cell sickling inhibit the Aquaporin-1 ion conductance

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4.1 Abstract

In sickle cell disease (SCD), the pathological shift of red blood cells (RBCs) into distorted morphologies under hypoxic conditions follows activation of a cationic leak current (P_{sickle}) and cell dehydration. Prior work showed sickling was reduced by 5-hydroxylmethyl-2-furfural (5-HMF), which stabilized mutant hemoglobin and also blocked the P_{sickle} current in RBCs, though the molecular basis of this 5HMF-sensitive cation current has remained a mystery. Work here is the first to test the hypothesis that Aquaporin-1 (AQP1) cation channels contribute to P_{sickle}. Human AQP1 channels expressed in *Xenopus* oocytes were evaluated for sensitivity to 5-HMF and four derivatives known to have differential efficacies in preventing RBC sickling. Ion conductances were measured by two-electrode voltage clamp, and osmotic water permeability by optical swelling assays. Compounds tested were: 5-HMF (5-hydroxymethylfurfural); 5-PMFC (5-(phenoxymethyl)furan-2-carbaldehyde); 5-CMFC (5-(4-chlorophenoxymethyl)furan-2-carbaldehyde); 5-NMFC (5-(2-nitrophenoxymethyl)-furan-2-carbaldehyde); and VZHE006 (tert-butyl (5-formylfuran-2-yl)methyl carbonate). The most effective anti-sickling agent, 5-PMFC, was the most potent inhibitor of the AQP1 ion conductance (99% block at 0.5 mM). The order of sensitivity of the AQP1 conductance to inhibition was 5-PMFC >VZHE006 >5-CMFC ≥ 5-NMFC at 0.5 mM, which corresponded with effectiveness in protecting RBCs from sickling. None of the

compounds altered AQP1 water channel activity. Selective inhibition of AQP1 ion channels is a serendipitous bonus of some effective anti-sickling agents. Future therapeutics aimed at combined hemoglobin modification and AQP1 ion channel inhibition could hold promise for improved treatments for SCD.

4.2 Introduction

Sickle cell disease (SCD) results from an inherited mutation in the oxygen-carrying molecule hemoglobin in red blood cells. Unlike wild type hemoglobin (Hb), hemoglobin carrying the SCD mutation (HbS) polymerizes more readily in low oxygen conditions into stiff strands which distort red blood cell morphology into diagnostic dysfunctional shapes. The SCD single point mutation in HbS converts a key glutamate residue to valine (Pauling et al., 1949, Eaton, 2020). When deoxygenated, HbS molecules aggregate into rigid polymers, changing red blood cell shape, and increasing fragility, solute loss and stickiness (Pauling et al., 1949, Joiner, 1993). Clinical concerns include chronic anemia, acute ischemia, severe pain episodes, and organ damage (Rees et al., 2010, Steinberg, 1999). Current interventions include blood transfusions to alleviate symptoms, and hydroxyurea treatment to reduce pain crises and anemia, but chronic side effects and variability in individual responsiveness to hydroxyurea limit its usefulness (Charache et al., 1987, Perutz and Mitchison, 1950, Segal et al., 2008). Transplanting stem cells from donor bone marrow is the only cure for SCD (Saraf and Rondelli, 2019), an option not available to most patients around the world.

New pharmacological treatment strategies have focused on identifying chemical modifiers to stabilize HbS. Promising compounds such as aromatic aldehydes and benzaldehydes have been tested in sickle cells (Zaugg et al., 1977, Abraham et al., 1991, Beddell et al., 1984). 5-Hydroxymethyl-2-furfural (5-HMF) forms a Schiff base with HbS, increasing oxygen affinity, reducing polymerization risk, and protecting red blood cells from sickling (Abdulmalik et al., 2005). Building on this discovery, Abdulmalik, Safo and colleagues (Xu et al 2017) engineered a group of ester and ether derivatives of 5-HMF which they tested for effectiveness in modifiying Hb, improving oxygen affinity, and preventing hypoxia-induced sickling of human SCD red blood cells. Of interest, four of the 12 synthesized compounds

conferred better protection from sickling than 5-HMF (all tested at 2 mM) and were effective in modifying hemoglobin, pointing to a new generation of candidate therapeutics. Curiously, some derivatives in the group such as 5-NFMC and 5-CMFC (despite yielding significant modification of Hb), were similar or worse than 5-HMF in preventing sickling (Xu et al., 2017, Zaugg et al., 1977). We considered this a clue that effective anti-sickling agents might serendipitously be affecting secondary targets, in addition to hemoglobin.

Volume regulation in RBCs involves a network of ion and water transport mechanisms which enable careful control of hemoglobin concentration within a narrow window (Gallagher, 2017). In the resting state, normal RBC membrane cation permeability is low, but increases in cation fluxes are evident during SCD cell sickling, mediated by the K^+-Cl^- cotransporter (KCC), the Ca^{2+}-activated K^+ (Gardos) channel, and a less well understood leak pathway known as "P_{sickle}" (Lew and Bookchin, 2005, Joiner, 1993). The involvement of a cation leak current in the sickling process has been appreciated for decades based on imbalances in K^+ and Na^+ levels in SCD cells (Tosteson et al., 1952). However, the mystery concerning the molecular basis of P_{sickle} has persisted as a profound gap in knowledge, despite widespread interest in finding a P_{sickle} inhibitor for expanding therapeutic strategies (Al Balushi et al., 2019, Kaestner et al., 2020, Lew et al., 1997, Hannemann et al., 2014a). Cation leak currents are activated in sickle cell RBCs during deoxygenation (Ma et al., 2012, Joiner et al., 1988, Joiner, 1993) and can be potentiated by membrane shear stress (Johnson and Gannon, 1990), such as that experienced in the microcirculation (McMahon, 2019).

AQP1 is expressed in red blood cells (Maeda et al., 2009, Ma et al., 1998b), and tissues including kidney, vascular system, heart, brain and others (Venero et al., 2001, Speake et al., 2003a, Papadopoulos et al., 2002, Badaut et al., 2002). It has been shown to serve dual

functions as a water channel and a non-selective cation channel regulated by cGMP acting at the loop D domain (Yu et al., 2006, Kourghi et al., 2018). Although initially controversial, multiple lines of evidence now show that AQP1 can function as a non-selective monovalent cation channel via the central pore of the tetramer, which is physically and pharmacologically distinct from the monomeric water-selective pores in the individual subunits (Zhang et al., 2007, Saparov et al., 2001, Boassa and Yool, 2003, Anthony et al., 2000, Yu et al., 2006). As reviewed previously, other classes of AQPs also have been shown to function as ion channels including AQP0, AQP1, AQP6, **Drosophila** Big Brain, soybean Nodulin (Yool and Campbell, 2012b) and Arabidopsis PIP2;1 (Byrt et al., 2017).

A possible role for AQP1 ion channels in the molecular mechanism of the P_{sickle} conductance has not previously been considered. Recent work from our group demonstrated that 5-HMF causes dose-dependent block of the AQP1 ion conductance, inducing 90±4 % block of the cGMP activated ion current at 10 mM, 74±5 % at 1 mM and 43% at 0.5 mM, with no effect on AQP1-mediated water fluxes (Chow et al., 2020; MS in review). These data served as a foundation for the hypothesis here that the furfural derivatives which are effective in reducing red blood cell sickling also act as pharmacological inhibitors of the AQP1 cation conductance. In summary, results here showed the two derivatives most effective in preventing SCD cell sickling, 5-PMFC and VZHE006, significantly inhibited the AQP1 cation conductance, as did the parent compound 5-HMF. No inhibition of the AQP1 ion conductance was observed with 5-NMFC or 5-CMFC, which were not effective in reducing sickling.

Results here are the first to identify AQP1 as a molecular component of the P_{sickle} current, and establish an additional therapeutic target for consideration in the development of anti-sickling

treatments. In practice, useful therapeutic agents would not need to prevent sickling completely, but simply would need to slow the process of HbS polymerization sufficiently that a larger proportion of RBCs could successfully pass through the microcirculation (Eaton, 2020). Novel therapeutic agents with combined actions on AQP1 ion channel inhibition and HbS stabilization could be of interest for expanding the range of affordable clinical options for treating sickle cell disease globally.

4.3 Material and methods

4.3.1Furan compounds

5-HMF (5-hydroxymethyl-2-furfural); 5-PMFC (5-(phenoxymethyl)furan-2-carbaldehyde); 5-CMFC (5-(4-chlorophenoxymethyl)furan-2-carbaldehyde); and 5-NMFC (5-(2-nitrophenoxymethyl)-furan-2-carbaldehyde) were purchased from Sigma Aldrich Chemicals (MO, USA). VZHE006 (tert-butyl (5-formylfuran-2-yl)methyl carbonate) was custom-synthesized at Virginia Commonwealth University (USA), as described previously (Xu et al., 2017). 5-HMF was dissolved in water and the other compounds were dissolved in dimethylsulfoxide (DMSO) to create 1000x stock solutions, which were diluted 1 µl/ml into experimental salines to final concentrations.

4.3.2 Oocyte preparation and injection

Unfertilized oocytes were harvested from *Xenopus laevis* frogs in accord with national guidelines (Australian Code of Practice for the Care and Use of Animals for Scientific Purposes), using a protocol approved by the University of Adelaide Animal Ethics Committee (#M2018-016). Oocytes were defolliculated with collagenase type 1A (2 mg/ml) in isotonic saline (100mM NaCl, 2mM KCl, 5mM $MgCl_2$, and 5mM HEPES; pH 7.6) at approximately 18°C for 1.5 hours. Oocytes were washed 3-4 times at ~10 min intervals with

isotonic saline and kept at 18°C in frog Ringers saline (isotonic saline supplemented with 0.6 mM $CaCl_2$, 5% horse serum (v/v), 100 units/ml penicillin, 0.1 mg/ml streptomycin, and 0.5 mg/ml tetracycline). Human AQP1 cRNA was synthesized in vitro (T3 mMessage mMachine kit, Ambion, Austin TX USA) from BamHI-linearized AQP1 cDNA (Genbank # NM_198098), which was subcloned into a *Xenopus* ß-globin expression vector (Anthony et al., 2000). RNA was resuspended in sterile water and stored at -80 °C. Oocytes were injected with 50 nl of sterile water (sham injection) or 50 nl of water containing 1 ng of AQP1 wild type cRNA. Injected oocytes were incubated in the dark in frog Ringers saline at 18°C for 48 h or more to allow time for protein expression. All chemicals were from Sigma-Aldrich (St. Louis, MO USA), unless otherwise indicated.

4.3.3 Quantitative swelling assays

Prior to swelling assays, sham injected (non-AQP1 controls) and AQP1-expressing oocytes were rinsed in isotonic saline (without serum, antibiotic-free) for at least 1 hour at room temperature. Swelling rates were measured in 50% hypotonic saline (isotonic Na^+ saline diluted with an equal volume of water, without test compounds present) and quantified by changes in the oocyte cross-sectional area imaged by videomicroscopy (Cohu, San Diego, CA) at 1 frame per second for 30-60 seconds, using ImageJ software (National Institutes of Health, Bethesda MD USA). In double-swelling assays, swelling was first measured without drug treatment (first swelling "S1"). Oocytes were then incubated for 2 hours in isotonic saline with vehicle or one of the test compounds, and reassessed in a second swelling assay ("S2"), as described previously (Migliati et al., 2009b). Swelling rates were measured from slopes of linear regression fits of relative volume as a function of time using Prism software (GraphPad Inc., San Diego, CA).

4.3.4 Electrophysiology

Two-electrode voltage clamp recordings of non-AQP1 control and AQP1-expressing oocytes in isotonic Na^+ saline were done with capillary glass electrodes (1–3 MΩ) filled with 1 M KCl, using a GeneClamp amplifier. Bath application of membrane permeable 8CPT-cGMP (8-(4-chlorophenylthio)-guanosine 3′,5′-cyclic monophosphate) at 10 µM final or SNP (sodium nitroprusside) at 1 mM final was used to activate the ionic conductance in AQP1-expressing oocytes, as described previously (Campbell et al., 2012, Kourghi et al., 2018). Changes in current as a function of time were monitored by repeated +40 mV voltage steps from a holding potential of -40 every 6 s. Conductance (slope) values were measured using a current-voltage protocol with steps from +60 to -110 mV. Recordings were filtered at 2 kHz, and stored to hard disk for offline analysis. Data were analyzed with Clampex 9.0 software (pClamp 9.0, Molecular Devices, Sunnyvale CA USA) and Prism software (GraphPad, San Diego CA USA).

4.3.5 In silico docking modeling

In silico modeling was carried out using methods reported previously (Pei et al., 2016b). The human AQP1 protein crystal structure (PDB ID:1FQY) was obtained from the National Institutes of Health NCBI Structure database (available at www.ncbi.nlm.nih.gov/Structure/pdb/1FQY). Structures for 5-HMF and the derivatives were downloaded from PubChem (pubchem.ncbi.nlm.nih.gov) and converted into software-compatible 3D structures in .pdb format using the online SMILES Translator and Structure File Generator (National Cancer Institute, U.S. Department Health and Human Services, Washington DC). MGLtools was used for preparing both AQP1 and ligand docking coordinates. The docking was performed using Autodock Vina (Trott and Olson, 2010), setting the docking grid to cover the intracellular face of the tetrameric pore.

4.3.6 Data analysis and statistics

Results compiled from replicate experiments are presented as box plots to show the full range of data points. Boxes represent 50% of the data points; the error bars indicate the full ranges; horizontal bars are median values. Statistical differences were evaluated using two-way ANOVA and post-hoc Students T-tests, and reported as * ($p < 0.01$), or NS (not significant; $p > 0.05$).

4.4 Results

4.4.1 Effects of 5-HMF and structural derivatives on AQP1 ion channel conductance

Electrophysiological analyses tested the effects of four 5-HMF derivatives, 5-PMFC, VZHE006, 5-NMFC, and 5-CMFC at 0.5 mM each, on the cGMP-activated ionic conductance in human AQP1-expressing oocytes (**Figure 1**), and confirmed block by the parent compound 5-HMF at 0.5 mM. Currents were measured before (initial) and after activation by the cyclic GMP agonist (cGMP 1st), at approximately 30 min for 8CPT-cGMP (Campbell et al., 2012) or 10-15 min for sodium nitroprusside (Kourghi et al., 2018). Oocytes were then incubated 2 hours in isotonic saline with vehicle or the indicated agents, followed by voltage clamp recordings of responses to the second application of cGMP agonist (cGMP 2nd). Incubation with vehicle did not impair subsequent conductance responses of AQP1-expressing oocytes to cGMP agonists, which remained comparable in amplitude in repeated trials. Conductance responses were inhibited following incubation in 0.5 mM 5-HMF, 5-PMFC, or VZHE006. No appreciable changes in responsiveness to cGMP were observed after incubation with 0.5 mM 5-NMFC or 5-CMFC.

During the 2 h incubation period (without cGMP agonist present), the ionic conductance recovered completely, as confirmed in trend plots tracking the conductance values for individual oocytes through the treatment series (**Figure 2)**. Amplitudes of ion conductances after the incubation period ('post incub') in all treatment groups were comparable to those in the initial condition, showing responses were uniformly reversible, and ruling out toxicity or oocyte damage. Second applications of cGMP agonist in normal saline were used to assess the level of block established during the incubation period. The AQP1 conductance was fully reactivated in oocytes incubated in vehicle treatment, showing that repeated recordings were well tolerated. The profound lack of AQP1 reactivation by cGMP after 2 hour incubation in

0.5 mM 5-PMFC demonstrated effective block of the ion conductance. VZHE006 caused moderate inhibition, whereas 5-NMFC and 5-CMFC showed no appreciable blocking effect. No effect on the baseline ionic conductance by cGMP or furfural derivatives was observed in non-AQP1 expressing control oocytes (data not shown), as documented elsewhere (Chow et al,. 2020; MS in review).

Ion conductance values, calculated from linear slope values from of current-voltage recordings, were compiled in a box plot (**Figure 3**) to identify statistically significant differences in the amplitudes of cGMP-activated conductances in AQP1-expressing oocytes before and after the incubation with vehicle or 5-HMF and derivatives. Responses measured after treatment with 5-PMFC or VZHE006 were significantly reduced as compared to initial responses to cGMP agonist in the same oocytes (Fig 3A). Structures of the furfural derivatives are illustrated in Fig 3B.

Figure 3C illustrates results of in silico modeling of the predicted binding site for 5-PMFC on the AQP1 channel, suggesting that the most energetically favourable site for interaction is located at the intracellular side of the central pore of the tetramer, in the gating domain (loop D) at a highly conserved serine residue (Ser167 in human AQP1). The predicted interaction energy of 5-PMFC at this site is -5.1 kcal/mol, which is more favorable than the predicted energy of interaction of 5HMF (-4.9 kcal/mol), consistent with its greater potency in blocking the AQP1 ion conductance (Fig 3A). This region in the loop D domain appears to be important for channel activation; mutation of an adjacent highly conserved residue, glycine 166 to proline, was found previously to significantly augment cGMP activation of the AQP1 ion conductance (Kourghi et al., 2018).

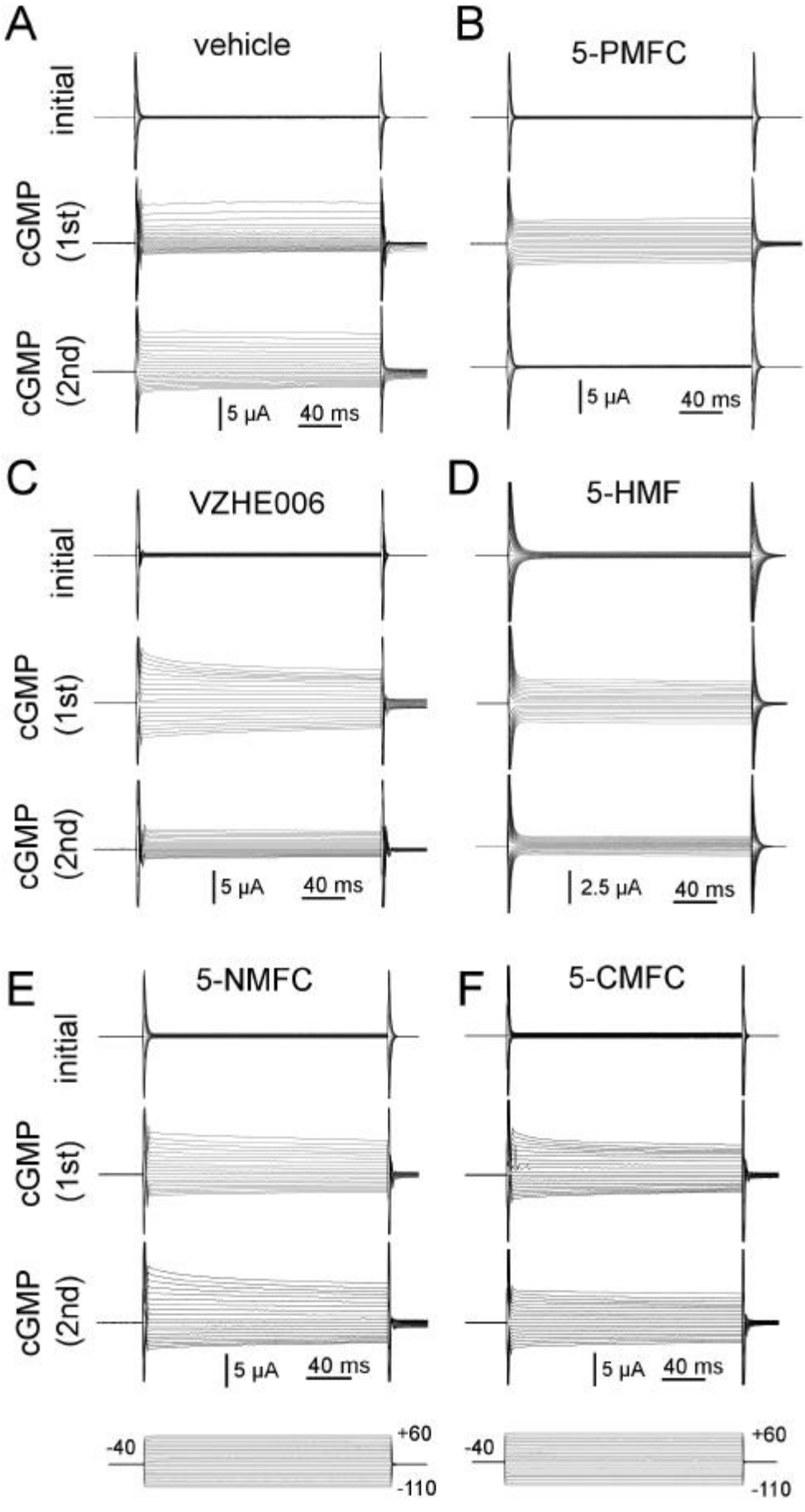
A
vehicle
initial
cGMP (1st)
cGMP (2nd)
5 µA
40 ms
B
5-PMFC
5 µA
40 ms
C
VZHE006
initial
cGMP (1st)
cGMP (2nd)
5 µA
40 ms
D
5-HMF
2.5 µA
40 ms
E
5-NMFC
initial
cGMP (1st)
cGMP (2nd)
5 µA
40 ms
-40
+60
-110
F
5-CMFC
5 µA
40 ms
-40
+60
-110

Figure 1. Electrophysiological recordings illustrating the effect of 5HMF and structural derivatives on the cGMP-activated AQP1 ion conductance. Representative sets of traces recorded by two-electrode voltage clamp of AQP1-expressing oocytes showing the initial conductance; the response induced by the first application of membrane-permeable cGMP; and the response to a second application of cGMP after 2 hours of incubation in isotonic saline containing 5HMF, 5HMFstructural derivatives and DMSO.

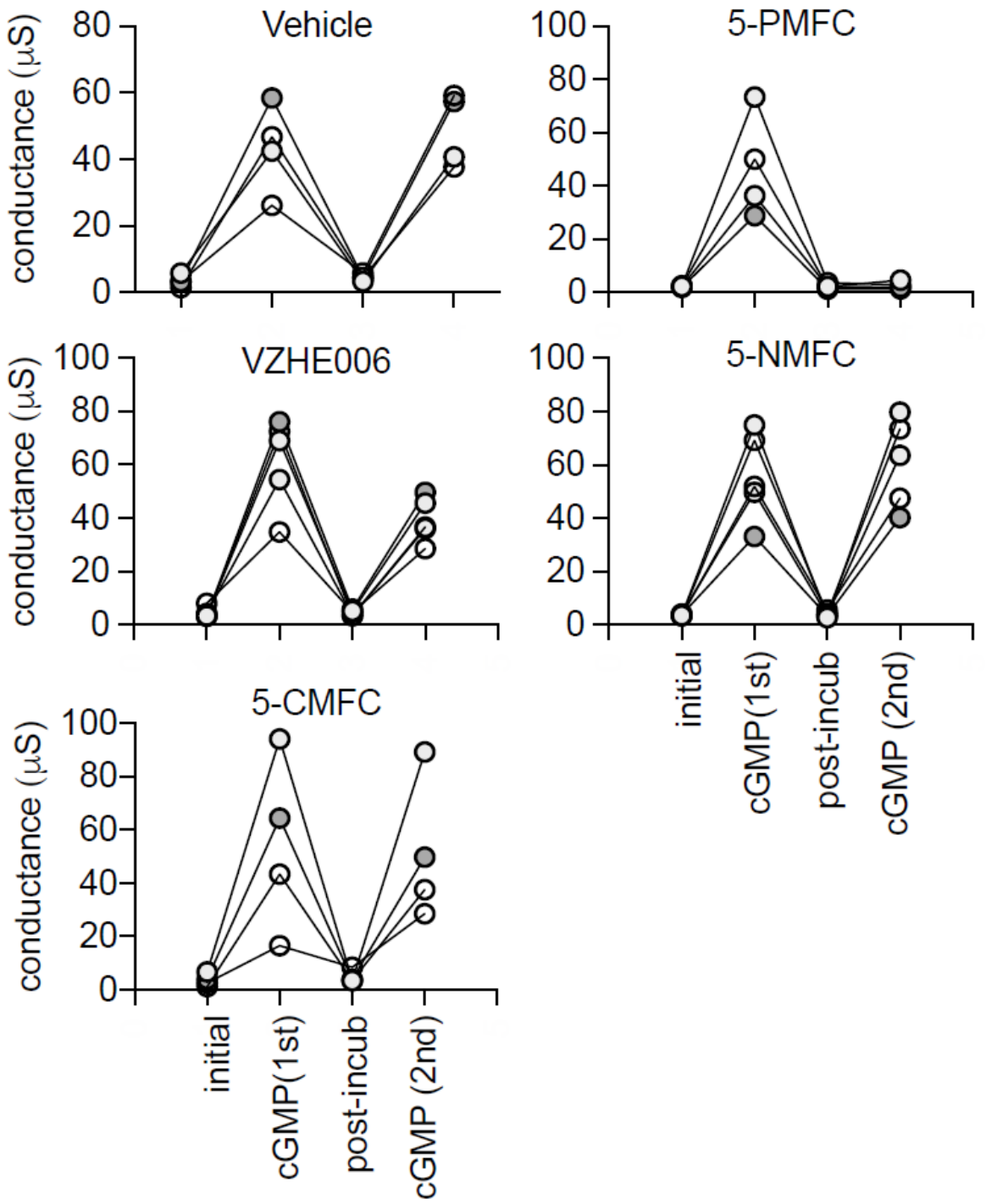

Figure 2. Trend plots showing the amplitude of the ionic currents, before and after the first activation by GMP, the recovery after incubation, and the response reactivated by a second cGMP application. Consistent recovery was seen after 5NMFC and 5CMFC but not after incubation with 5PMFC or VZHE006 indicating ion channel inhibition. The n values are as shown; each line represents a series of recordings from one oocyte.

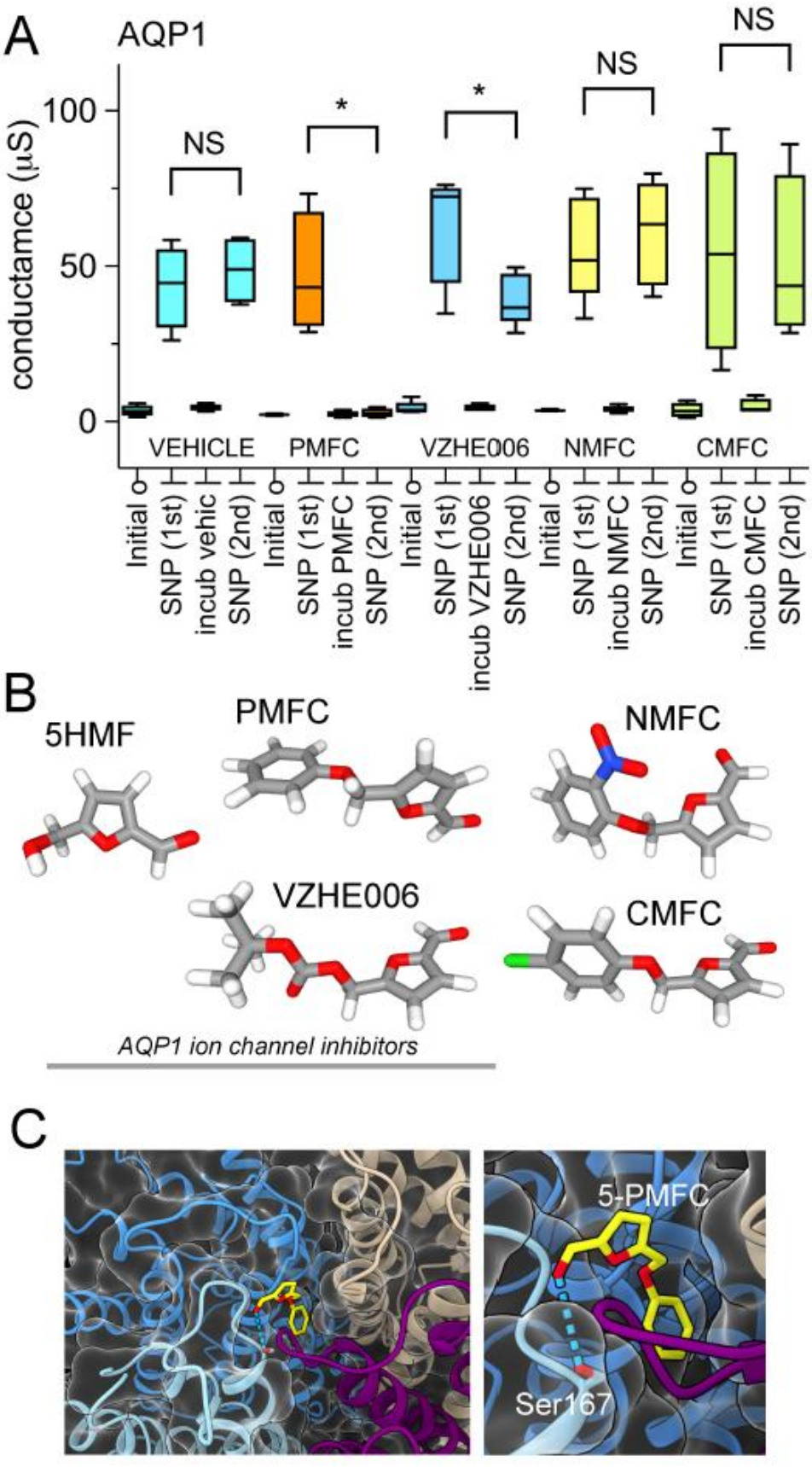
A
AQP1
conductamce (μS)
100
50
0
NS
*
*
NS
NS
VEHICLE
PMFC
VZHE006
NMFC
CMFC
Initial o
SNP (1st)
incub vehic
SNP (2nd)
Initial o
SNP (1st)
incub PMFC
SNP (2nd)
Initial o
SNP (1st)
incub VZHE006
SNP (2nd)
Initial o
SNP (1st)
incub NMFC
SNP (2nd)
Initial o
SNP (1st)
incub CMFC
SNP (2nd)
B
5HMF
PMFC
NMFC
VZHE006
CMFC
AQP1 ion channel inhibitors
C
5-PMFC
Ser167

Figure 3. Compiled data showing the effects of furfural derivatives on the amplitude of the cGMP-activated AQP1 ion conductance.

(A) Compiled box plot data showing statistically significant inhibition of the AQP1 ion conductance by 5-PMFC or VZHE006, but not with vehicle, 5-NMFC or 5-CMFC. (B) Space filling structures of the compounds tested. (C) In silico docking model illustrating the predicted site for the most favorable interaction of 5-PMFC with AQP1 is located in the central pore vestibule of the tetrameric channel in the loop D gating domain (left). Shifted close up view of the predicted hydrogen bonding interaction between 5-PMFC and Ser167 (right).

4.4.2 Effects of 5-HMF structural derivatives on AQP1 osmotic water fluxes

The 5-HMF derivatives had no effect on AQP1-mediated osmotic water permeability (**Figure 4**). Oocytes expressing AQP1 and non-AQP-expressing control oocytes, after 2 h incubation in saline with the indicated compounds (2 mM) or equivalent vehicle, were assessed for osmotic water permeability using an optical swelling assay (Fig 4A). All AQP1 expressing oocytes showed strong osmotic swelling responses; in contrast, non-AQP1 control oocytes showed little osmotic water permeability. AQP1-mediated swelling showed no significant effects of vehicle or 5-HMF derivative treatments, as summarized in compiled box plot data (Fig 4B). AQP1-expressing oocytes were tested in double swelling assays, in which each oocyte was tested before and after 2 hour incubation in isotonic saline containing vehicle or the 5-HMF derivative compounds at 2 mM. There were no significant differences between the first (S1) and second (S2) swelling rates for individual oocytes in any of the treatment groups (Fig 4C), as indicated by the slope values near 1.0 in plots of S1 versus S2 swelling rates. These results showed that none of the 5-HMF related compounds affected AQP1 osmotic water permeability, demonstrating the AQP1 channels remained intact and localized in oocyte plasma membrane through the experimental treatments.

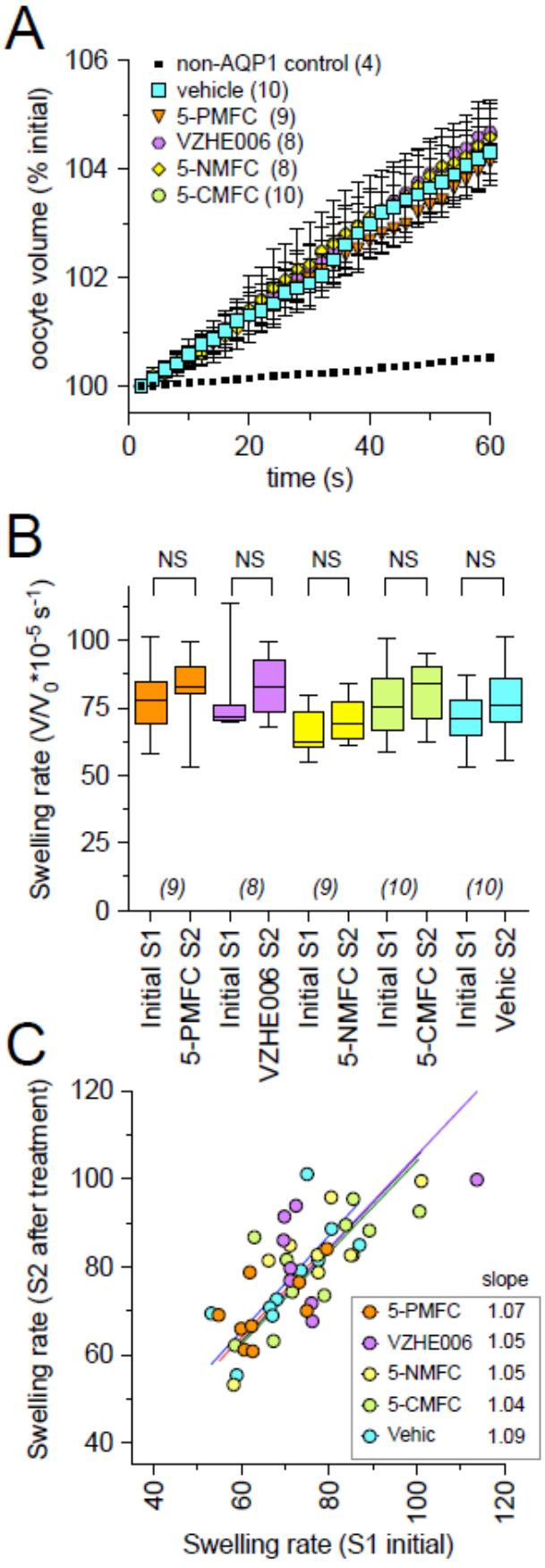
A
non-AQP1 control (4)
vehicle (10)
5-PMFC (9)
VZHE006 (8)
5-NMFC (8)
5-CMFC (10)
oocyte volume (% initial)
106
104
102
100
0
20
40
60
time (s)
B
NS
NS
NS
NS
NS
Swelling rate (V/V0*10-5 s-1)
100
75
50
25
0
(9)
(8)
(9)
(10)
(10)
Initial S1
5-PMFC S2
Initial S1
VZHE006 S2
Initial S1
5-NMFC S2
Initial S1
5-CMFC S2
Initial S1
Vehic S2
C
Swelling rate (S2 after treatment)
120
100
80
60
40
slope
5-PMFC 1.07
VZHE006 1.05
5-NMFC 1.05
5-CMFC 1.04
Vehic 1.09
40
60
80
100
120
Swelling rate (S1 initial)

Figure 4. Osmotic water permeability of AQP1-expressing oocytes is not altered by treatment with furan derivatives related to 5-HMF. (A) Mean swelling responses of AQP1 expressing oocytes in 50% hypotonic saline were not affected after 2 hours of preincubation in the furan derivatives. Data are mean ± S.E.M.; n values are as shown. (B) Compiled box plot data showing comparable swelling rates in the first (S1) and second (S2) assays, measured in the same oocytes before and after 2-hour incubation in saline with treatments as indicated. (C) A plot of the first (S1) versus the second (S2) swelling rates for individual oocytes shows a linear relationship with slope values near 1.0 in all treatment conditions, indicating no change in oocyte membrane integrity, function activity or levels of channel expression during the pharmacological incubation and repeated assays.

4.4.3 The effect of 5HMF and structural derivatives on AQP 1 ion conductance, haemoglobin modification and sickling

A summary of the effects of 5HMF and structural derivatives on the amplitude of the AQP 1 ion conductance, the level of hemoglobin modification, and the percentage of SCD cell sickling in hypoxic conditions is shown in **Figure 5**. The potency of sickle cell inhibition was 5-PMFC > VZHE006 > 5-HMF > 5-NMFC > 5-CMFC (Xu et al., 2017). The agents 5-PMFC, VZHE006 and 5-HMF significantly inhibited the AQP1 ion channel, whereas 5-NMFC and 5-CMFC were not effective. Interestingly, 5-PMFC and VZHE006 yielded similar levels of Hb modification, as did 5-HMF and 5-NMFC, yet these agents differed in their abilities to inhibit RBC sickling, showing outcomes consistent with their inhibitory effects on the AQP ion channel conductance.

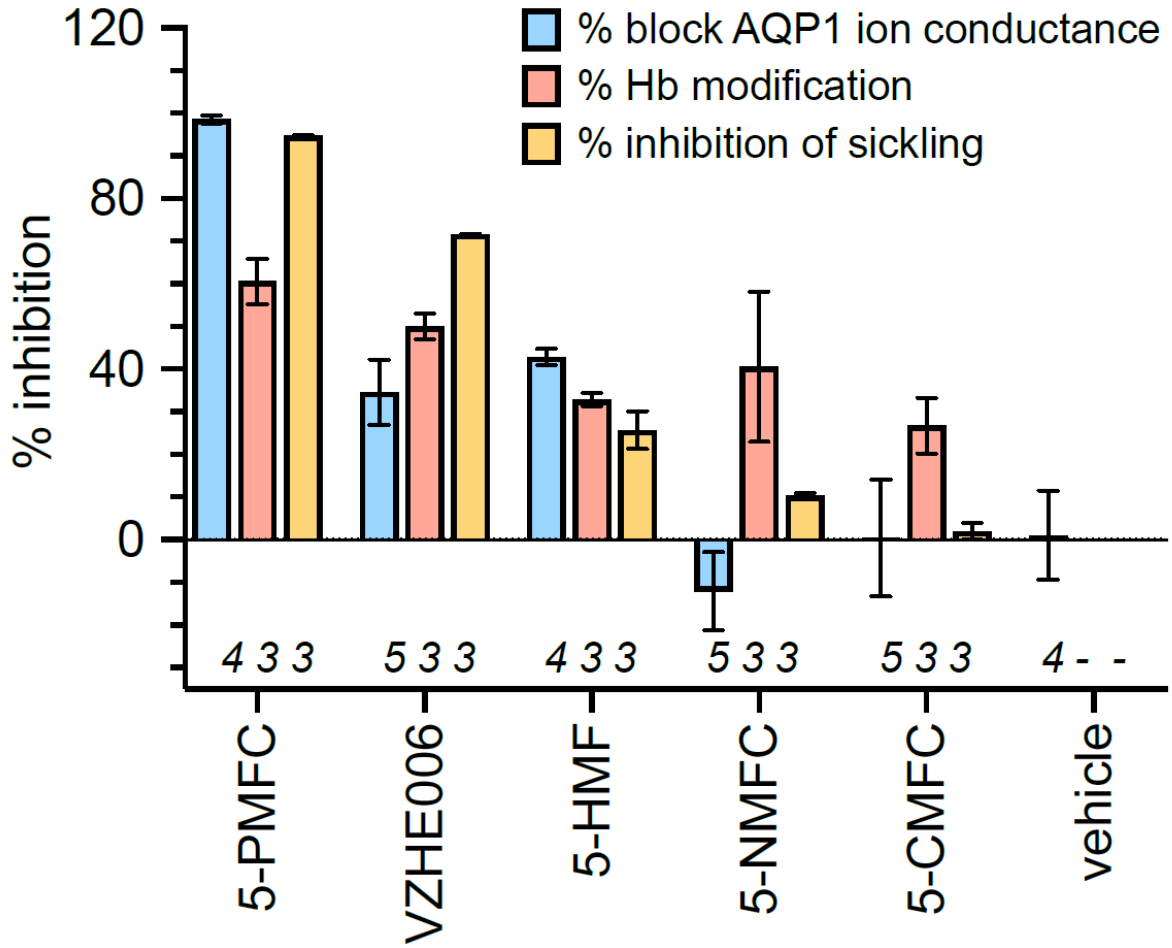

Figure 5. Effects of selected furan compounds on percent block of AQP1 ion conductance amplitudes, percent modification of haemoglobin (Hb), and percent inhibition of sickling SCD red cells exposed to low oxygen. Test of AQP1 block were done with agents at 0.5mM each; tests for haemoglobin modification and sickling risk were done at 2mM each. Data for Hb modification and red blood cell sickling were based on results presented in published tables (Xu et al., 2017).

4.5 Discussion

Cellular loss of K^+, Cl^{--} and water drives dehydration and increases the concentration of HbS in red blood cells of patients with SCD, leading to cell sickling (Lew and Bookchin, 2005, Joiner, 1993, Gibson and Ellory, 2002). Compounds structurally related to 5-HMF were shown previously by Abdulmalik and colleagues to protect SCD cells from sickling, with differences in efficacies attributed mainly to levels of modification of HbS and oxygen affinity (Xu et al., 2017). The observations that 5-HMF inhibited the P_{sickle} cation leak (Hannemann et al., 2014a), and blocked the AQP1 ion conductance (Chow et al., 2020, MS in review) suggested a link between the cation conductance pathways. Results here demonstrate that the most effective of the anti-sickling 5-HMF derivatives, 5-PMFC, was the most potent inhibitor of the cationic conductance of AQP1. AQP1 has not previously been considered as a candidate for P_{sickle}, but could account for the component that is pharmacologically distinct from Piezo1, KCC and Gardos, and important as one of the early steps in the dehydration cascade leading to the sickling phenomenon.

5-HMF is known to decrease sickling of RBCs from disease-affected patients, with effects originally attributed to decreased hemoglobin crosslinking (Safo et al., 2004). Of interest to us was the observation that 5-HMF blocked the P_{sickle} current at doses comparable to those used to reduce RBC sickling (Hannemann et al., 2014a). At millimolar concentrations, 5-HMF reduced the P_{sickle} component as measured by Rb^+ uptake in SCD cells; the magnitude of the monovalent ion flux compared for treated and untreated cells correlated strongly with the percentage of cell sickling. In contrast, Gardos activity showed a small reduction by 5-HMF, and no evidence of direct block (Hannemann et al., 2014a). SCD cell membranes show increased permeability to K^+, Na^+, Cs^+, Rb^+ and Li^+ (Joiner et al., 1993), and an accumulation of intracellular radiolabelled Ca^{2+} attributed to increased membrane Ca^{2+} permeability (Etzion et al., 1993). The idea that P_{sickle} was a single pathway for permeation of

all mono- and divalent cations was advanced, with the caution that the idea was unproven (Lew et al., 1997), leaving open the possibility that the cation leak currents rely on more than one mechanism (Kaestner et al., 2020).

Multiple lines of evidence now support the originally controversial concept that AQP1 is a gated ion channel (Yool et al., 1996, Agre et al., 1997), and is permeable to Na^+, K^+, Cs^+ and Li^+ but not divalent cations, as demonstrated by electrophysiology experiments, structure-function analyses, molecular dynamic modeling, and real-time visualization with a photoswitchable optical probe (Anthony et al., 2000, Campbell et al., 2012, Kourghi et al., 2017, Yool and Stamer, 2004, Yu et al., 2006, Pei et al., 2019). The proportion of AQP1 available for ion channel gating in the total water channel population is modulated by tyrosine phosphorylation in the carboxyl terminal domain (Campbell et al., 2012) and potentially by other factors such as membrane and cytoskeletal protein interactions (Cowan et al., 2000).

A number of properties of AQP1 ion channels are consistent with its contribution to the P_{sickle} conductance. P_{sickle} currents are found in red blood cells, in which AQP1 is expressed (Agre et al., 1995). The P_{sickle} current is blocked by 5-HMF (Hannemann et al., 2014a), which also blocks the the AQP1 ion conductance. SCD cells show elevated levels of cGMP as compared with controls, consistent with an increased activity of the cGMP-dependent AQP1 ion conductance (Conran et al., 2004, Almeida et al., 2020). The stochastic behavior of P_{sickle} suggests a dependence on low-probability channel activation events (Lew and Bookchin, 2005). The probability of AQP1 channel opening is low, and the kinetics of channel gating are slow (Anthony et al., 2000, Campbell et al., 2012), consistent with a low amplitude

background current that could escape detection in normal cells and account for the stochastic nature of P_{sickle} in hypoxic SCD cells.

In the SCD red blood cell, optimal anti-sickling agents could be acting in parallel to reduce HbS polymerization, countering rigid deformations of cell shape, and to block the AQP1 ion channel, countering part of the P_{sickle} leak. Results here are the first to identify AQP1 as one of the molecular components of the P_{sickle} current. In addition to RBCs, AQP1 water channels are expressed in endothelial and epithelial membranes of many tissues (Maeda et al., 2009, Ma et al., 1998b, Agre, 2004) including vascular endothelium, which is a key player in SCD vaso-occlusion events. The endothelial cell is a additional potential site of action for AQP1 pharmacological inhibitors, and will be a topic of interest for further research. AQP1 merits consideration as an additional therapeutic target for the development of anti-sickling treatments. Candidate drugs, perhaps similar to 5-PMFC with combined actions on both HbS stabilization and AQP1 ion channel inhibition, could be valuable starting points for generating affordable clinical options for treating sickle cell disease globally.

4.6 Acknowledgements

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Chapter 5: Thesis General Discussion and Future Considerations

5.1 Thesis main findings and innovative contributions

Aquaporin -1 (AQP1) is a integral transmembrane protein that facilitates water movement across cell membranes (Preston et al., 1992, Reizer et al., 1993, Calamita et al., 1995), and it mainly expressed in epithelia such as kidney tubules, choroid plexus and ciliary epithelium for fluid absorption and secretion (Verkman, 2011). Upregulation of AQP-1 found in a diverse type of aggressive cancers and thought to enhance cell migration and metastasis (Papadopoulos et al., 2008, Verkman et al., 2008). In addition to its water function, AQP-1 is also an ion channel (Yool and Campbell, 2012a, Yu et al., 2006, Sui et al., 2001), which is rare among AQPs, and the physiological role of the ion channel remains elusive. AQP-1 is not the only AQP that upregulated in cancer cells, for example, in malignant astrocytes, both AQP1 and AQP4 is upregulated, however the role of AQP1 in cell migration is not replaceable by AQP-4, suggesting the function of AQP1 is unique in cell migration (McCoy and Sontheimer, 2007a). Although there is a gap in knowledge on how exactly AQP1 enhances cell migration, the water channel and ion channel of AQP1 are involved. Current models predicted that during cell migration, water and ion movement triggers local cell volume changes that enable process extension (Schwab et al., 2007, Stroka et al., 2014, Frede et al., 2013). AQP1 is proposed to promote Na^+ influx which creates an osmotic driving force that allows water to move across the AQP1 water channel at the leading edge of cells, resulting in local cell volume changes hence cell migration (Verkman 2012). Thus the first part of this thesis is to further investigate the role of AQP-1 water and ion channel in cell migration by discovering novel inhibitors of ion channel and water channels.

Work from this thesis documents the discovery of new AQP-1 ion channel and AQP-1 water channel inhibitors. In chapter 2, furfural derivatives has been demonstrated to have inhibitory effect on AQP-1 ion channel The efficacy of the furfural derivatives is based on the size of

the side chain at position 5 on the furan ring; 5-hydroxymethylfurfural (5HMF) with the smallest side change has the highest efficacy whereas 5-acetoxymethyl-2-furaldehyde 9 (5AMF) with the largest side chain has the lowest efficacy. These results were consistent with molecular docking studies, supporting the idea that the size of the side change affects the binding energy of the furfural derivatives. Pharmacological targeting of AQP1 ion channel with furfural derivatives slowed cell migration and invasion in high AQP1 expressing cancer cell lines but not in low AQP1 expressing cancer cell lines. Furfural derivatives significantly inhibited cell migration and invasion in colorectal cancer cell line HT29 and breast cancer cell line MDA-MB-231, following the order of potency in AQP1 ion channel inhibition. Surprisingly, 5-nitro-2-furoate, the only furfural derivative that did not affect AQP1 channel activity, showed significant inhibition in cell migration and invasion in all tested cell line including colorectal cancer, breast cancer, and glioma (unpublished data) suggesting it inhibited a mechanism that all cell lines share with. In chapter 3, a commercial supplement KeenMind isolated from Bacopa monnieri was tested for its effects on cancer cell migration. Results here showed that KeenMind extracts significantly inhibited colon cancer cell migration through impairing AQP1 water channel activity. This observation consistent with previous work from our group, which reported the inhibitory effect of bacopaside II (Pei et al., 2016a), one of the major components of KeenMind.

The final part of this thesis investigated the role of the AQP1 ion channel as a therapeutic target in sickle cell disease (SCD). SCD is an inherited disease that resulted from the mutation in the haemoglobin in red blood cells (Bunn, 1997, Bauer, 1940).Cation leakage is key in the pathogenesis of sickle cell and involves a pathway known as P_{sickle}, for which the molecular identity remains as a gap in knowledge. 5HMF has been previously reported to inhibit red blood cell sickling and reduce cation leak (Abdulmalik et al., 2005, Hannemann et al., 2014b). Interestingly, as shown here, 5HMF also blocked the AQP1 ion conductance,

suggesting a possible role of AQP1 in P_{sickle}. In chapter 4, new anti-sickling agents developed from 5HMF were tested for effects on AQP1 ion channel conductance and water channel activity. The new agent 5-PMFC had a higher inhibition rate in red blood cell sickling than 5HMF, mirroring their relative effectiveness in blocking the AQP1 ion conductance. For the first time, works here identify AQP1 as one of the likely components of the P_{sickle} current.

5.2 Future considerations

5.2.1 Role of AQP1 ion conductance in cancer invasion and metastasis

The physiological significance of AQP1 ion channel has been reported in the choroid plexus production of cerebral spinal fluid (Boassa et al., 2006) and colon cancer cell migration (Kourghi et al., 2016b). Work from this thesis is consistent with the previous proposal from our group that pharmacological targeting of the AQP1 ion channel conductance impairs cancer migration and invasion. Future work could include further investigation of the idea of dual inhibition of the AQP1 ion and water channel in cancer cells, in order to develop an AQP1 based therapy in cancer. Dual treatment of AQP1 with pharmacological agents has been reported to have a synergistic inhibitory effect in reducing cancer cell migration and invasion (De Ieso et al., 2019b). AQP1 ion channel inhibitors tested in this thesis showed different efficacies in blocking AQP1 ion channels. Thus, testing different combinations of AQP1 ion channel inhibitors with AQP1 water channel inhibitors will provide additional information on the role of AQP 1 in cancer migration. It would also be intriguing to test the pharmacological sensitivity to AQP1 ion channel inhibitor in non-AQP1 expressing cell lines after transfection with wild-type AQP1. A possible outcome is that transfected cell lines will show increased sensitivity to the ability of AQP1 ion channel inhibitors to reduce cell migration and invasion. Another future experiment would be to transfect non-AQP1 expressing cell lines with AQP1 constructs that have been mutated so that the AQP ion

channel can be deactivated, and tested with AQP1 ion channel blockers. The Loop D domain in AQP1 is the gating site for the ion channel (Kourghi et al., 2018). Site-directed mutagenesis in the loop D domain with selected mutation such as alanines substituted for two arginines (R159A+R160A); aspartic acid for proline (D158P); or threonine for proline (T157P) were shown to reduce AQP1 ion conductance activation (Kourghi et al., 2018), and we would predict that cells transfected with these AQP1 mutants would migrate slower than cells transfected with wild type AQP1. Alternately, site mutations such as glycine substituted for proline (G166P) are known to enhance the AQP1 ion conductance, and we would predict that cells expressing this mutant would migrate faster and perhaps be more sensitive to AQP1 ion channel inhibitors.

5.2.2 Investigate the effect of AQP1 pharmacological agents in vivo

Number of AQP1 pharmacological agents has discovered and tested in vitriol, however, there is limited research investigating the efficacy of these pharmacological agents in vivo. Genetic knockdown of AQP1 in a murine melanoma tumor model resulted in a smaller and fewer micro-vessels tumour as compared to AQP1 wild type mice (Saadoun et al., 2005, Nicchia et al., 2013), suggesting AQP1 pharmacological agent could slow down cancer development. Therefore future work should test the effect of the AQP1 pharmacological agents using animal models. The other possibility would be testing the AQP1 pharmacological agents with chemotherapy treatments in vivo. AQP1 expression has been associated with the sensitivity to chemotherapy in colon cancer (Imaizumi et al., 2018), and knocking down AQP1 expression in bladder cancer cells enhances the efficacy of chemotherapy (Zhang et al., 2018b). The prediction would be that the combination of an AQP1 inhibitor with chemotherapy would increase the effect of chemotherapy.

5.2.3 Identifying the mechanism of methyl 5-nitro-2-furoate in cell migration.

Methyl 5-nitro-2-furoate (M5N2F) is a compound structurally related to 5HMF; however M5N2F did not block the AQP1 ion channel or water channel in the oocyte expression system. For the first time, M5N2F was tested here in cancer 2D cell migration and invasion assays, and shown to significantly inhibit cell migration and invasion in all tested cell lines, including colorectal, breast and brain cancer cell lines (unpublished data). Cell viability assays indicated no reduction in cell viability at the doses tested, suggesting the inhibitory effects observed were not due to decreased cell viability. Since M5N2F inhibited cell migration in multiple cell lines and had no effect on AQP1 ion or water channels, one of the hypotheses would be that M5N2F interferes with a more widespread mechanism of motility in the cancer cells. Work here showed that M5N2F reduced signal intensities in actin-dyed cancer cells, suggesting the inhibitory effects of M5N2F involved actin disorganization. Future work should further test effects of M5N2F in more cell lines, including non-cancer cell lines. Another testable hypothesis would be that M5N2F inhibits chemotactic machinery in cancer cells. Chemotaxis is a cellular response which directs cell movement toward an attractant in a chemical gradient. Pilot data from our group showed that the inhibitory effects of M5N2F remained consistent when the chemotactic gradient was increased, whereas the inhibitory effects of 5HMF were reduced when the chemotactic gradient was increased. Future work could knock down or inhibit the downregulating receptors for chemotactic factors in the cancer cells and test whether M5N2F is still able to impair cell migration and invasion. If M5N2F lost its inhibitory effects in any of the knocked down or inhibited conditions, it would suggest that the targeted receptor in that condition is one of the pharmacological targets of the inhibitor.

5.2.4 Role of AQP1 ion channel in sickle cell diseases

Red blood cell sickling is the result of polymerization of mutated hemoglobin when deoxygenated, which leads to anemia, pain crisis, acute chest syndrome, and eventually organ damage (Bunn, 1997). The only approved drug for treatment of sickle cell disease is hydroxyurea; however, chronic side effects such as dose-dependent myelosuppression indicate there is a need for better treatments (Platt, 2008, Steinberg et al., 2003). P_{sickle} is a cation leaking pathway in sickle cell that contributes to the probability of sickle cell formation (Joiner et al., 1986). Work here considered the AQP1 ion channel as one of the molecular pathways by showing the inhibitory effect of two antisickle agents in AQP1 ion channel. It would be interesting to test the effects of other AQP1 ion channel inhibitors such as AqB011 in sickle cell disease. The prediction would be that AqB011 partially inhibits sickle cell formation, but not completely beacause P_{sickle} is only one of the factors that leads to dehydeation and shape change. Thus the combination of AQP1 ion channel inhibitor with hydroxyurea could reduce the dosage of hydroxyurea needed for a benfical effect. It would be also interesting to test the effects of AQP1 water channel inhibitors in sickle cell. Future work should also consider using patch clamping to confirm AQP1 ion channel activity in sickle cells.

5.2.5 AQP1 pharmacological modulators for the treatment of other pathologies

AQP1 is also involved in several other diseases and pharmacological agents might be useful in these conditions. For instance, AQP1 pharmacological agents could be useful for the treatment of elevated intracranial pressure (ICP) by reducing cerebrospinal fluid production. Following stroke or traumatic brain injury, the blood- brain barrier undergoes breakdown, which results in an increase in permeability of small vessels, leading to the accumulation of cerebrospinal fluid within the brain (Greve and Zink, 2009, Ropper, 1984). AQP1 is highly

expressed in the choroid plexus epithelium and responsible for cerebrospinal fluid production (Longatti et al., 2004, Speake et al., 2003b). AQP1 null mice exhibited a significant reduction in cerebrospinal fluid secretion and a reduced ICP (Speake et al., 2003b). Thus, inhibiting the AQP1 function in choroid plexus could be a useful tool to lower the ICP. In contrast, AQP1 agonists might be useful in treating Alzheimer's disease. The exact pathogenesis is not fully understood, but recent studies indicated that the Alzheimer's condition is correlated with the excess production and deposition of the β-amyloid peptide (Aβ)(Gouras et al., 2015).Transgenic mouse model revealed that excess Aβ is likely to surround choroidal capillaries which is highly express AQP1; this build up impairs the clearance from CSF to blood (González-Marrero et al., 2015). Therefore AQP1 agonists could have potential benefits in increasing the CSF production, improving CSF circulation, and preventing the accumulation of Aβ to slow disease progression.

AQP1 inhibitors might also be useful in non-cerebral oedema. Various forms of oedema such as pulmonary oedema and ascites result from conditions such as nephrotic syndrome (NS), cirrhosis, and congestive heart failure (Qavi et al., 2015, Doucet et al., 2007, Warren and STEAD, 1944). Common treatments for non-cerebral oedema include thiazide diuretics, which target the sodium-chloride transporter in the kidney distal convoluted tubules (DCTs) and loop diuretics which targeting sodium-potassium-chloride cotransporter in the ascending limb of the loop of Henle, both of the treatments reduce the reabsorption of salts in kidney and therefore remove excess water through urine (Morrison, 1997, O'Brien et al., 2005). However, some patients with advanced conditions might have resistance to diuretic (Elwell et al., 2003). There is a need for alterantive treaments to reduce blood volume. AQP1 is highly expressed in plasma membranes of the proximal tubule and thin descending limb of Henle, responsible for over 80% of water reabsorption. Genetic knockdown of AQP1 significantly reduced water permeability in the proximal tubule and thin descending limbs of Henle (Ma et

al., 1998a, Schnermann et al., 1998, Chou et al., 1999). Therefore future work should test the AQP1 inhibitors together with thiazide diuretics and loop diuretics in a mouse model to see possible effects on body water reabsorption. A prediction would be that combined treatments will exert a greater inhibition of water reabsorption than with thiazide diuretics or loop diuretics alone.

5.3 Thesis conclusion

Work in this thesis supported the hypothesis that AQP1 ion and water channels play a role in diseases involving fluid imbalance, and that pharmacological targeting of both the water and ion channel could slow down disease development. Work here illustrated that AQP1 ion and water channels facilitated cancer cell motility, showed a potential role for AQP1 in sickle cell formation and documented discovery of new AQP1 ion and water channel inhibitors, and testing their efficacy using oocyte system and cancer models. Work here has paved the way for developing AQP1-based therapies for diseases including including cancer, sickle cell disease, cerebral oedema, and non-cerebral oedema. Future investigations should utilize these findings to further explore pharmacological modulators of AQP1 as novel therapies.

5.4 References

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