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Aquaporin water channels affect the response of conventional anticancer therapies of 3D grown breast cancer cells

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Aquaporin (AQP) water channels facilitate water transport across cellular membranes and are essential in regulation of body water balance. Moreover, several AQPs are overexpressed or ectopically expressed in breast cancer. Interestingly, several in vitro studies have suggested that AQPs can affect the response to conventional anticancer chemotherapies. Therefore, we took a systematic approach to test how AQP1, AQP3 and AQP5, which are often over-/ectopically expressed in breast cancer, affect total viability of 3-dimensional (3D) breast cancer cell spheroids when treated with the conventional anticancer chemotherapies Cisplatin, 5-Fluorouracil (5-FU) and Doxorubicin, a Combination of the three drugs as well as the combination plus the Ras inhibitor Salirasib. Total viability of spheroids overexpressing AQP1 were decreased by all treatments except for 5-FU, which increased total viability by 20% compared to DMSO treated controls. All treatments reduced viability of spheroids overexpressing AQP3. In contrast, only Doxorubicin, Combination and Combination + Salirasib reduced total viability of spheroids overexpressing AQP5. Thus, this study supports a significant role of AQPs in the response to conventional chemotherapies. Evaluating the role of individual proteins that contribute to resistance to chemotherapies is essential in advancing personalized medicine in breast carcinomas.

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1. Introduction

In 2020, The International Agency for Research on Cancer estimated the number of new breast cancer cases to 2.3 million, making female breast cancer the most commonly diagnosed cancer worldwide [1]. Molecular subtypes of breast cancer are based on expression of key proteins (estrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor receptor 2 (HER2) and the proliferation marker Ki67) and are classified as Luminal A, Luminal B, enriched HER2 (HER2+), and Triple negative breast cancer (TNBC). Conventional treatment of breast cancer includes surgery, radiation therapy, chemotherapy and hormone therapy. Chemotherapy is mainly used to treat the TNBC subtype [2]. However, chemotherapy is also highly recommended for women with stage III, ER-positive breast cancer, four or more affected lymph nodes, including those with lobular carcinoma and/or grade 1 or luminal A breast cancers [3]. A major problem in treatment is drug resistance which can be both inherit or intrinsic, thus the cancers can be resistant before treatment or acquire resistance. Resistance is observed for all conventional breast cancer chemotherapeutics like Doxorubicin [4] (an anthracycline that causes cell death by DNA–adduct formation and topoisomerase II inhibition [5]), Cisplatin [6] (an alkylating platinum based drug that forms DNA adducts thus triggering apoptosis [7]) and 5-Fluorouracil (5-FU) [8] (an antimetabolite designed to achieve chemotherapeutic effects by inhibiting thymidine synthase [9]). Crosstalk between the tumor microenvironment and the cancer cells as well as alterations of downstream signaling pathways impacting cell death/survival, including the Ras signaling pathway [10], are major factors in development of drug resistance [11].

Aquaporins (AQPs) and aquaglyceroporins are channel proteins that facilitate transport of water, small solutes, such as glycerol and urea, as well as certain gasses across cellular membranes. Thus, AQPs are important in regulation of body water homeostasis and their dysregulation is associated with a wide range of water balance disorders. Beside their canonical function in body water balance, ectopic AQP expression and overexpression are associated with numerous malignancies, including breast cancer (see reviews [12,13]). For instance, AQP1 expression in invasive ductal carcinoma...
was associated with metastasis, recurrence, and decreased 5-year survival [14]; AQP3 expression was associated with poor recurrence-free survival in patients with HER2-positive breast cancer [15] and ectopic AQP5 expression in early ER/PR-positive and/or HER2-overexpressed subtype breast cancer patients correlated with spread to lymph nodes and poor prognosis [16]. In TNBC, overexpression of both AQP3 and AQP5 was significantly associated with tumor size, lymph node status and local relapse/distant metastasis [17].

AQPs have been shown to affect multiple cellular processes involved in cancer development and spread, including cell proliferation and migration [18–21], cell-cell adhesion [22–24] and expression of matrix metalloproteases (MMPs) [25,26]. While the mechanisms underlying these regulatory functions are still being elucidated, it is known that AQPs mediated water influx at the leading edge and alterations in cellular signaling affect cancer cell migration [for review [13]]. AQP regulation of cell-cell adhesion was, at least in part, mediated by the C-terminal tail of the AQPs, and is presumed to involve protein–protein interactions [22–24]. Moreover, in vitro studies have shown that AQP alteration of signaling pathways like PI3K/Akt and Erk1/2, can alter expression of several MMPs [25,26]. Uniquely for AQP5, it contains a serine in position 156 (S156) in the intracellular loop D which upon phosphorylation activates the Ras signaling pathway [27–29]. This activation is involved in AQP5 mediated cancer cell proliferation [27], migration and cell dissemination from migrating cell sheets [22]. Interestingly, recent discoveries indicate a potential role of AQPs in drug resistance. For instance, overexpression of AQP1 increased sensitivity to anthracycline treatment in MDA-MB-231 breast cancer cells [30] and AQP1 depletion using shRNA in bladder cancer cells resulted in increased sensitivity to Mitomycin C [31]. AQP3 upregulation mediated Cisplatin resistance in gastric cancer cells [32] and selective shRNA mediated silencing of AQP3 increased 5-FU mediated cell death of breast cancer cells [19]. Moreover, in colorectal cancer, shRNA mediated AQP5 knockdown increased chemosensitivity of cells to 5-FU by facilitating 5-FU mediated apoptosis [33], siRNA mediated AQP5 knockdown in breast cancer cells increased sensitivity to Adriamycin (Doxorubicin) [34] and siRNA mediated AQP5 knockdown in colon cancer cells improved efficacies of 5-FU and Cisplatin [35].

So far, a systematic analysis of how different AQP isoforms affect drug sensitivity in breast cancer cells is lacking. Thus, we took a methodical approach to investigate how AQP1, AQP3 and AQP5 overexpression affects drug sensitivity. As a model system, we used AQP overexpressing breast cancer cells grown as 3D spheroids, which recapitulate the tumor microenvironment and structural organization with cellular layered assembling, which affects hypoxia and nutrient gradients [36]. We used the conventional breast cancer anticancer chemotherapies Cisplatin, 5-FU and Doxorubicin, either alone or as Combination, and the Combination with the Ras inhibitor Salirasib [37]; Salirasib was added to test the effect of AQP5 mediated Ras signaling.

2. Materials and methods

2.1. Constructs and cell cultures

AQP1-EGFP, AQP3-EGFP and AQP5-EGFP plasmids were from Prof. Peter Pohl (Johannes Kepler University, Austria (AQP1-EGFP)) and Prof. Anita Aperia (Karolinska Institutet, Sweden (AQP3-EGFP and AQP5-EGFP)), respectively and were previously characterized [23,38]. The generation of the point mutant AQP5S156A-EGFP is described in Ref. [38]. Cell lines were MCF7 [39] and MDA-MB-231 [40] breast cancer cells. For generation of stable cell lines, cells were transfected with Lipofectamine 2000 and selected with G418 (Merck) (manuscript accepted [41]) as previously described [38]. Cells with similar expression levels were chosen. Cells were grown at 5% CO2 at 37 °C in DMEM low with 1 g/L D-glucose (Thermo Fisher Scientific), 10% fetal bovine serum (FBS) (Thermo Fisher Scientific), and antibiotics as listed: 0.5 μg/ml penicillin G sodium (Merck), 0.5 mg/ml streptomycin sulfate (Thermo Fisher Scientific), and 1 mg/ml kanamycin sulfate (Thermo Fisher Scientific). Cells were allowed to grow to a maximum of 80% confluency and passaged.

2.2. Spheroid growth and drug treatment assay

Spheroids were generated following a previously published protocol [42]. In brief, 1000 cells were seeded per well in round-bottomed, ultra-low attachment 96-well plates (Thermofisher Scientific, 174925) in media supplemented with 1.5% GelTrex LDEV-Free Reduced Growth Factor Basement Membrane Matrix (Thermo Fisher, A1413202). All reagents including plates and media were kept on ice before and after seeding to avoid solidification of the GelTrex. Plates were centrifuged for 25 min at 750 RCF. After 2 days of spheroid growth, the anti-cancer treatments Cisplatin (18.75 nM, P4394 Sigma), 5-Fluor Uracil (5-FU) (0.0625 nM, F6627 Sigma) and Doxorubicin (18.75 nM, D2438 Sigma) were added either alone, as Combination or as Combination with the Ras inhibitor, Salirasib (S-farnesylsiosalylicylic 75 μM, SML1166 Sigma) [22]. The Combination of the above-mentioned drugs used was previously described [43]. As control, DMSO was added to an equivalent volume of the highest chemotherapy treatment. For spheroids generated from MCF7 cells, the medium was replaced on day 1, day 3, day 6 and day 8 and for spheroids generated from MDA-MB-21 cell lines, the medium was replaced on day 1, day 3, day 5 and day 7 (100 μl including anticancer drugs and inhibitor). Images of spheroids were acquired on the days of media replacement (see below).

2.3. Total viability assay

Spheroid growth was terminated on day 8 and day 7 for MCF7 and MDA-MB-231 cell lines, respectively. For total viability assay, spheroids were transferred to black walled, clear, flat bottom plates (3631, Corning). The 100 μl medium was replaced with 100 μl CellTiter-Glo 3D Reagent (G9683, Promega) as in Ref. [43], Medium with 100 μl CellTiter-Glo 3D reagent was used as baseline setting. The plates were shaken for 5 min at 240 rpm, incubated for 25 min at room temperature and the luminescent signal was recorded using the FLUOStar Optima (BMG).

2.4. Microscopy and image analysis

Images of spheroids on day 1 were acquired using an Olympus Leica MZ16 microscope with a phase contrast 10x air objective. Subsequently, images were captured using a Nikon Eclipse Ti2 microscope equipped with a 20x air objective. All images were analyzed using ImageJ Fiji software from NIH [44]. Area was quantified with the polygon drawing tool. Total viability data were analyzed using Excel and GraphPad.

2.5. Statistical analysis

All experimental conditions were carried out as duplicates. To evaluate spheroid size, average area (μm²) of spheroids on day 7 (MDA-MB-231) and day 8 (MCF7) was measured and normalized to the untreated group. Average area from 3 independent experiments were plotted as bar graphs to represent spheroid size. For quantification of spheroid total viability on day 7 (MDA-MB-231) and day 8 (MCF7), the baseline value was subtracted from all readings and
average total viability (arbitrary units) was calculated and normalized to the untreated group. Average total viability from 3 independent experiments were plotted as bar graphs. In all the above-mentioned assays, Two-way ANOVA followed by Dunnett's post hoc test was carried out to evaluate significant differences between the DMSO treated and different experimental conditions. Error bars represent standard error of mean. For comparing size and viability between untreated cell lines, One-way ANOVA followed by Dunnett’s post hoc test was used.

3. Results and discussion

AQPs, especially AQP1, AQP3 and AQP5, are overexpressed in breast cancer [14,45–47]. Thus, we aimed to test if these AQPs affected sensitivity to clinically relevant, breast cancer chemotherapeutics. MCF-7 cells [48,49], which is a model of human luminal A breast cancer, stably overexpressing AQP1-EGFP, AQP3-reduced spheroid size (70% of DMSO treated) and Combination and therapeutics. MCF-7 cells [48,49], which is a model of human affected sensitivity to clinically relevant, breast cancer chemo-

breast cancer [14,45

and viability between untreated cell lines, One-way ANOVA fol-

Error bars represent standard error of mean. For comparing size (Fig. 1A and D-E).

no effect on neither spheroid size, morphology nor total viability between untreated cell lines, One-way ANOVA fol-

(untreated group. Average total viability from 3 independent experiments were plotted as bar graphs. In all the above-mentioned assays, Two-way ANOVA followed by Dunnett's post hoc test was carried out to evaluate significant differences between the DMSO treated and different experimental conditions. Error bars represent standard error of mean. For comparing size and viability between untreated cell lines, One-way ANOVA followed by Dunnett’s post hoc test was used.

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The cells were grown for 2 days as spheroids, followed by treat-

ment with a chemotherapeutic regiment consisting of 18.75 nM Cisplatin, 18.75 nM Doxorubicin, 0.0625 nM 5-FU and a combination of the three. The combination mimics the clinical TNBC-treatment and treatment of spheroids with these drugs have previously been described [43]. Since AQP5 can activate the Ras signaling pathway, we also included the combination with the Ras inhibitor Salirasib [37] at a concentration of 75 μM; as determined in a previous study [22]. Treatments are hereafter referred to as Cisplatin, 5-FU, Doxorubicin, Combination and Combination + Salirasib. All spheroids were analyzed for size and total viability on day 8 (MCF7) or day 7 (MDA-MB-231).

3.1. Overexpression of AQP1, AQP3 and AQP5 in MCF7 spheroids differentially affects sensitivity to chemotherapeutics

AQP overexpressing spheroids: First, we compared spheroid growth of untreated spheroids, which showed that spheroids overexpressing AQP5 were significantly smaller than the wild type MCF7 spheroids (77% of control MCF7), whereas AQP5S156A, AQP1 and AQP3 overexpression did not alter spheroid size (Fig. 1A and B). Next, we measured total viability of spheroids to evaluate the cytotoxic effect of the drugs, which showed that total viability was unchanged for all AQP overexpressing spheroids compared to MCF7 spheroids (Fig. 1A and C). In analysis of collective cell migration, AQP5 mediate cell detachment and dissemination from migrating sheets of both normal epithelial cells [22] as well as of MCF7 cells (manuscript accepted [41]). Specifically, the cell detachment and dissemination was dependent of AQP5 mediated Ras activation. Thus, since total viability was unaltered and AQP5S156A overexpressing spheroids were comparable to control spheroids in respect to size, this suggests that the smaller size of the AQP5 overexpressing spheroids are due to cell detachment and dissemin-

ation from the spheroids.

Since there was an initial difference in size of AQP over-

expressing spheroids, all data were normalized to the untreated control for each cell line to evaluate the effect of drugs on the different spheroid types. The solvent for the drugs, DMSO, had no effect on neither spheroid size, morphology nor total viability (Fig. 1A and D-E).

MCF7 spheroids (Fig. 1A and D-E): Doxorubicin significantly reduced spheroid size (70% of DMSO treated) and Combination and

Combination + Salirasib caused an even larger reduction (23 and 22% of DMSO treated, respectively). In contrast, the individual drugs Cisplatin and 5-FU did not significantly affect spheroid size (105% and 98% of DMSO treated, respectively). Total viability was significantly reduced for Cisplatin and 5-FU (83% and 85% of DMSO treated, respectively) and even more reduced by Combination and Combination + Salirasib (41% and 40% of DMSO treated, respecti-

ve). In contrast, Doxorubicin did not significantly affect total viability (105% of DMSO treated). Thus, Combination and Combination + Salirasib had the biggest effects on total viability and spheroid size.

AQP1 overexpressing MCF7 spheroids (Fig. 1A and D-E): Cisplatin, Doxorubicin, Combination and Combination + Salirasib all significantly reduced spheroid size (37%, 45%, 32% and 27% of DMSO treated, respectively). In contrast, 5-FU did not significantly affect spheroid size (103% of DMSO treated). Total viability was significantly reduced for Cisplatin and Combination (67% and 67% of DMSO treated, respectively) and to a larger degree by Doxorubicin and Combination + Salirasib (57% and 43% of DMSO treated, respectively). In contrast, total viability was significantly increased by 5-FU (120% of DMSO treated).

A previous study in MDA-MB-231 cells grown in 2D found that overexpression of AQP1 increased sensitivity towards Epirubicin [30], which is an epimer of Doxorubicin [50]. Moreover, breast cancer patients with high AQP1 expression responded better to anthracycline treatment than patients with lower AQP1 expression [30]. Cell culture experiments revealed that mechanistically, AQP1 competed with glycogen synthase kinase-3β for binding to β-catenin, thus stabilizing β-catenin, which subsequently induced transcription of Topoisomerase IIα, which increased sensitivity to anthracycline [30]. Our in vitro studies confirm this sensitivity to Doxorubicin and indicate that an even larger response can be ob-

tained by the Combination + Salirasib, indicating a role of the Ras signaling pathway. Interestingly, AQP1 siRNA knockdown reduced Ras signaling in gastric cancer cells (AGS and MKN45), indicating a possible link between AQP1 and Ras activation [51].

AQP3 overexpressing MCF7 spheroids (Fig. 1A and D-E): Doxorubicin, Combination and Combination + Salirasib all significantly reduced spheroid size (44%, 38% and 38% of DMSO treated, respecti-

ve). In contrast, Cisplatin and 5-FU did not significantly affect spheroid size (97% and 96% of DMSO treated, respectively). Total viability was significantly reduced by all the treatments Cisplatin, 5-

FU, Doxorubicin, Combination and Combination + Salirasib (82%, 80%, 65%, 73% and 68.91% of DMSO treated, respectively).

Another study investigated AQP3 silencing in MCF7 cells and found that 48 h after a short (90 min) exposure to 5-FU, cell viability was decreased in the AQP3 silenced group [52], which is in contrast to our data showing that 5-FU treated AQP3 overexpressing spheroids had decreased total viability. A similar result was found in a study with AQP3 silencing in MDA-MB-231 cells, where silencing induced an increase in cell death upon 48 h of 5-FU treatment [19]. Several differences exist between these two studies and this. First, we worked with AQP3 overexpression whereas the other two used AQP3 silencing. The short treatment time and 48 h recovery in the MCF7 study [52] as well as the difference between the two studies being conducted in 2D cell cul-
tures compared to the spheroid setup, which closely recapitulates in vivo conditions, may explain this discrepancy.

In AGS colon cancer cells, AQP3 overexpression decreased cisplatin mediated cell toxicity while silencing of AQP3 in the colon cancer cell lines, MGC803 and SGC7901, increased the cytotoxic action of Cisplatin [32]. Thus, cell lines, model systems and drug doses used seem to influence the drug response.

AQP5 overexpressing MCF7 spheroids (Fig. 1A and D-E): 5-FU caused a small but significant reduction in size (87% of DMSO
Fig. 1. Overexpression of AQPs increases Doxorubicin sensitivity of MCF7 spheroids. Spheroids were treated with Cisplatin (18.75 nM), Doxorubicin (18.75 nM) and 5-FU (0.0625 nM), a combination of drugs and combination of drugs + Salirasib (75 nM) on day 1, day 3, day 6 and day 8. DMSO served as vehicle for the anti-cancer therapy. Images were acquired on above-mentioned days and total viability assays were performed on day 8. Experiments were performed in duplicates and repeated 3 times. (A) Representative images of MCF7 cell lines spheroids on day 8. Scale bar is 100 μm. (B) Bar graph represents average area of untreated spheroids at day 8. Significant values were determined by One-way ANOVA, followed by Dunnett’s post hoc test. *** represents p < 0.0001. Error bars represent standard error of mean. (C) Bar graph represents average total viability of untreated spheroids at day 8. Significant values were determined by One-way ANOVA, followed by Dunnett’s post hoc test. Error bars represent standard error of mean. (D) Bar graph represents average area of spheroids at day 8. Significant values were determined by Two-way ANOVA, followed by Dunnett’s post hoc test. * and **** represent p < 0.05 and 0.0001. Error bars represent standard error of mean. (E) Bar graph represents average total viability of spheroids at day 8. Significant values were determined by Two-way ANOVA, followed by Dunnett’s post hoc test. **** represents p < 0.0001. Error bars represent standard error of mean.
Fig. 2. Overexpression of AQP5 increases sensitivity to Doxorubicin, Combination and Combination + Salirasib of TNBC spheroids. Spheroids were treated with Cisplatin (18.75 nM), Doxorubicin (18.75 nM) and 5-FU (0.0625 nM), a Combination of drugs and Combination of drugs + Salirasib (75 μM) on day 1, day 3, day 5 and day 7. DMSO served as vehicle for the anti-cancer therapy. Images were acquired on above-mentioned days and total viability assays were performed on day 7. Experiments were performed in duplicates and repeated 3 times. (A) Representative images of MDA-MB-231 cell line spheroids on day 7. Scale bar is 100 μm. (B) Bar graph represents average area of untreated spheroids at day 7. Significant values were determined by One-way ANOVA, followed by Dunnett’s post hoc test. Error bars represent standard error of mean. (C) Bar graph represents average total viability of untreated spheroids at day 7. Significant values were determined by One-way ANOVA, followed by Dunnett’s post hoc test. * and ** represent p < 0.05 and 0.001. Error bars represent standard error of mean. (D) Bar graph represents average area of spheroids at day 7. Significant values were determined by Two-way ANOVA, followed by Dunnett’s post hoc test. **** represents p < 0.0001. Error bars represent standard error of mean. (E) Bar graph represents average total viability of spheroids for day 7. Significant values were determined by Two-way ANOVA, followed by Dunnett’s post hoc test. * and **** represent p < 0.05 and 0.0001. Error bars represent standard error of mean.
treated) and Doxorubicin, Combination and Combination + Salirasib caused larger reductions in size (59%, 38% and 32% of DMSO treated, respectively). In contrast, Cisplatin did not significantly affect spheroid size (92% of DMSO treated). Total viability was significantly reduced for Doxorubicin, Combination and Combination + Salirasib (90%, 78% and 45% of DMSO treated, respectively), whereas Cisplatin and 5-FU did not affect total viability (103% and 105% of DMSO treated, respectively). AQP5 and AQP5S156A was unique to MCF7 spheroids, we also tested 3.2. Overexpression of AQP5 in TNBC spheroids increases sensitivity to Doxorubicin, Combination and Combination + Salirasib

To further explore if the sensitivity difference observed between AQP5 and AQP5S156A was unique to MCF7 spheroids, we also tested how overexpression affected drug sensitivity in the invasive TNBC cell line, MDA-MB-231 [49].

AQP overexpressing MDA-MB-231 spheroids: First, we compared spheroid size in the untreated controls. This revealed that AQP5 and AQP5S156A overexpression did not alter spheroid size compared to that of MDA-MB-231 spheroids (78% and 81% of MDA-MB-231). Although AQP5 overexpressing spheroids seemed smaller than control, the difference was not significant, which may be due to the larger variation of AQP5S156A overexpressing spheroids (Fig. 2A and B). In respect to total viability, AQP5 and AQP5S156A overexpressing spheroids had significantly reduced total viability compared to MDA-MB-231 spheroids (73% and 74% of MDA-MB-231) (Fig. 2A and C). This is in contrast to MCF7 cells (Fig. 1A–B), where AQP5 overexpressing spheroids were smaller and AQP5 and AQP5S156A spheroids and unchanged total viability. Thus, the effect on viability seems cell type specific.

Since there was an initial difference in total viability, all data were normalized to the untreated control for each cell line to evaluate the effect of the different drugs. This revealed that the dissolvent for the drugs, DMSO, had no effect on spheroid size or total viability (Fig. 2A and D-E).

MDA-MB-231 spheroids (Fig. 2A and D-E): Doxorubicin, Combination and Combination + Salirasib all significantly reduced spheroid size (56%, 49% and 41.67% of DMSO treated, respectively). In contrast, Cisplatin and 5-FU did not significantly affect spheroid size (106% and 101% of DMSO treated, respectively). This was mirrored by total viability, where Doxorubicin, Combination and Combination + Salirasib all caused reduced total viability (33%, 34% and 28% of DMSO treated, respectively) whereas Cisplatin and 5-FU did not significantly affect total viability (95% and 95% of DMSO treated, respectively).

This is consistent with a study of MDA-MB-231 spheroids where treatment with Combination, reduced spheroid viability [43].

AQP5 overexpressing MDA-MB-231 spheroids (Fig. 2A and D-E): Doxorubicin, Combination and Combination + Salirasib all significantly reduced spheroid size (45%, 39% and 40% of DMSO treated, respectively). In contrast, Cisplatin and 5-FU did not significantly affect spheroid size (100% and 102% of DMSO treated, respectively). This was mirrored by total viability, where Doxorubicin, Combination and Combination + Salirasib all significantly reduced total viability (29%, 21% and 16% of DMSO treated, respectively). In contrast, Cisplatin and 5-FU did not significantly affect total viability (94% and 105% of DMSO treated).

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Data and materials availability
All data and materials used in the analysis are available in some form upon request to any researcher for purposes of reproducing or extending the analysis.
Declarations of competing interest

The authors declare no conflict of interest.

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