Long-term treatment with gadopentetic acid or gadodiamide increases TRPC5 expression and decreases adriamycin nuclear accumulation in breast cancer cells

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Abstract

Gadopentetic acid and gadodiamide are paramagnetic gadolinium-based contrast agents (GBCAs) that are routinely used for dynamic contrast-enhanced magnetic resonance imaging (MRI) to monitor disease progression in cancer patients. However, growing evidence indicates that repeated administration of GBCAs may lead to gadolinium (III) cation accumulation in the cortical bone tissue, skin, basal ganglia, and cerebellum, potentially leading to a subsequent slow long-term discharge of Gd\(^{3+}\). Gd\(^{3+}\) is a known activator of the TRPC5 channel which is implicated in breast cancer resistance to chemotherapy. Here we found that gadopentetic acid (Gd-DTPA, 1 mM) enhanced the inward and outward currents through TRPC5 exogenously expressed in HEK293 cells. Gd-DTPA (1 mM) also activated the Gd\(^{3+}\) sensitive R593A mutant of TRPC5, which exhibits a reduced sensitivity to GPCR-G\(_q/11\)-PLC dependent gating. Conversely, Gd-DTPA had no effect on TRPC5-E543Q, a Gd\(^{3+}\) insensitive TRPC5 mutant. Long-term treatment (28 days) of human breast cancer cells (MCF-7) and adriamycin-resistant MCF-7 cells (MCF-7/ADM) with Gd-DTPA (1 mM) or gadodiamide (GDD, 1 mM) did not affect cell survival in the presence of ADM. However, the treatment with Gd-DTPA or GDD significantly increased TRPC5 expression and decreased the accumulation of ADM in the nuclei of MCF-7 cells, increasing the risk of the breast cancer cell chemoresistance. The antagonist of TRPC5, AC1903 (1 µM), reversed the Gd-DTPA-treatment mediated changes in ADM nuclear accumulation. We propose that clinically, repeated administration of GBCAs should be minimized in breast cancer patients to reduce the risk of drug resistance.

Introduction

Gadolinium-based contrast agents (GBCAs) are clinically utilized during dynamic contrast-enhanced magnetic resonance imaging (MRI) to diagnose and monitor the growth of various tumor and cancer lesions, including metastatic cancers (Li et al., 2021). Although GBCAs are contraindicated in patients with renal failure because of a high risk of developing the nephrogenic systemic fibrosis (NSF) (Cheong et al., 2022, Rah et al., 2018, Zheng et al., 2022), these contrast agents are mostly considered safe and are associated with only rare adverse acute effects, such as: headache, dizziness, and nausea. Therefore, GBCAs are widely used for routine clinical MRIs. However, there have been alarming reports of gadolinium (3+) cation deposition in the cortical bone tissue (Turyanskaya et al., 2020), skin, liver, basal ganglia, and cerebellum (Ramalho et al., 2016) of the patients with normal renal function following repeated administration of GBCAs (Ariyani et al., 2022, Funke et al., 2022) (Nakamura et al., 2022, Richter et al., 2021). Notably, Gd\(^{3+}\) can be detected in the human tissues for years after the initial exposure, and it continues accumulating there with each subsequent GBCA-enhanced MRI scan. Likely, over time, the accumulated Gd\(^{3+}\) may be slowly leaking into the interstitial fluid.

Animal studies revealed that high doses of GBCAs may have a substantial toxicity. For example, one study in the zebra fish model demonstrated that high concentration of GBCAs can cause a significant reduction in sensory hair counts accompanied by increased apoptosis and mitochondrial damage (Rah, Han, 2018). On the other hand, to date, the clinical significance of Gd\(^{3+}\) accumulation in the human
tissues is unclear. No association between Gd\(^{3+}\) accumulation and occurrences of side effects has been conclusively demonstrated (Pasquini et al., 2018, Ramalho, Semelka, 2016), thus far. However, gadavist, one of widely used GBCAs, is reported to cause seizures in human patients, if it is used inappropriately (Lee et al., 2016, Muldoon and Neuwelt, 2015).

Remarkably, low micromolar concentrations of trivalent cations of gadolinium increase the activity of the TRPC5 channel (Chen et al., 2017, Semtner et al., 2007). TRPC5 is predominantly expressed in the nervous system (Strubing et al., 2001). Early studies indicated that TRPC5 activation is implicated in regulating neurite outgrowth and dendritic morphogenesis (Davare et al., 2009, Kumar et al., 2012) and in pilocarpine-induced seizure genesis (Zheng and Phelan, 2014). More recent studies have demonstrated that dysregulation of TRPC5 in cancer cells is highly associated with cancer progression, especially cancer chemoresistance (Cai et al., 2021, He and Ma, 2016, Ma et al., 2014, Zou et al., 2019). Ma et al. analyzed the role of TRPC5 in adriamycin-resistant human breast cancer cells (MCF-7/ADM) and provided evidence that upregulated TRPC5 plays a key role during extracellular vesicle (EV) formation and release. Uniquely, TRPC5 on EVs can be carried intercellularly to non-chemoresistant breast cancer cells, be transferred to the cells, conferring chemoresistance to them due to increased activity of TRPC5 (Ma, Chen, 2014). Follow-up studies revealed that increased TRPC5 activity correlates with chemoresistance to several other chemotherapeutic agents in breast and colorectal cancers in vitro and in vivo (Wang et al., 2018, Zou, Chen, 2019). Thus, it appears that the factors upregulating TRPC5 expression may increase the risk of anticancer drug resistance in cancer patients.

Gd\(^{3+}\) deposits in human tissues following repeated GBCAs application may become the depot of slowly releasing Gd\(^{3+}\) into the blood plasma over time. Escaping Gd\(^{3+}\) may tonically increase the TRPC5 signaling cascade linked to conferring chemoresistance to breast cancer cells. In this study, we determined that Gd-DTPA, a major component of magnevist, a GBCA, can potentiate TRPC5 activity. Additionally, we found that long time treatment (4 weeks) of MCF-7 and MCF-7/ADM cells with Gd-DTPA and gadodiamide upregulated TRPC5 expression and decreased the accumulation of ADM in breast cancer cell nuclei, which was abolished by TRPC5 antagonist, AC1903. Our results suggest that GBCAs may increase the cells ADM resistance likely via targeting TRPC5 channels.

Materials And Methods

Cell culture and transfection

HEK cells were obtained from American Type Culture Collection and cultured in Eagle’s Minimum Essential Medium supplemented with 10% fetal bovine serum. HEK cells were transfected using the Lipofectamine 3000 reagent (Invitrogen, Carlsbad, CA) in accordance with the manufacturer’s instruction. The mouse wild type TRPC5 (NM_009428) and two TRPC5 mutants (TRPC5-E543Q and TRPC5-R593A) were used during this study. The details of mTRPC5 cloning and introduction of mutations are described elsewhere (Chen, Li, 2017, Jung et al., 2003). The transfection mixture contained 4 \(\mu\)g of each channel cDNA and 0.25 \(\mu\)g of the histamine H1 receptor. The transfected cells were cultured for 24–48 hours
before electrophysiological experiments. MCF-7 and MCF-7/ADM cells were purchased from China National Laboratory Cell Resource Sharing Service Platform. MCF-7 human breast cancer cells were cultured in DMEM medium, 10% FBS, 1% Penicillin/Streptomycin, while MCF-7/ADM human breast cancer resistant cells were cultured in DMEM medium, 10% FBS, 1% Penicillin/Streptomycin, 500 ng/mL ADM, 0.01 mg/mL bovine insulin.

**Patch-clamp Electrophysiology**

TRPC5 currents were recorded with an Axopatch 200B amplifier and Digidata 1550A digitizer (Molecular Devices, CA, USA) in the whole-cell patch-clamp mode. Series resistance compensation was set to 50–70%, and the currents were filtered at 3 kHz. Acquisition control and data analyses were performed using the pCLAMP 10 software package. Cells were voltage-clamped at a holding potential of -60 mV, and the voltage ramps from -100 to +100 mV were applied with 2 second intervals. TRPC5 activity was activated either by 10 µM histamine or by a dialysis with 500 µM GTPγS added directly into the pipette solution. Current traces from cells where the leak current exceeded 100 pA and/or the access resistance was greater than 10 MΩ were excluded from analysis. The current densities were calculated by dividing the current amplitude values by the cell capacitance. All of the electrophysiological experiments were performed at room temperature (22–25 ºC).

**Solutions**

The standard external solution contained (in mM): 145 NaCl, 2.5 KCl, 2.0 CaCl₂, 1 MgCl₂, 10 HEPES, and 5.5 glucose (pH 7.2 adjusted with NaOH). The standard pipette solution contained (in mM): 125 CsMeSO₃, 3.77 CaCl₂, 2 MgCl₂, 10 EGTA (100 nM free Ca²⁺), and 10 HEPES (pH 7.2 adjusted with Trisma base). The NMDG⁺ solution contained (in mM): 150 NMDG-Cl, 10 HEPES, and 5.5 glucose (pH 7.2 adjusted with Trisma base). The 150NaCl solution contained (in mM): 150 NaCl, 10 HEPES, 0.5 EGTA and 5.5 glucose (pH 7.2 adjusted with Trisma base). The osmolarity of all solutions was adjusted to 300–305 mOsm with mannitol.

**Cell Counting Kit-8 Assay**

MCF-7 and MCF-7/ADM cells were subdivided into the control group, gadodiamide group (1 mM), Gd-DTPA group (1 mM), and GdCl₃ group (100 µM). After 28 days of treatment, MCF-7 and MCF-7/ADM cells at the logarithmic growth stage were detached from the substrate using the trypsin treatment and then resuspended in the fresh medium. Cell suspension (100 µL, 5×10³ cells) was inoculated in each well of 96-well plates and cultured overnight at 37 ºC in a 5% CO₂ incubator. The blank wells contained the plain DMEM complete medium. For the MCF-7 group, the DMEM complete medium was supplemented with the corresponding concentration of ADM (0.01, 0.03, 0.1, 0.3, 1, 3, 10, 30, 100 µM), while for the MCF-7/ADM group the DMEM complete medium was supplemented with the following concentration of ADM (0.1, 0.3,
1, 3, 10, 30, 100, 300, 1000 µM) and cultured for two days. Cells were then washed twice with PBS, and the fresh DMEM medium containing cell counting kit-8 solution (CCK8; Selleck Chemicals, USA) was added to each well. The absorbance was measured at 450 nm using a Tecan Spark instrument (Mannedorf).

**Immunofluorescence**

The coverslips with MCF-7 or MCF-7/ADM cells were washed with PBS and fixed with 4% PFA. The primary TRPC5 (1C8) mouse monoclonal antibody (#SC-293259, Santa Cruz Biotechnology, USA, 1:100 in PBS) was added to the fixed and permeabilized cells. Cell culture plate was placed horizontally in a wet box at 4°C and incubated overnight. After washing, a PBS diluted Alexa Fluor 488 Donkey Anti-mouse Antibody (Thermo Fisher Scientific, USA, 1:500) or Alexa Fluor 594 Goat Anti-Rabbit Antibody (Immunoway, USA, 1:500) was added to the fixed and permeabilized cells and incubated at room temperature for 1h, then washed with PBS. After drying, anti-fluorescence quenching sealing tablets (containing DAPI; Beyotime, China) were added to seal the samples. After an incubation for 10 min at the room temperature, away from light, the fluorescence was observed using a confocal microscope (Olympus FluoView™ FV300, Japan).

**Quantitative Real-time Polymerase Chain Reaction**

Total RNA was extracted from MCF-7 and MCF-7/ADM cells lysed in the TRIzol reagent (Invitrogen), using an OMEGA kit. High-quality extracted RNA (5 µg/sample) was used for synthesis of single-strand cDNA with a TaKaRa reverse transcription kit. Quantitative real-time PCR was conducted with 45 ng of cDNA using a BIO-RAD real-time quantitative PCR instrument CFX96. Primers used to quantify TRPC5 are shown below: the forward primer was 5'-CCCTTTCCCTGTGTGCTCATCC-3', and the reverse primer was 5'-TGCAGAAATCCTGAGCCAAGT-3'. The GADPH primer pair was as follows, the forward primer was 5'-AACTGCTTAGCACCCCTGGC-3', and the reverse primer was 5'-ATGACCTTGCCACAGGCTT-3'. The thermal cycling protocol consisted of 3 min at 94°C, followed by 40 cycles of 94°C for 30 seconds, 53°C for 30 seconds, and 72°C for 1 minute. The reactions were quantified by selecting the amplification cycle when the PCR product of interest was first detected (threshold cycle, Ct). Each reaction was performed in quadruplicate, and the average Ct value was used in all analyses. To account for variability in total RNA input, expression of each transcript was normalized to GAPDH RNA in the samples.

**The Adm Accumulation Assay**

Confocal microscopy (Olympus FluoView™ FV300, Japan) was used to observe the distribution of ADM in MCF-7 and MCF-7/ADM cells. The cells on coverslips were treated with ADM (1 µM or 10 µM for MCF-7 and MCF7/ADM cells, respectively) for 48 h, and the ADM fluorescence was observed under a confocal microscope. The excitation wavelength was 478 nm, and the emission wavelength was 596 nm. The ratios of nuclear to cytoplasmic ADM autofluorescence intensities were determined and recorded.
Calcium Imaging

After 28 days of treatment with GDD (1 mM) or GdCl₃ (100 µM), the cells were detached using trypsin and resuspended in fresh culture medium. The cell suspension was distributed among wells of a 96-well plate (100 µl, 1×10⁴ cells per well) and cultured in an incubator with 5% CO₂ at 37 °C overnight. The culture medium was discarded on the next day, and the cells were washed twice with HPSS. 50 µL Calcium 6 Assay Kit Loading Buffer was added into each well and incubated for 1 h. The fluorescence changes were monitored using a calcium imaging reader (BioTek Cytation5, USA). Calcium 6 fluorescence was excited at 485 nm and emitted light was collected using a 595 nm band-pass filter.

Drugs

GDD and Gd-DTPA were purchased from Selleck. Magnevist and Gadavist were purchased from Bayer Healthcare Pharmaceutical (Whippany, NJ, USA). GdCl₃, Histamine, AC1903, KN93 and other salts used in the experimental solutions were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Statistical Methods

SigmaPlot 12.5 (Systat software, Inc., California, USA) was used for the statistical analyses. The unpaired t-test was used to determine whether there is a statistically significant difference between two groups. The one-way ANOVA test followed by the Student-Newman-Keuls post hoc all pair-wise multiple comparison test was used to compare the experimental groups when the data sets were normally distributed populations with equal variances. The data sets were considered significantly different if the p value was less than 0.05. The data are presented as mean ± standard error of the mean (SEM).

Results

Gd-DTPA potentiated TRPC5 currents in HEK cells

Figure 1A shows the structural formulas of magnevist and gadavist. They are the most clinically used GBCAs. We first employed the single cell patch-clamp approach to determine the effect of clinical formulations of magnevist and gadavist on TRPC5 currents in HEK cells expressing TRPC5. The formulations contained all excipients, including free unliganded Gd³⁺ chelators. Our data revealed that clinical formulations of magnevist and gadavitst had no effect on TRPC5 currents induced by 1 µM histamine (Fig. 1C and 1D). Since magnevist exhibited a trend to potentiate TRPC5 currents, we next decided to establish whether the major constituent of magnevist, Gd-DTPA, can affect TRPC5 activity. Surprisingly, we found that Gd-DTPA (1 mM) significantly potentiated histamine-induced TRPC5 activity (Fig. 1E). However, its potency was lower compared to that of Gd³⁺ (100 µM, Fig. 1A). Gd-DTPA also significantly potentiated TRPC5 currents elicited by the dialysis of GTPγS (500 µM) through the patch
pipette (at -100 mV: from \(-51.8 \pm 12.3\) to \(-117.1 \pm 16.9\) pA/pF and at +100 mV: from \(80.4 \pm 9.2\) to \(100.4 \pm 11.3\) pA/pF; \(n = 14\); Fig. 1F and 1G).

After identifying that Gd-DTPA increases TRPC5 activity, we next tested the ability of Gd-DTPA to modulate histamine-activated currents through TRPC5-E543Q, a mutant of TRPC5 that can be activated downstream the Gq/11-PLC signaling but is insensitive to Gd\(^{3+}\) (Jung, Muhle, 2003) because it lacks the critical Glu-543 residue in the Gd\(^{3+}\)-binding site of TRPC5. Figure 2B and-C show that Gd-DTPA had no effect on the TRPC5-E543Q currents induced by either histamine or GTP\(\gamma\)S, suggesting that the Gd-DTPA potentiating effect requires a functional Gd\(^{3+}\)-binding site of TRPC5. We next investigated whether Gd-DTPA would affect the TRPC5-R593A mutant that was reported to be less sensitivity to Gq/11-PLC activation in the absence of Gd\(^{3+}\) but could be activated downstream Gq/11-PLC in the presence of Gd\(^{3+}\) (Chen, Li, 2017). We determined that consistently, Gd-DTPA also potentiated TRPC5-R593A currents (Fig. 2D and 2E).

**Effect Of Gbcas On Mcf-7 And Mcf-7 /adm Cell Viability In The Presence Of Adm**

Upregulation of TRPC5 activity has been recently implicated in conferring chemoresistance to breast cancer cells. Herein, we demonstrated that the major component of contrast agent magnevist can potentiate TRPC5 currents. Therefore, we next asked if breast cancer cells incubated with a GBCA would acquire drug-resistance. To determine this, we cultured MCF-7 and adriamycin (ADM) resistant MCF-7/ADM breast cancer cells in the presence of vehicle- (PBS), Gd-DTPA (1 mM), GDD (gadodiamide, 1 mM), or GdCl\(_3\) for 4 weeks, with the GdCl\(_3\) (0.1 mM) treated MCF-7 cells and the vehicle-treated MCF-7 cells serving as controls. We then performed the ADM-resistance assay where MCF-7 cells were treated with 0.01, 0.03, 0.1, 0.3, 1, 3, 10, 30, and 100 \(\mu\)M concentrations of ADM for 48 hours, whereas MCF-7/ADM cells were treated with 0.1, 0.3, 1, 3, 10, 30, 100, 300, and 1000 \(\mu\)M concentrations of ADM for 48 hours. The survival of breast cancer cells decreased in the presence of higher ADM concentrations with the half maximal effective concentration (EC\(_{50}\)) values for ADM of 0.16 \(\pm\) 0.04 \(\mu\)M, 0.42 \(\pm\) 0.17 \(\mu\)M, 0.75 \(\pm\) 0.11 \(\mu\)M, and 0.17 \(\pm\) 0.05 \(\mu\)M in GDD-, Gd-DTPA-, and GdCl\(_3\)-treated MCF-7 cells, respectively, and 20.8 \(\pm\) 3.1 \(\mu\)M, 31.9 \(\pm\) 5.7 \(\mu\)M, 33.4 \(\pm\) 9.9 \(\mu\)M, and 17.1 \(\pm\) 3.1 \(\mu\)M in GDD-, Gd-DTPA-, and GdCl\(_3\)-treated MCF-7/ADM cells, respectively. There was no significant difference in the EC\(_{50}\) values for ADM among the tested MCF-7 groups or MCF-7/ADM groups (Fig. 3A and 3B). However, GDD, Gd-DTPA, and GdCl\(_3\)-treated MCF-7 cells exhibited an increased viability in the presence of 1 \(\mu\)M ADM as compared to control MCF-7 cells (Fig. 3C), and GDD, Gd-DTPA, and GdCl\(_3\)-treated MCF-7/ADM cells exhibited an increased viability in the presence of 10 \(\mu\)M ADM compared to control MCF-7/ADM cells (Fig. 3D).

The nuclear accumulation of ADM was increased in MCF-7 and MCF-7/ADM cells treated with GCBAs for 4 weeks.
To further investigate whether GCBAs can promote drug resistance in breast cancer cells, we measured the accumulation of ADM in the cytosol and nucleoplasm of MCF-7 and MCF-7/ADM cells by detecting ADM autofluorescence. As in the previous experiments, MCF-7 and MCF-7/ADM cells were first treated with GDD (1 mM), Gd-DTPA (1 mM), or GdCl₃ (0.1 µM) for 4 weeks, and then ADM was added into the culture medium for 48 h. Figure 4 shows that the autofluorescence of ADM decreased in the nucleus and increased in the cytoplasm in GCBA-treated MCF-7 cells compared to the control MCF-7 cells. The ratios of nuclear to cytoplasmic fluorescence intensities were 0.53 ± 0.07, 0.31 ± 0.06, 0.31 ± 0.04 and 0.15 ± 0.01 for control, GDD-, Gd-DTPA-, and GdCl₃-treated MCF-7 cells, respectively. Conversely, the amount of ADM accumulation in the nuclei of MCF-7/ADM cells treated with GCBAs or GdCl₃ was not different from that in the control MCF-7/ADM cells (Fig. 4B and 4D). Notably, the ratio of nuclear to cytoplasmic fluorescence intensities was significantly smaller in MCF-7/ADM cells as compared to MCF-7 cells (0.11 ± 0.02, 0.1 ± 0.02, 0.11 ± 0.01 and 0.1 ± 0.01 for control, GDD, Gd-DTPA, and GdCl₃ treated MCF-7/ADM cells).

We next performed immunofluorescence experiments and used quantitative real time polymerase chain reaction (qRT-PCR) to test the effect of 4-week treatment with GCBAs on the expression of TRPC5 in MCF-7 cells. Figure 5A and B show that the immunofluorescence intensities were significantly greater by 1.7, 1.3, and 2.1 folds in GDD, Gd-DTPA, and GdCl₃-treated MCF-7 cells probed with the anti-TRPC5 antibody as compared to the vehicle-treated MCF-7 cells. Subsequent qRT-PCR quantification further confirmed that the expression of TRPC5 was significantly up-regulated in MCF-7 cells treated with GDD, Gd-DTPA, and GdCl₃ (Fig. 5C). The immunofluorescence intensity of TRPC5 was also significantly increased by 1.2 and 1.8 times in GDD and GdCl₃ treated MCF-7/ADM cells compared to the vehicle-treated MCF-7/ADM cells (Fig. S1).

**Long-term treatment with GDD decreased intracellular calcium transients induced by histamine and bradykinin in MCF-7 cells.**

We next set out to determine whether elevated TRPC5 expression correlates with increased TRPC5 activity in MCF-7 cells treated with GDD or GdCl₃ for 4 weeks. TRPC5 can be activated by ligands of membrane receptors coupled to G₉/₁₁-Phospholipase C, and MCF-7 cells express two of such receptors: the histamine and bradykinin receptors. Therefore, we measured histamine and bradykinin-elicted [Ca²⁺]ᵢ changes in GDD-, GdCl₃-, and vehicle-treated MCF-7 cells. Unexpectedly, we found that histamine and bradykinin-induced [Ca²⁺]ᵢ rises were smaller in GDD-treated MCF-7 cells compared to vehicle-treated MCF-7 cells (Fig. 6).

**Gd-DTPA-dependent up-regulation of TRPC5 expression in MCF-7 cells reduces nuclear ADM accumulation, whereas downregulation of TRPC5 activity decreases chemoresistance in Gd-DTPA-treated MCF-7 cells**
TRPC5 expression can be controlled through the CaMKII-CREB signaling pathway (He et al., 2012). Therefore, we next investigated whether treatment with CaMKII inhibitor, KN93, would prevent Gd-DTPA-induced nuclear ADM accumulation. MCF-7 and MCF-7/ADM cells were treated with Gd-DTPA (1 mM) alone or with a combination of Gd-DTPA (1 mM) and KN93 (1 µM). Cell survival rates were then determined using the CCK8 assay. During the ADM survival assay, MCF-7 cells were treated with the following concentrations of ADM, 0.01, 0.03, 0.1, 0.3, 1, 3, 10, 30, 100 µM, whereas MCF-7/ADM cells were treated with a higher concentration gradient of ADM, 0.1, 0.3, 1, 3, 10, 30, 100, 300, 1000 µM. ADM treatments lasted for 48 h. We found that the EC₅₀ values for ADM in MCF-7 cells were 0.33 ± 0.05, 0.74 ± 0.11, 0.48 ± 0.37 µM in control, Gd-DTPA, Gd-DTPA + KN93-treated MCF-7 cells, and the EC₅₀ values for ADM were 20.8 ± 3.1, 33.4 ± 9.9, 22.5 ± 9.4 µM in control, Gd-DTPA, Gd-DTPA + KN93-treated MCF-7 cells (Fig. 7A and 7B), respectively. These EC₅₀ values for ADM were not significantly different between the Gd-DTPA and Gd-DTPA + KN93 groups in MCF-7 and MCF-7/ADM cells. KN93 treatment did not significantly improve the survival rate of MCF-7 and MCF-7/ADM cells (Fig. 7C and 7D).

We next assessed the accumulation of ADM in nuclei of MCF-7 cells and quantified whether KN93 treatment affects nuclear ADM accumulation. MCF-7 cells were treated with Gd-DTPA in the absence or presence of KN93 for 4 weeks. Then, 1 µM ADM was added to detect ADM accumulation in the nuclei of MCF-7 cells. Some of the MCF-7 cells were also treated with 1 µM AC1903, a specific selective inhibitor of TRPC5, for 10 minutes before the application of ADM. Compared with the control group, the ratios of nuclear to cytoplasmic autofluorescence intensities were significantly decreased in the Gd-DTPA treatment group and the effect was reversed by KN93 or AC1903 treatment (Fig. 8A and 8B). Thus, AC1903 and KN93 increase ADM accumulation into the nucleus of MCF-7 cells promoting its anti-cancer activity.

**Discussion**

In this study, we tested the hypothesis whether long-term treatment with GBCAs may increase ADM resistance of breast cancer MCF-7 cells by upregulating TRPC5 expression. Upregulated TRPC5 expression in MCF-7 cells has been linked to increased chemotherapy resistance of the cells (Ma et al., 2012, Ma, Chen, 2014). We indeed found that 28 day-treatment with GDD or Gd-DTPA, two of the most clinically used GBCAs, significantly increased TRPC5 expression in MCF-7 cells. Long-term treatment with GBCAs also reduced ADM (Adriamycin; also known as Doxorubicin) accumulation in the nuclei of MCF-7 cells. ADM exhibits its anti-cancer effects in part because it can intercalate into cancer cell DNA and can disrupt topoisomerase-II-dependent DNA repair (Thorn et al., 2011). Although ADM does have cytosolic anticancer effects, ADM should accumulate into the nuclei of cancer cells to slow down their proliferation, and a reduced nuclear ADM accumulation may lead to a decreased anticancer efficacy of ADM.

TRPC5 channels have many critical physiological roles in the human body, especially the channels are highly expressed in the nervous system (Chen et al., 2020). Recently, the role of TRPC5 in breast cancer cell resistance to chemotherapy has been discovered (Ma, Chen, 2014, Santoni et al., 2020, Singh et al.,...
Several research groups demonstrated that polyvalent cations, such as La\(^{3+}\) and Gd\(^{3+}\), can markedly enhance TRPC5 activity (Chen, Li, 2017; Semtner, Schaefer, 2007). Gd\(^{3+}\) is not a usual constituent of the human extracellular fluids or blood plasma. However, there is a possibility that the escape of Gd\(^{3+}\) from its chelating cages of the clinically used formulations utilized for contrast enhanced MRI would modulate TRPC5 activity in breast cancer cells to promote breast cancer cell chemoresistance.

GBCAs are routinely used for multiple repeated dynamic MRIs to monitor disease progression in cancer patients. Here, we found that Gd-DTPA significantly potentiated TRPC5 currents (Fig. 1). However, magnevist and gadavist had no effect on TRPC5 current. It is possible that Gd-DTPA potentiated TRPC5 currents due to the spontaneous release of Gd\(^{3+}\) from its chelating complexes. Indeed, clinical magnevist and gadavist formulations include additional unliganded chelators to reduce the harmful effect of Gd\(^{3+}\) on the human body. However, after the MRI scans the chelators will be filtered by the kidney, whereas Gd\(^{3+}\) may accumulate in several tissues. According to “Bayer,” 1 mL of magnevist contains: 0.5 mmol Gd-DTPA, 5 micromole meglumine, and 1 micromole of pentetic acid, an analog of EDTA that chelates Gd\(^{3+}\). A 70 kg patient usually receives 14 ml of the solution or 7 mmol of Gd-DTPA. It means that the patient will have a highest final concentration of about 2.5 mM/L of magnevist in the blood plasma during each MRI scan. We used 1 mM concentration of Gd-DTPA that is lower than the peak concentration of the GBCA in the blood plasma. Gadavist (gadobutrol) excipients also include: Tris (Hydroxymethyl)aminomethane-HCl and calcobutrol sodium, which is another Gd\(^{3+}\) chelator. Interestingly, magnevist is formulated so that it is not supposed to cross the blood brain barrier, but it appears that it does, since it accumulates in the nervous tissue is reported even in subjects with normal kidney function (Ringler et al., 2021).

Our mutagenesis experiments involving the Gd\(^{3+}\) binding site of TRPC5 demonstrated that Gd-DTPA modulates TRPC5 activity via the same Gd\(^{3+}\)-binding site on TRPC5 as free Gd\(^{3+}\) does (Fig. 2). Considering the role of TRPC5 in breast cancer chemoresistance (Ma, Cai, 2012) and the repeated use of Gd-DTPA in cancer patient, we investigated the effect of long-term Gd-DTPA or GDD treatment on the expression of TRPC5 in MCF-7 and MCF-7/ADM. Although the treatment of Gd-DTPA or GDD did not alter the ADM EC\(_{50}\) values in both MCF-7 and MCF-7/ADM, Gd-DTPA or GDD significantly improved MCF-7 or MCF-7/ADM cell survival in the presence of ADM comparing to the vehicle control (Fig. 3). Remarkably, four-week treatment with Gd-DTPA or GDD significantly increased TRPC5 expression in the breast cancer cell line (Fig. 4).

We noticed that TRPC5 proteins were localized to the cytosol of some Gd-DTPA or GDD-treated cells rather than to the plasma membrane as may be expected (Fig. 5A). It was indeed reported that TRPC5 may accumulate in small vesicles held in reserve near the plasma membrane to be later inserted to the plasma membrane in a growth factor-PI\(_3\)K-Rac1-PIP\(_5\)Kalpha dependent manner (Bezzerides et al., 2004). Such unique cellular distribution of TRPC5 may explain our unexpected findings. Consistently, our functional test revealed that receptor-operated Ca\(^{2+}\) signaling was downregulated in GDD treated cells (Fig. 6). It is possible that the observed reduction in receptor-operated Ca\(^{2+}\) signaling, an indirect measure
of TRPC5 activity in breast cancer cells, represents a negative feedback mechanism geared towards reducing chronic Gd$^{3+}$-dependent stimulation of TRPC5 activity. The activation of TRPC5 is confirmed by the positive effect of TRPC5 inhibitor, AC1903, treatment that reduced ADM accumulation in the nuclei of MCF-7 cells. Notably, upregulation of TRPC5 protein expression is crucial for P-gp induction and the development of chemoresistance in breast cancer cells (Ma, Cai, 2012, Zhang et al., 2017).

ADM exhibits a natural red autofluorescence and its beneficial antitumor effect depends on the drug delivery to the nucleus (Hittelman and Rao, 1975). Altered accumulation of ADM in MCF-7 and MCF-7/ADM following the treatment with Gd-DTPA or GDD was detected by measuring its autofluorescence (Fig. 5). As expected, ADM mainly accumulated inside the nuclei in control MCF-7/WT cells but was redistributed to the cytoplasm in MCF-7 treated with Gd-DTPA or GDD. In MCF-7/ADM, the ADM resistant cell line, ADM accumulation was reduced, with the residual drug accumulation being mostly detected in the cytoplasm, and Gd-DTPA or GDD having no effect on ADM accumulation (Fig. S2). Thus, our data suggest that Gd-DTPA or GDD may upregulate TRPC5 expression in MCF-7 cell and decrease ADM accumulation in the nuclei of MCF-7 cells.

Many studies revealed that repeated administration of GBCAs may lead to gadolinium (III) accumulation in various human tissues. However, the association between gadolinium (III) accumulation and occurrences of side effects and toxic damage has not been conclusively demonstrated. Our findings reveal a possible adverse effect of the excessive administration of GBCAs for monitoring breast cancer progression. It may be worthwhile to minimize repeated administration of GBCAs in breast cancer patients to reduce the potential of promoting chemotherapy resistance in the patients.

**Declarations**

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**Ethical Approval:** not applicable.

**Availability of data and materials:** not applicable.

**References**


**Figures**

**Figure 1**

Effects of GBCAs on histamine or GTPγS-induced currents in TRPC5 expressing HEK cells. **A** Molecular structures of magnevist (Gd-DTPA) and gadavist. **B-E** Sample traces of TRPC5 currents induced by histamine (His, 10 µM). **F** Sample trace of TRPC5 inward (blue) and outward (red) currents induced by dialysis of GTPγS (500 µM) via the patch pipette. Gd³⁺ (100 µM), Gd-DTPA (1 mM), magnevist (1 mM) and gadavist (1 mM) were added at the times indicated by the horizontal bars. The dotted lines indicated
the zero current. The upper and lower traces represented the outward and inward whole cell current recorded at +100 mV and -100 mV, respectively. Inserts show the current-voltage relations acquired during the voltage ramps from -100 mV to +100 mV in the absence (solid lines) and presence (broken lines) of GBCAs or Gd$^{3+}$ at the time points indicated with “a” and “b” in the same experiment. G Comparison of the mean current densities of GTP$\gamma$S-activated currents measured at the time points of “a” and “b” at the holding potentials of -100 mV and +100 mV in (F). ***P < 0.001.

**Figure 2**

Effects of Gd-DTPA on histamine or GTP$\gamma$S-induced currents in TRPC5-E543Q and TRPC5-R593A expressing HEK cells. A-D Sample traces of time courses for currents via TRPC5-E543Q or TRPC5-R593A. Histamine (10 µM), Gd$^{3+}$ (100 µM), and Gd-DTPA (1 mM) were added at the times indicated by the horizontal bars. The dotted lines indicate the level of the zero current. The upper traces represent the outward whole cell currents recorded at +100 mV, whereas the lower traces represent the inward currents recorded at -100 mV. Insets show the current-voltage relationships acquired during the voltage ramps from -100 mV to +100 mV in the absence (solid lines) and presence (broken lines) of Gd$^{3+}$ or Gd-DTPA in the same experiment. E Comparison of the ratios of current densities measured at the time points of “a” and “b” at the holding potentials of -100 mV (C and D). *P < 0.05.
Figure 3

MCF-7 and MCF/ADM cell survival in the presence of ADM. A and B Cell viability-concentration curves were used to determine the half-maximal effective concentration (EC$_{50}$) values for ADM in MCF-7 and MCF-7/ADM cells treated with GDD, Gd-DTPA and GdCl$_3$ for 4 weeks. C and D Relative cell survival of MCF-7 and MCF-7/ADM cells treated with 1 µM ADM for 48 h. The survival rates were analyzed using the cell counting kit-8 assay (n=6). The one-way ANOVA test followed by the Student-Newman-Keuls post hoc all pair-wise multiple comparison test was used to compare the data in the treatment groups with the control group. All values are mean ± SEMs. NS, no significant difference. *P < 0.05, **P < 0.01.
Figure 4

The accumulation of ADM in MCF-7 and MCF-7/ADM cells treated with GDD, Gd-DTPA, or GdCl₃ for 4 weeks. A and B Confocal fluorescence images of MCF-7 and MCF-7/ADM cells (ADM autofluorescence – red; DAPI nuclear stain - blue). C and D Summary data of ADM accumulation in MCF-7 and MCF-7/ADM cells (n=4). One-way ANOVA test followed by the Student-Newman-Keuls post hoc all pair-wise multiple comparison test was used to compare the data sets to the control group. All values are mean ± SEMs. *P < 0.05, **P < 0.01. NS, no significant difference. Scale bars: 30 µm
Figure 5

TRPC5 expression was greater in MCF-7 treated with GDD, Gd-DTPA, and GdCl$_3$ for 4 weeks. A and B Representative confocal fluorescence images of TRPC5 protein immunostaining and summary data in MCF-7 cells (TRPC5, green fluorescence, n=6). C RT-PCR results for TRPC5 expression in MCF-7 cells (n=5). The One-way ANOVA test followed by the Student-Newman-Keuls post hoc all pair-wise multiple comparison test was used to compare the treatment groups with the control group. All values are mean ±SEMs. *P < 0.05 **P < 0.01. Scale bars indicates 30 µm.
Figure 6

Histamine and Bradykinin-induced intracellular calcium increases are smaller in MCF-7 cells long-term treated with GDD (1 mM) or GdCl₃ (0.1 mM) compared to control MCF-7 cells. A and B Averaged traces show fluorescence intensity ratio changes in MCF-7 cells. The blue arrows indicate the time when histamine or bradykinin is added to the bath. C and D Summary data comparing the peak fluorescence intensity ratio values shown in (A and B), n=4 independent experiments. The One-way ANOVA test followed by the Student-Newman-Keuls post hoc all pair-wise multiple comparison test was used to compare the data sets. All values are mean ± SEMs. *P < 0.05, **P < 0.01. NS, no significant difference.
Figure 7

MCF-7 and MCF-7/ADM cell survival in the presence of ADM. A and B Response-concentration curves were used to determine the half-maximal effective concentration (EC$_{50}$) values for ADM in MCF-7 and MCF-7/ADM cells treated with Gd-DTPA, Gd-DTPA and KN93 for 4 weeks. C and D Relative cell survival of MCF-7 and MCF-7/ADM cells treated with 1 µM and 10 µM ADM for 48 h. The survival rates were analyzed by the cell counting kit-8 (CCK-8) assay (n=6). The one-way ANOVA test followed by the Student-Newman-Keuls post hoc all pair-wise multiple comparison test was used to compare the treatment groups with the control group. All values are mean ± SEMs. NS, no significant difference. *$P < 0.05$, **$P < 0.01$. 

Figure 8

Inhibitors of TRPC5 and CaMKII decrease the efflux of ADM from MCF-7 cell nuclei. A The nuclear ADM accumulation assay. Shown are the representative confocal micrographs of MCF-7 cells treated with Gd-DTPA alone or in combination with AC1903 or KN93 and in the presence of 1 µM ADM (autofluorescence - red fluorescence). B Summary data for nuclear ADM accumulation in MCF-7 cells (n=4). The One-way ANOVA test followed by the Student-Newman-Keuls post hoc all pair-wise multiple comparison test was used to compare the treatment groups with the control group. All values are mean ± SEMs. *P < 0.05 **P < 0.01. NS, no significant difference. Scale bars indicate 30 µm.

Supplementary Files

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