The gold compounds auranofin (AUR) and gold sodium thiomalate (GST) are disease-modifying antirheumatic drugs (DMARDs). AUR and GST were widely used for the treatment of rheumatoid arthritis (RA) until low-dose methotrexate (MTX) was introduced as a treatment option. Both AUR and GST are still used for patients with RA who do not respond to MTX (21). Moreover, gold compounds have been recently used as a family of promising experimental agents for cancer treatment because they have potent antiproliferative actions (8, 23). The precise mechanisms through which gold compounds produce their antitumor effects are still unknown. However, one mechanism may involve the ability of gold compounds to potently inhibit thioredoxin reductase (TrxR) (8, 11, 33).

Stimulation of human TRPA1 channels by clinical concentrations of the antirheumatic drug auranofin

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Hatano N, Suzuki H, Muraki Y, Muraki K. Stimulation of human TRPA1 channels by clinical concentrations of the antirheumatic drug auranofin. Am J Physiol Cell Physiol 304: C354–C361, 2013. First published December 5, 2012; doi:10.1152/ajpcell.00096.2012.—Gold compounds, which are widely used to treat rheumatoid arthritis, have been recently used as experimental agents for tumor treatment. Transient receptor potential (TRP) ankyrin repeat 1 (TRPA1) is a Ca2+-permeable ion channel that senses acute and inflammatory pain signals. Electrophilic compounds such as mustard oil and cinnamaldehyde activate TRPA1 by interacting with TRPA1 cysteine residues. Here we investigate the effects of the gold compound auranofin (AUR) on TRPA1 channels. Intracellular Ca2+ and whole cell patch-clamp recordings were performed on human embryonic kidney cells transiently expressed with TRPA1, TRP melastatin 8 (TRPM8), and vanilloid type TRP (TRPV1–4) channels. AUR stimulated TRPA1 in a concentration-dependent manner with a half-maximum potency of around 1.0 μM. The AUR-induced response was effectively blocked by HC030031, a TRPA1 antagonist. On the other hand, AUR failed to activate TRPM8 and TRPV1–4 channels, which are highly expressed in sensory neurons as nociceptors. The stimulatory effect on TRPA1 channels depended on the C414, C421, C621, and C633 cysteine residues and not on the inhibition of thioredoxin reductase by AUR. Moreover, AUR effectively activated TRPA1 channels expressed in human differentiated neuroblastoma cell lines. The study shows that AUR is a potent stimulator of TRPA1 channels.

Ca channel; transient receptor potential; gold compounds

THE GOLD COMPOUNDS auranofin (AUR) and gold sodium thiomalate (GST) are disease-modifying antirheumatic drugs (DMARDs). AUR and GST were widely used for the treatment of rheumatoid arthritis (RA) until low-dose methotrexate (MTX) was introduced as a treatment option. Both AUR and GST are still used for patients with RA who do not respond to MTX (21). Moreover, gold compounds have been recently used as a family of promising experimental agents for cancer treatment because they have potent antiproliferative actions (8, 23). The precise mechanisms through which gold compounds produce their antitumor effects are still unknown. However, one mechanism may involve the ability of gold compounds to potently inhibit thioredoxin reductase (TrxR) (8, 11, 33).

Transient receptor potential (TRP) ankyrin repeat 1 (TRPA1) is a Ca2+-permeable ion channel that senses acute and inflammatory pain signals as a nociceptor (4, 22, 32). TRPA1 is activated by environmental irritants and oxidative- and thiol-reactive compounds, some of which are endogenously produced under oxidative stress conditions (19, 25, 36). In particular, mutagenesis studies have identified that cysteine 414, cysteine 421, and cysteine 621 of human TRPA1 are targeted by electrophilic compounds such as mustard oil (MO) and cinnamaldehyde (CA) to activate TRPA1 (14, 25). On the other hand, nonelectrophilic compounds like Δ9-tetrahydrocannabinol, nicotine, and menthol (Men) activate TRPA1 via unknown mechanisms (19, 20, 38). Moreover, clinical drugs such as acetaminophen, dihydropyridines, and general anesthetics stimulate TRPA1, which may account for the clinical and adverse actions of these drugs (1, 10, 28, 30). Also, the antitumor drugs cisplatin (CIS) and oxaliplatin (OXA) activate TRPA1 via the generation of reactive oxygen species, which in turn may lead to peripheral neuropathy (PN) (29). It is therefore noteworthy to investigate the effects of clinical drugs and experimental agents on the human TRPA1 channel.

Here we report that AUR activates only human TRPA1 but not five other human TRP channels (TRPM8 and TRPV1–4) that are predominantly expressed in sensory neurons as putative nociceptors.

MATERIALS AND METHODS

Cell culture, plasmids, mutagenesis, and transfection. Human embryonic kidney cell lines (HEK cells) and IMR-32 human neuroblastoma cells (IMR-32 cells) were obtained from the Health Science Research Resources Bank and were maintained in Dulbecco’s modified minimum essential medium (Sigma) supplemented with 10% heat-inactivated FCS (JRS Biosciences), penicillin G (100 U/ml), and streptomycin (100 μg/ml). Partially confluent HEK cells were transfected with pcDNA3.1/neo(+)–human TRPA1 plasmid DNA, pcDNA3.1/neo(+)–human TRPM8 plasmid DNA, and pcDNA3.1/neo(+)–human TRPV1–4 plasmid DNAs using the calcium phosphate-DNA coprecipitation method. The TRPA1 mutations were constructed by PCR using mutant oligonucleotide primers in which cysteine residues at 414, 421, 621, 633, and 641 were changed to serine (Agilent Technologies), and all constructs were verified by sequencing. All experiments were performed within 48 h of transfection. To identify transfected cells, we applied 30 μM MO before finishing recording of Ca2+ concentration and membrane currents. We discarded cells without response to MO. When differentiated to neuronal cells (hIMR-32), IMR-32 human neuroblastoma (IMR-32) cells were treated with 5 μM 5-bromo-2′-deoxyuridine (BrdU) and were then cultured for 12 days in BrdU-containing medium that was exchanged every 2 days (24).

Whole cell patch-clamp experiments. Whole cell patch-clamp experiments were performed as described previously (18). The resistance of electrodes was 3–5 MΩ when filled with the pipette solution [in mM: 110 Cs-aspartate, 30 CsCl, 1 MgCl2, 10 HEPES, 1 EGTA, 2 Na2ATP (pH 7.2 by CsOH)]. Membrane currents and voltage signals were digitized onto a computer using an analog-digital converter (PCI6229, National Instruments Japan). Data acquisition and analysis of whole cell currents were performed using WinWCP3.7, developed by Dr. John Dempster (University of Strathclyde, UK). The liquid junction potential between the pipette and bath solutions (~10 mV) was corrected. A ramp voltage protocol from −150 mV to +50 mV of 400 ms was applied every 5 s from a holding potential of −50 mV. A leak current component was not subtracted from the recorded...
currents. A standard HEPE S-buffered bathing solution (HEPE S solution) was used with the following composition (in mM): 137 NaCl, 5.9 KCl, 2.2 CaCl₂, 1.2 MgCl₂, 14 glucose, 10 HEPE S (pH 7.4 by NaOH). All experiments were performed at 25 ± 1°C.

**Measurement of the Ca²⁺ fluorescence ratio.** HEK cells and IMR-32 cells were loaded with 5 μM Fura-2 acetoxyethyl ester (Fura-2) in the HEPE S solution for 30 min at room temperature. After cells were superfused with the HEPE S solution for 10 min, each drug was applied to the bathing solution. Fura-2 fluorescence signals were measured using the Argus/HisCa imaging system (Hamamatsu Photo nics, Hamamatsu) driven by Imagework Bench (version 6.0, Indec). The frequency of image acquisition was selected as 0.2 Hz. Efficacy of gene transfection was similar but not identical from cell to cell. To reduce the variety, we collected 30–60 single cells from one coverslip for analysis and repeated the same experiment to use separate coverslips. In each analysis, a whole cell area was chosen as a region of interest to average fluorescence ratio of the area. The ratios of fluorescence intensity were transformed into Ca²⁺ concentration (Ca²⁺) using the following equation (12):

\[
\text{Ca}^{2+} = \frac{224 \times B \times \left( R - R_{\text{min}} \right)}{\left( R_{\text{max}} - R \right)}
\]

where R is the ratio of 340/380 nm; R_max and R_min are fluorescence intensity determined by addition of 1 mM EGTA and 2.2 mM Ca²⁺, respectively, after the permeabilization of cells with 10 μM ionomycin; and B is the ratio of the fluorescence proportionality coefficients obtained at 380 nm under R_min and R_max conditions. In the Fura-2 experiments, the number of independent experiments performed is shown as the n number and the total number of cells in parentheses.

**Quantitative PCR.** Real-time quantitative PCR was performed with the use of SYBR Green Chemistry on a Thermal Cycler Dice Real Time System (Takara Bio) as described previously (18). Transcrip tional quantification of gene products was normalized to β-actin. Each cDNA sample was tested in triplicate. After an initial 30-s activation period of Ex Taq DNA polymerase at 95°C, the program used for quantitative PCR amplification included a 15-s denaturation step at 95°C and a 60-s annealing and extension step at 60°C (for 45 cycles), followed by a final dissociation step (15 s at 95°C, 30 s at 60°C, and 15 s at 95°C). The oligonucleotide sequences of primers specific for human TRPA1 and β-actin (sense and antisense: 5' to 3') were TGCATGTGTCATTTTCCAGAAG and TTGAGGGCTGTAAGCAGAG, and ACCGACCGCGGTCA and CAACCCGTGGCATCTTTT, respectively.

**Western blot analysis.** To isolate wild and mutant TRPA1 protein, HEK cells were lysed in 50 μl lysis buffer [in mM: 50 Tris·HCl (pH 8.0), 150 NaCl, 5 EDTA, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, and protease inhibitors; (13)] in each 6-well plate. The cell lysates were incubated on ice for 30 min with vortexing every 5 min and then centrifuged at 20,000 g for 30 min at 4°C. Each lysate (50 μg protein) was separated on a 8% polyacrylamide gel. Proteins were then transferred to a PVDF membrane and blocked for 2 h in Tris-buffered saline (TBS) containing 5% skim milk and 0.1% Tween 20. The PVDF membrane was incubated with the first antibody of TRPA1 (host: goat, 1:1,000 dilution, Santa Cruz Biotechnology) for overnight at 4°C. The blot was washed three times with washing buffer (TBS containing 0.1% Tween 20) and then secondary antibody (goat-HRP, 1:10,000 dilution) was added to the PVDF membrane. Blots were washed again, and detection reagents (Millipore) were added to generate a chemiluminescence product. To determine the relative quantities of TRPA1 protein against β-actin protein in each sample, the PVDF membrane was also exposed to β-actin monoclonal antibody (host: mouse, 1:10,000 dilution, Sigma). Gels were scanned with LAS-3000mini (Fujiﬁlm, Japan).

**Chemicals and reagents.** The following drugs were used: MO (allyl isothiocyanate, Kanto Chemical), 15-deoxy-Δ prostaglandin J2 (PGJ2, Enzo Life Sciences), ZnSO₄, (Zn²⁺, Wako), HCO3003 (HC, Enzo Life Sciences), MTX (Wako), d-penicillamine (Pen, Sigma), salazosulphapyridine (SAL, Sigma), AUR (Enzo Life Sciences), GST (Sigma), actarit (ACT, Santa Cruz Biotechnology), mizoribine (MIZ, Sigma), cyclophosphamide (CPP, Enzo Life Sciences), hydroxychloroquine (HCQ, Toronto Research Chemicals), CBS (Wako), capsaicin (Cap, Sigma), cannabi diol (CBD, Sigma), 2-aminoethoxydiphenyl borate (2-APB, Sigma), and mizoribine (MIZ, Sigma). Each drug was dissolved in the vehicle recommended by the manufacturer.

**Data analysis.** The data are expressed as means ± SE. The statistical difference between two or multiple groups was examined using Student’s t-tests or Dunnett’s and Tukey’s multiple-comparison tests, respectively. Statistical significance at P < 0.05 and P < 0.01 is indicated in the figures.

**RESULTS**

**Auranofin stimulates TRPA1 channels.** TRPA1 expression in HEK cells transfected with TRPA1 plasmid (HEK-TRPA1 cells) was conﬁrmed by three assays: TRPA1 mRNA transcript expression, TRPA1 protein expression, and TRPA1 channel function. HEK-TRPA1 cells had expression of TRPA1 at the mRNA (Fig. 1A) and protein level (Fig. 1B). Moreover, as shown in Fig. 1C, application of MO (10 μM), a TRPA1 agonist, induced elevation of intracellular Ca²⁺ concentration (Ca²⁺) in HEK-TRPA1 cells but not in HEK cells (HEK) and HEK cells transfected with a control plasmid (vector only, HEK-vector). Using these HEK-TRPA1 cells, we examined the effects of nine DMARDs on TRPA1. According to the literature (6, 7, 9, 11, 16, 17, 26, 34, 37, 40), DMARDs except AUR and CPP were also applied at concentrations of 3 and 30 μM. AUR and CPP were also applied at relatively lower concentrations (1 and 10 μM). As shown in the representative time series plots (Fig. 1D), eight DMARDs failed to induce a Ca²⁺ response of HEK-TRPA1 cells, but application of 1 and 10 μM AUR substantially induced the response (Fig. 1E). For comparison, we also examined the effects of AUR on other types of human TRP channels expressed in HEK cells. Here, we chose TRPV1–4 and TRPM8 because these TRPs are functionally important for nociception in sensory neurons. To show the presence of the channels, we ﬁrst used Cap (1 μM) as a TRPV1 agonist, CBD (30 μM) for TRPV2, 2-APB (30 μM) for TRPV3, GSK (3 nM) for TRPV4, and Men (10 μM) for TRPM8 to evoke TRPV1, TRPV2, TRPV3, TRPV4, and TRPM8 activity in Ca²⁺ measurement assays (Fig. 1F). However, in HEK cells transfected successfully with TRPV1, TRPV2, TRPV3, TRPV4, or TRPM8, AUR at 1 and 10 μM failed to evoke a Ca²⁺ response (Fig. 1F). These data show that AUR selectively activates only the TRPA1 channels among six TRPs.

In Fig. 2, the characteristics of the AUR-evoked response were pharmacologically and electrophysiologically proﬁled. After construction of a concentration-response curve of Ca²⁺ measurements, the EC₅₀ of AUR needed to activate TRPA1 channels was ~1 μM (Fig. 2A). In contrast, as much as 10 μM AUR failed to evoke a Ca²⁺ response in control HEK cells (open circles, Fig. 2A). It has been reported that concentration of AUR in plasma in patients is ~20 μM (11). In the following experiments, we used 3 μM AUR as a typical concentration, which causes a maximum activation of TRPA1 and is within the therapeutic concentration. To further conﬁrm that AUR activates Ca²⁺-permeable TRPA1, we applied 3 μM AUR to HEK-TRPA1 cells with or without 30 μM HCO30031 (HC), a TRPA1 antagonist. The treatment signiﬁcantly inhibited the Ca²⁺ response induced by 3 μM AUR (Fig. 2, B and C). In the
whole cell recording mode, application of 3 μM AUR to HEK-TRPA1 cells induced inward and outward currents at −100 and +40 mV, respectively (Fig. 2, D–F). The evoked currents had a similar current-voltage (I-V) relationship to that induced by a typical TRPA1 agonist, MO [Fig. 2E; (13)]. After withdrawal of AUR, these evoked currents were abolished (Fig. 2, D–F). These data suggest that out of the nine DMARDs tested, only AUR is a potent stimulator of TRPA1.

Activation mechanism of TRPA1 channels by auranofin.

The conservative mutations of cysteine residues to serine (C414S, C421S, C621S, C633S, and C641S) in the predicted NH2-terminal ankyrin repeat heptad repeats have been shown to change the activation of human TRPA1 channels by selective agonists (14, 25). These results were confirmed in our studies in HEK cells transfected with wild-type TRPA1 (HEK-wTRPA1) or mutant TRPA1 (HEK-muTRPA1) channels. The substantial expression of the TRPA1 mutants was confirmed by Western blotting (Fig. 3A). The Ca2+ responses evoked by MO, CA, Zn2+, and PGJ2 were tested in HEK-wTRPA1 and HEK-muTRPA1 cells and compared with the Ca2+ responses evoked by AUR (Fig. 3, B–F). When these agonists were applied to HEK-wTRPA1 and HEK-muTRPA1 cells, the Ca2+ responses of cells with C414S and C421S mutant TRPA1 channels were substantially affected (Fig. 3, B–F), which suggests that these cysteines are critical for activation of TRPA1 (2, 14, 25, 31). On the other hand, the Ca2+ responses to Zn2+ and PGJ2 were partially inhibited in cells with C641S mutant TRPA1 channels (Fig. 3, B–F). In contrast, HEK-muTRPA1 cells with the C623S mutation had a relatively smaller Ca2+ response to AUR, which was <30% of wild-type TRPA1 (Fig. 3F). Moreover, the Ca2+ responses of cells with C633S mutant TRPA1 channels were sensitive to only AUR (Fig. 3F). Thus, C621 and C633 play critical roles in activation of TRPA1 by AUR. The importance of cysteine at 621 for activation of TRPA1 by AUR was also confirmed with the patch-clamp technique. As shown in Fig. 3G, HEK-muTRPA1 cells with the C621S mutation had smaller current densities induced by AUR than HEK-wTRPA1.

All cysteine residues involved in activation of TRPA1 by AUR in this study are located intracellularly (25). Therefore, we examined whether intracellular application of AUR through the patch pipette can cause activation of TRPA1. As shown in Fig. 4, A and B, internal application of 3 μM AUR to HEK-TRPA1 cells had little effect on current densities at 0, 3, and 7 min after the break-through of the patch membrane. In contrast, exposure to the bathing solution containing 3 μM AUR evoked
suggesting that AUR stimulates TRPA1 in human neuronal cells.

and

shown in Fig. 4, the effects of CIS on the Ca\(^{2+}\) responses of HEK-TRPA1 cells. With or without 30 \(\mu\)M HC, cells were exposed to 3 \(\mu\)M AUR. A representative trace of change in Ca\(^{2+}\) and summary of the \(\Delta\)Ca\(^{2+}\), are shown in B and C, respectively. D: time course change in membrane currents at \(-100\) and \(+40\) mV before and during application of 3 \(\mu\)M AUR and after the washout in a representative HEK-TRPA1 cell. E: current-voltage relationships of membrane currents before (control) and during application of 3 \(\mu\)M AUR and after the washout in a representative HEK-TRPA1 cell. Ramp waveform voltage pulses from \(-150\) to \(+50\) mV for 400 ms were applied every 5 s. F: the mean change in current amplitudes normalized with cell capacitance (pA/pF) at \(-100\) and \(+40\) mV evoked by 3 \(\mu\)M AUR in HEK-TRPA1 cells (n = 5). Statistically significant difference: **P < 0.01 vs. control.

AUR is a potent inhibitor of TrxR [IC\(_{50}\) = 4 nM; (11)]. To examine whether the inhibition of TrxR by AUR is involved in the activation of TRPA1, we used CIS, another TrxR inhibitor [IC\(_{50}\) = \(~10\) \(\mu\)M; (35)]. Since the inhibitory effect of TrxR by CIS was irreversible and highly specific (3), we examined the effects of CIS on the Ca\(^{2+}\) response of HEK-TRPA1 cells. As shown in Fig. 4, C and D, 10 \(\mu\)M CIS failed to evoke a Ca\(^{2+}\) response in HEK-TRPA1 cells while 10 \(\mu\)M AUR was able to evoke the response. These results strongly suggest that AUR can stimulate TRPA1 independently of enzyme activity of TrxR.

Auranofin stimulates TRPA1 channels in human neuroblastoma cells. To test whether AUR could stimulate TRPA1 channels expressed in human neuronal cells, we differentiated IMR-32 cells to neuronal cells (nIMR-32) where TRPA1 is functionally expressed (24). Based on real-time PCR, we confirmed the mRNA expression level of TRPA1 in IMR-32 cells (Fig. 5A). The expression of TRPA1 mRNA transcripts in nIMR-32 cells was significantly higher than that in nonneuronal IMR-32 cells. Moreover, the application of 10 \(\mu\)M MO evoked Ca\(^{2+}\) responses in nIMR-32 cells while not in nonneuronal IMR-32 cells (Fig. 5B), showing that TRPA1 is functional in nIMR-32 cells. AUR (3 \(\mu\)M) was applied to nIMR-32 cells and evoked Ca\(^{2+}\) responses that were significantly inhibited by 30 \(\mu\)M HC (Fig. 5, C and D), suggesting that AUR stimulates TRPA1 in human neuronal cells.

**DISCUSSION**

The present study shows that AUR stimulates TRPA1 channels via a mechanism that depends on the cysteine residues at 414, 421, 621, and 633 in the NH\(_2\) terminus of TRPA1. To our knowledge, this is the first report of ion channel sensitivity to AUR and the first study to identify that TRPA1 is the channel activated by AUR. This study raises the possibility that TRPA1 senses adverse actions caused by AUR which may be relevant in the treatment of RA.

TRPA1 is stimulated by electrophilic compounds, such as MO and CA, or by endogenous compounds like PGJ\(_2\) and hydrogen peroxide, which all activate the channel by covalent modification of cysteines and lysines in the NH\(_2\) terminus (2, 14, 25, 31). Although it is not yet known whether other nucleophilic sinks are involved in channel activation, the importance of C414, C421, and C621 to the activation of human TRPA1 by electrophilic compounds has been identified by mutagenesis experiments. As shown in previous studies and in the present study, HEK-muTRPA1 cells with the C414S mutation did not have a Ca\(^{2+}\) response to AUR, or to MO, CA, Zn\(^{2+}\), or PGJ\(_2\). Thus, C414 is critical for TRPA1 channel activation. C621 was crucial for channel activation by AUR, which suggests that AUR is the most sensitive to this cysteine among electrophilic compounds tested in this study. Moreover, C633 is also important for channel activation by AUR. On the other hand, it has been proposed that the Cys-495/SeCys-496 center of TrxR is a target of AUR (11, 42). MO is also a potent irreversible inhibitor of TrxR (15) and easily
forms conjugates at the active site of the enzyme. Therefore, AUR is an electrophilic compound that is sensitive to specific cysteine residues of TRPA1 and activates the channel. Although gold compounds are potent inhibitors of TrxR [e.g., the $K_D$ of AUR = $\sim 4$ nM; (11)], the correlation between AUR-induced TrxR inhibition and the activation of TRPA1 is negligible. This is supported by the evidence that GST and CIS at concentrations $<$30 $\mu$M, which are also potent inhibitors of TrxR (3, 35, 39), failed to activate TRPA1. On the other hand, internal application of AUR through the patch pipette had little effects on TRPA1. Since all cysteine residues sensitive to AUR in this study are located intracellularly, it is not clear why AUR applied internally was not effective to TRPA1 in this study. It is likely that AUR cannot approach TRPA1 channel protein within several minutes due to its high lipophilicity and/or relatively low concentration tested when applied through the patch pipette.

Protein expression of TRPA1 was relatively lower in HEK-muTRPA1 cells with C421S, C621S, and C633S mutations. In contrast, $\mathrm{Ca}^{2+}$ response to both CA and $\mathrm{Zn}^{2+}$ of cells with the C621S and the C633S mutation was comparable to that in HEK-wTRPA1 cells, hence excluding the possibility that lower protein expression of TRPA1 mutants with C621S and C633S causes smaller $\mathrm{Ca}^{2+}$ response to AUR. On the other hand, it is possible that protein expression of TRPA1 with C421S is not enough to produce relatively large $\mathrm{Ca}^{2+}$ response to all TRPA1 agonists employed. Since expression of TRPA1 with C414S was slightly lower than that of wild TRPA1 but substantial, this mutation may cause dysfunction of channel sensitive to these TRPA1 agonists (2, 14, 25, 31).

Although CIS failed to activate TRPA1 in our study, the antitumor agents CIS, OXA, and paclitaxel indirectly activate TRPA1, which causes the adverse side effect of PN in mice (27, 29). The clinically used concentrations of AUR are measured as 20 $\mu$M in plasma (11), which raises the concern that AUR could also cause PN. In fact, AUR worsened mechano-allodynia and mechano-hyperalgesia in paclitaxel- and OXA-treated rats (41).

Gold compounds have been recently used as a family of promising experimental agents for tumor treatment. To be effective antitumor agents, new gold compounds should not interact with at least TRPA1, TRPM8, and TRPVs.
On the other hand, the high potency of AUR to activate the TRPA1 channel and the selectivity against TRPM8 and TRPVs are preferable features of TRPA1 agonists. Moreover, AUR effectively evoked TRPA1-dependent Ca\(^{2+}\) responses in human differentiated neuroblastoma cells. Thus, AUR is a potential lead TRPA1 agonist for humans. However, dramatic differences of TRPA1 responses among species and between native neurons and heterologous cells expressing cloned channel have been reported (5, 6) and need further exploration.

In summary, the data in the present study show that TRPA1 channels can be potently stimulated by clinically relevant concentrations of AUR. AUR is a specific agonist for TRPA1 channels without affecting the other five TRP channels. Although previous studies have reported stimulatory effects of

![Fig. 4. Mechanisms of activation of TRPA1 by AUR. A and B: AUR at 3 \(\mu\)M was internally applied to HEK-TRPA1 cells through the patch pipette. Ramp waveform voltage pulses from \(-150\) to \(+50\) mV for 400 ms were applied every 5 s to get the current density at each time. A representative time course change in current density at \(-100\) and \(+40\) mV is shown in A. The current recording started just after the break-through of patch membrane (0 min). To check TRPA1 expression, cells were exposed to the bathing solution containing 3 \(\mu\)M AUR. The current density at \(+40\) mV is summarized at 0, 3, and 7 min after the break-through of patch membrane in B (n = 3). The peak current density evoked by external 3 \(\mu\)M AUR was also determined. The arrows in A indicate corresponding times when data in B were sampled. C: the Ca\(^{2+}\), evoked by AUR at 10 \(\mu\)M was compared with that of another thioredoxin reductase (TrxR) inhibitor, cisplatin (CIS), at 10 \(\mu\)M in HEK-TRPA1 cells. D: a summary of \(\Delta\text{Ca}^{2+}\), is shown as a bar graph (n = 4, 225 cells). Statistically significant difference: \(*P < 0.05\) vs. 0 min in B, \(**P < 0.01\) vs. CIS in D.

![Fig. 5. Expression of TRPA1 and the effects of AUR on the Ca\(^{2+}\) response in differentiated human neuroblastoma cells. Nonneuronal IMR-32 neuroblastoma cells were differentiated to neuronal cells (nIMR-32) with 5-bromo-2\'-deoxyuridine (BrdU) treatment (see MATERIALS AND METHODS). To confirm expression of TRPA1, TRPA1 mRNA transcripts (A, n = 6 for each) and the Ca\(^{2+}\) response (\(\Delta\text{Ca}^{2+}\)) to 10 \(\mu\)M MO [B, n = 4 (141 cells) and 5 (110 cells) for IMR-32 and nIMR-32 cells, respectively] were compared between IMR-32 and nIMR-32 cells. C: the \(\Delta\text{Ca}^{2+}\) induced by 3 \(\mu\)M AUR was compared between IMR-32 (n = 4, 116 cells) and nIMR-32 cells (n = 4, 91 cells). D: the AUR-induced \(\Delta\text{Ca}^{2+}\) in nIMR-32 cells in the absence and presence of 30 \(\mu\)M HC is summarized (n = 3, 21 cells). Statistically significant difference: \(**P < 0.01\) vs. IMR-32 and +30 \(\mu\)M HC in A–C and D, respectively.]}
electrophilic compounds on TRPA1, this is the first study to identify the specific cysteine residues on TRPA1 channels that are critical components of the molecular mechanism mediating the effect of AUR on TRPA1.

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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS
N.H., H.S., and Y.M. performed the experiments; N.H., H.S., and Y.M. analyzed the data; N.H. and H.S. interpreted the results of the experiments; N.H. and H.S. prepared the figures; N.H., H.S., and K.M. edited and revised the manuscript; N.H., H.S., and Y.M., and K.M. approved the final version of the manuscript; K.M. conception and design of the research; K.M. drafted the manuscript.

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7. C360 AURANOFIN IS A POTENT STIMULATOR OF TRPA1


