Novel Small Molecule MEK Inhibitor URML-3881 Enhances Cisplatin Sensitivity in Clear Cell Ovarian Cancer

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Abstract

Advanced clear cell ovarian cancer (CCOC) is a highly fatal malignancy with a scarcity of effective treatment options. CCOC is inherently chemotherapy resistance, but the exact mechanism of this resistance has yet to be established. Prosurvival signaling, such as through the MAPK cascade, is one way in which cancer cells can evade chemotherapy. We have determined that CCOC exhibits baseline elevated levels of MAPK activity, which increase further upon cisplatin exposure. We have developed a novel MEK inhibitor, URML-3881, to test the effect of MAPK inhibition in CCOC. URML-3881 was found to reduce in vitro CCOC viability through apoptosis and proliferation inhibition, yet it failed to induce in vivo tumor regression. Similarly, cisplatin alone had minimal impact on tumor growth, but remarkably, the combination of MEK inhibition and cisplatin led to a significant and prolonged tumor regression. These studies confirm that the combination of MEK inhibition with URML-3881 and cisplatin is superior to either agent alone in CCOC. Our data support the design of future preclinical and clinical studies into the combination of MEK inhibition and platinum-based chemotherapy as a treatment strategy for CCOC.

Introduction

Ovarian cancer is the fifth leading cause of cancer death among women in the United States, resulting in approximately 14,240 deaths annually [1]. Epithelial ovarian cancers (EOCs) are a heterogeneous collection of diseases including serous, endometrioid, mucinous, and clear cell subtypes [2]. The incidence of clear cell ovarian cancer (CCOC) varies geographically. In the United States, it accounts for only about 10% of all ovarian cancers [3]; yet in Japan, it comprises up to 25% of all ovarian cancers [4]. CCOC is histologically and phenotypically distinct from the other classes of EOC [4] and has been comparatively understudied. CCOC is highly resistant to traditional chemotherapy [5], and due to the lack of other effective treatments, advanced CCOC portends a worse prognosis [6,7]. There is clearly a strong need for new and innovative therapies to improve outcomes in this disease.

The mitogen-activated protein kinase (MAPK) pathway is vital to the survival of tumor cells and is an appealing potential target in cancer therapeutics. It is a signaling cascade initiated by the activation of receptor tyrosine kinases at the cell surface. After this initial step, intracellular kinases are activated sequentially leading to a phosphorylated kinase entering the nucleus where it regulates several processes including transcription and proliferation. The classical, and best characterized, MAPK pathway involves Ras, Raf, MEK, and ERK and is known to play a role in tumor cell survival, proliferation, migration, and angiogenesis [8–10]. Literature on the role of MAPK pathway signaling in CCOC is relatively sparse. However, vascular endothelial growth factor, epidermal growth factor receptor, and hepatocyte growth factor receptor are overexpressed in this cancer type, and all signal through the MAPK pathway [4,11]. In the last decade, four MAPK inhibitors have been approved by the FDA and are now in clinical use in melanoma and lung cancers with activating mutations [12,13]. While CCOCs rarely carry activating mutations in the MAPK pathway, such mutations are not predictive of response to MAPK inhibition in ovarian cancer [14].
In addition to the tumor-promoting features of MAPK activation discussed above, this pathway is known to confer a chemotherapy-resistant phenotype to tumor cells by mediating prosurvival signaling [15–17]. Interestingly, MEK inhibition has been shown to overcome platinum chemotherapy resistance in several cancer cell types [18,19]. The ability of the MAPK pathway to contribute to cytotoxic escape makes it an attractive candidate for combination treatment with chemotherapy in CCOC. Encouragingly, early phase clinical trials looking at combined MEK inhibition and chemotherapy in other cancer types are proving this to be a tolerable treatment strategy [20].

Here we investigate the role of the MAPK pathway in CCOC viability and chemotherapy resistance and assess its potential as a therapeutic target. Inhibition of the kinase MEK is of particular interest to us since it represents an important bottleneck in the pathway and directly activates p-ERK, the master regulator of downstream nuclear events. Additionally, attempts at blocking further upstream have led to paradoxical pathway overactivity in RAF wild-type tumor cells [21]. We have designed a novel MEK inhibitor, coined URML-3881, with the intention of improving therapeutic options for patients with CCOC. In the present study, the efficacy of MEK inhibition with URML-3881 in CCOC was tested both as a single agent and as an adjunct to platinum chemotherapy.

Materials and Methods

Cell Culture

Patient-derived clear cell ovarian cancer cell lines OVMANA, OVAS, OVTOKO, HCH-1, and RMG-1 were provided by Dr. Hiroaki Itamochi at Tottori University School of Medicine, Japan. ES-2 was purchased from ATCC (CRL-1798). ES-2 was grown in FBS-supplemented McCoy media (Gibco, 16600-082), while all other cell lines were grown in FBS-supplemented RPMI (Gibco, 22400-089).

Western Blot

OVMANA cells were plated (5×10^5) after adherence media were replaced with fresh media containing either DMSO (control), URML-3881 at varying concentrations, R05126766 at 10 μM, or sorafenib at 10 μM (all drugs except URML-3881 were obtained from Selleck Chemicals). Cell lysis buffer (Cell Signaling, 98035), supplemented with Halt protease and phosphatase inhibitors (Thermo Scientific, 1862209 and 1862495), was utilized for protein extraction. Animal tumor tissue was snap frozen and stored in liquid nitrogen. It was then thawed and mechanically homogenized in cell lysis buffer. Protein concentrations were determined using the DC Protein Assay (Bio-Rad, 5000111). Western blot analysis was performed as previously described [22]. Primary antibodies against p-ERK (4370), ERK (9102), p-MEK (2338), MEK 1/2 (4694), p-AKT (9271), AKT (9272), pPEA-15 (2776), and PEA-15 (2780) were all obtained from Cell Signaling Technology and used at the recommended dilutions.

URML-3881 Synthesis

Schematic of synthesis is pictured in Figure S1A. 3((2-fluoro-4-iodophenyl)amino)isonicotinic acid [1] [Ark Pharm Inc., Catalog No: AK118913] (0.1 mM) was coupled with 4-aminomorpholine 1,1-dioxide [2] [TCI Inc., A1798] (0.1 mM) in dry dichloromethane (10 ml) using DCC (Sigma Aldrich, Cat. No: 36650) (0.12 mM). Isonicotinic acid [2] was stirred with DCC at 0°C in argon atmosphere for 10 minutes; a white suspension was formed. Amine [2] was added, and a catalytic amount of dimethyl aminopyridine (DMAP) (Sigma Aldrich, 39,405) was added. The reaction mixture was stirred overnight over a temperature range of 0°C to room temperature. The precipitated product was filtered and washed repeatedly with hexane (4×5 mL) and dried under vacuum. The final product, URML-3881, was synthesized multiple times in over 60% yields and was characterized by mass spectrometry.

In Silico Prediction of Drug-Like Qualities

The polar surface area, molar mass, LogP, cLogP, and LogS of drugs were predicted using the property prediction feature of ChemDraw software.

Cell Free Kinase Assay

Kinase inhibition studies were performed by Reaction Biology Corp. In brief, purified kinases were incubated with known substrate, radiolabeled ATP, and test drug or a predetermined reference compound. Enzyme activity was then determined by the presence of radiolabeled-phosphorylated substrate and calculated as a percentage of control. Full details for each tested kinase assay can be found on the company’s publically available data sheets.

Immunohistochemistry

A human ovarian tumor array was purchased from US Biomax (OV8011). The paraffin-embedded slides were baked, deparaffinized in Americare (Adwin Scientific, 72060044), and rehydrated in decreasing concentrations of ethanol. Antigen retrieval was performed by incubation in heated Dako target retrieval solution. Slides were then permeabilized by adding Triton-X100 (0.15%) to wash solution, blocked in 50% horse serum, and incubated overnight with anti-p-ERK primary antibody (Cell Signaling, 4370) or isotype control per manufacturer’s recommendation. Slides were washed and incubated with an anti-rabbit secondary antibody conjugated to Dylight 488 (Invitrogen, 35,552). Tissue sections on the slide were immersed in Vectashield with DAPI and coverslipped. Slides were imaged at 60× on an Olympus BX43F fluorescence microscope utilizing CellSens software.

Cell Viability Assays

The viability of cells after drug treatment was determined using the 96 Aqueous-One-Solution Assay (Promega, G3580). Briefly, 5×10^5 cancer cells were exposed to serial dilutions of URML-3881 and incubated at 37°C for 24 hours. Media were then replaced with MTS reagent (1:10 dilution) and incubated for 2 hours. Absorbance was measured at 490 nm on a Bio-Rad iMARK microplate reader. Experiments were performed in triplicate; data are expressed as the mean percentage (+ standard deviation) compared to control (DMSO) treated cells. In cells that were treated with both URML-3881 and cisplatin, URML-3881 was added 24 hours before cisplatin, and viability was determined 24 hours after the addition of cisplatin.

Apatosis Assays

Annexin V Apoptosis Detection Kit (eBioscience, 88-8007) was used. In brief, 5×10^5 OVMANA cells/well were plated and allowed to adhere; then the media were replaced with fresh complete media containing URML-3881 (5 or 20 μM) or DMSO control and incubated for 24 hours at 37°C. In order to avoid losing dead/dying
cells, the media were collected and centrifuged, and the adherent cells were collected by trypsinization and combined with the nonadherent cells. Cells were stained with Annexin-V-APC followed by 7-AAD according to the manufacturer’s instructions. Cells were then immediately analyzed by flow cytometry on a BD LSR Fortessa cytometer.

**Proliferation Assay**

Proliferation of cells was determined by BrdU incorporation using a Cell Proliferation Assay Kit (Cell Signaling Technology, 6813). A total of $5 \times 10^3$ were plated into a 96-well flat-bottom plate and allowed to adhere; serial dilutions of URML-3881 or DMSO control, and BrdU were added to wells. Cells were incubated for 48 hours at 37°C and then washed, fixed, denatured, and stained with anti-BrdU-HRP according to manufacturer’s instructions. TMB substrate exposure time was 30 minutes; then stop solution was added, and the absorbance was measured at 450 nM on a Bio-Rad iMARK microplate reader. Experiments were performed in replicates of nine. Data are expressed as the mean percentage (± standard deviation) compared to control (DMSO) treated cells.

**Xenograft Animal Model**

The University of Rochester Animal Care and Use Committee authorized the animal experiments. NOD scid gamma (NSG) mice were injected subcutaneously in the right flank with $1 \times 10^6$ OVMANA cells in 100 μl of Matrigel (Corning, 356235). Tumors were allowed to establish for 8 weeks until they averaged approximately 100 mm$^3$ in volume, and animals were randomized. Treatment with URML-3881 was delivered daily via oral gavage for 21 days at either 10-mg/kg dosing or 30-mg/kg dosing (PBS control). When administered in the combination groups, cisplatin chemotherapy or control (PBS) was started 24 hours after URML-3881 treatment and was given intraperitoneally every 7 days (4 mg/kg) for three doses. Animals were monitored for signs of distress at least twice weekly throughout the course of the experiment. Tumor volume and animal weight were recorded weekly.

**Statistical Analysis**

Kinase inhibition, proliferation, and cell viability were evaluated using a one-way ANOVA with multiple comparisons. Apoptosis assay and animal studies were compared using a two-way ANOVA (GraphPad Prism 5.0 Software). $P$ values <.05 were considered statistically significant.

**Results**

**MAPK Activity in CCOC**

MAPK pathway overexpression in cancer is known to enhance proliferation and angiogenesis, thereby promoting tumor growth. The importance of the MAPK pathway has been shown in other EOC subtypes, such as low-grade serous carcinomas which often carry a KRAS mutation. However, the importance of this pathway in
clear cell ovarian cancer has not been heavily studied. While RAS/RAF activating mutations are rare in CCOC, overexpression of hepatocyte growth factor receptor, vascular endothelial growth factor, and epidermal growth factor receptor, all which signal through the MAPK pathway, is frequently identified [4]. ERK is the substrate of MEK, and when activated by phosphorylation, it is considered to be a crucial effector in the MAPK pathway. Consequently, phosphorylated-ERK (pERK) levels are commonly used to quantitate MAPK pathway activity. We stained patient CCOC, high-grade serous ovarian cancer, and adjacent normal ovarian tissues for p-ERK. While there is clearly variability between samples, the CCOC specimens expressed higher p-ERK than control ovary or high-grade serous cancer (Figure 1). These data suggest that MAPK overactivity is a previously unrecognized feature of CCOC.

URML-3881, a Novel, Specific MEK1/2 Inhibitor

Our lab has developed a novel MEK inhibitor coined URML-3881 (Figure 2A). This class of drugs has been plagued by issues such as poor solubility and high molecular weight which lead to suboptimal pharmacokinetics/pharmacodynamics. In fact, the most commonly used MEK inhibitor, trametinib, requires the potentially toxic molecule dimethyl sulfoxide (DMSO) as a solvate, a feature that is unusual among drugs in clinical use. It is unclear to what degree oral DMSO is enhancing the side effect profile of trametinib, but intravenously delivered DMSO can cause a variety of serious and potentially life-threatening side effects [23]. URML-3881 has been designed to possess optimized drug-like qualities compared to existing MEK inhibitors in an effort to enhance pharmacokinetics and pharmacodynamics. Additionally, URML-3881 structurally

Figure 2. URML-3881 is a novel MEK inhibitor that reduces MAPK pathway activity in CCOC. (A) Chemical structure of URML-3881, a novel MEK inhibitor. (B) Bar graph showing percent kinase activity in the presence of URML-3881 (30 nM) compared to kinase activity at baseline. (C) CCOC cell lines (OVMANA and OVTOKO) were cultured in the presence of increasing concentrations of URML-3881 for 24 hours; cell lysates were then obtained, and p-ERK, ERK, p-MEK and MEK protein expression was determined by Western blot. (D) Schema of the MAPK pathway at baseline, with MEK inhibition, when overactivated (as in CCOC) and when overactivated with MEK and RAF inhibition. ** = \( P < .01 \).
incorporates a tertiary nitrogen (Figure 2A) that, when converted to salt forms, will enhance its aqueous solubility. A comparison of the predicted pharmacologic properties of URML-3881 in comparison to other MEK inhibitors shows that it possesses more favorable drug-like characteristics in terms of molar mass, LogP, CLogP, and Log S (Table S1). We have confirmed URML-3881 as a potent inhibitor of MEK 1/2 (Figure 2B), with an IC50 of 30 nM in a cell-free kinase inhibition assay. This assay also established the drug’s specificity for MEK 1 and 2, as we saw no inhibition among a panel of other kinases including MEK 3 and MEK5, which are activated in alternative MAPK signaling pathways.

**URML-3881 Activity In Vitro**

Two human-derived CCOC cell lines, OVMANA and OVTOKO, were subjected to increasing concentrations of URML-3881, and MAPK activity was determined by ERK phosphorylation. Western blot analyses revealed that increasing concentrations of MEK inhibitor resulted in less p-ERK expression by CCOC (Figure 2C), while p-MEK conversely increased with URML-3881 concentration. Increased upstream MAPK activity confirms URML-3881 as a specific functional MEK inhibitor *in vitro* since p-MEK would not be increased if the drug was working further upstream. This upstream MAPK compensatory overactivity occurs due to the loss of negative feedback regulatory mechanisms [24] (Figure 2D). P-ERK inhibition and p-MEK accumulation were confirmed with another known specific MEK inhibitor (AS703026), while a combination MEK/RAF inhibitor (R05126766) resulted in reduction of both p-ERK and p-MEK (Figure S1B). Sorafenib, a RAF inhibitor with no known activity against MEK1/2, did not cause a reduction in either p-ERK or p-MEK and actually increased the levels of both phosphorylated kinases. This finding is consistent with the recognized paradoxical activation of the MAPK pathway by RAF inhibitors in non-RAF mutant cancer cells [21,25], depicted in Figure 2D.

**URML-3881 Induces Apoptosis and Inhibits Proliferation**

The ability of URML-3881 to inhibit tumor cell viability was next determined. We found that 20 μM of drug resulted in a significant reduction in the viability of six human-derived CCOC cell lines compared to DMSO control (Figure 3A). This inhibition of CCOC viability was determined to be dose-dependent in 48-hour cell co-cultures with serial drug dilutions (Figure 3B), with some cell lines showing a significant reduction in viability at concentrations as low as 1.25 μM. Previous work has suggested that pPEA-15 regulates the ability of ovarian cancer cells to respond to MEK inhibition [26]. We assessed pPEA-15 levels in our various CCOC cell lines and found that there was no clear correlation to MEK inhibitor response (Figure 3C).

![Figure 3](image-url)

**Figure 3.** URML-3881 causes reduced CCOC viability due to induction of tumor cell apoptosis and inhibition of proliferation. (A) Six CCOC cell lines were cultured in the presence of 20 μM URML-3881 for 24 hours, and viability was determined as percentage of control tumor cell growth (DMSO control). (B) The same six cell lines were cultured in serial dilutions of URML-3881 for 24 hours, and viability was determined as percentage of control tumor cell growth. (C) Six clear cell ovarian cancer cell lines were lysed and assessed for p-PEA-15 and PEA-15 protein expression (per 10 μg of protein from cell lysate) by Western blot. (D) OVMANA cells were cultured in the presence of serial dilutions of URML-3881 for 48 hours, and proliferation was assessed by BrdU incorporation; proliferation rate is expressed as a percentage with DMSO (control) treated cells used as a reference. (E) OVMANA cells were cultured with URML-3881 (5 and 20 μM) for 24 hours, and apoptotic status of cells was assessed by flow cytometry. 7-AAD uptake = dead cells. Annexin V staining = actively dying or dead cells. * = P < .05, ** = P < .01, *** = P < .001.
Inhibition of cell viability, with apoptosis occurring at an increased rate at higher doses.

**URML-3881 Single Agent In Vivo Activity**

Tumor-bearing animals were treated with a single bolus of 30 mg/kg URML-3881 and sacrificed 2.5 hours later. This time point was chosen based on the known short Tmax values of other MEK inhibitor compounds [27,28]. Tumors were harvested, and p-ERK level was determined by Western blot. As expected, treatment with URML-3881 reduced p-ERK in tumors (Figure 4A), thus confirming in vivo efficacy as a MEK inhibitor. Animals were then treated with URML-3881 at doses of either 10 mg/kg or 30 mg/kg daily (by oral gavage) for 21 days, and tumor volumes were measured throughout. While URML-3881 was well tolerated by the animals at both doses, as evidenced by stable weight (Figure 4B), it was ineffective at inducing tumor regression or delaying tumor growth (Figure 4C).

**URML-3881 Inhibits Chemotherapy Induced Prosurvival Signaling**

Platinum-based chemotherapy, a staple in ovarian cancer treatment regimens, is known to increase ovarian cancer’s reliance on the prosurvival MAPK pathway [19]. We sought to determine the potential of URML-3881 as a combinatorial agent with platinum chemotherapy. We first confirmed that there is an increase in MAPK pathway activity in CCOC upon exposure to platinum chemotherapy (10 and 30 μM) and found this to be the case in five of six cell lines (Figure 5A). The baseline p-ERK in OVAS was very faint and did not clearly change with chemotherapy. We next performed dual drug culture experiments where cells were pretreated with URML-3881 for 24 hours and then exposed to cisplatin chemotherapy. We found that URML-3881 is able to abrogate chemotherapy-induced MAPK signaling (Figure 5B) and reduce tumor cell viability (Figure 5C) in vitro. The URML-3881 10-μM combination treatments also caused a decrease in ERK expression in the unphosphorylated form. AKT was run on the same blot (shown in the bottom panel of Figure 5B) and confirms that there were consistent amounts of protein loaded into each lane. CCOC is known to have overactivity of the PI3K/AKT pathway [19]. We sought to determine the potential for URML-3881 to inhibit the PI3K/AKT pathway, which is known to increase survival upon exposure to platinum chemotherapy. We next performed dual drug culture experiments where cells were pretreated with URML-3881 for 24 hours and then exposed to cisplatin chemotherapy. We found that URML-3881 is able to abrogate chemotherapy-induced MAPK signaling (Figure 5B) and reduce tumor cell viability (Figure 5C) in vitro. The URML-3881 10-μM combination treatments also caused a decrease in ERK expression in the unphosphorylated form. AKT was run on the same blot (shown in the bottom panel of Figure 5B) and confirms that there were consistent amounts of protein loaded into each lane. CCOC is known to have overactivity of the PI3K/AKT pathway [19].

**Combination Cisplatin and URML-3881 In Vivo Activity and Tolerability**

To further validate combination MEK inhibition with URML-3881 and cisplatin as a treatment strategy for CCOC, we treated NSG mice bearing established OVMANA CCOC xenografts with URML-3881 daily (starting on day 1) and cisplatin at 4 mg/kg weekly for 3 weeks (starting on day 0) (Figure 5D). A significant and prolonged reduction in tumor volume was seen with combination URML-3881 and cisplatin compared to both control (P = .02) and URML-3881 alone (P = .0003) (Figure 5E). URML-3881 + cisplatin was not significantly different from cisplatin alone (P = .09), likely due to variability within the cisplatin group. The lower error bars have been left out of this multigroup comparison graph for clarity. Animal weight was recorded for the cisplatin group. The lower error bars have been left out of this multigroup comparison graph for clarity. Animal weight was recorded for the cisplatin group. The lower error bars have been left out of this multigroup comparison graph for clarity. Animal weight was recorded for the cisplatin group. The lower error bars have been left out of this multigroup comparison graph for clarity.

Figure 4. URML-3881 inhibits the MAPK pathway in CCOC in vivo but is not effective at controlling tumor growth as a single agent. (A) NSG mice with established OVMANA xenografts were treated with one dose of oral URML-3881 at 30 mg/kg or control and sacrificed 2.5 hours later. Protein was isolated from the tumors, and p-ERK and ERK were analyzed by Western blot. (B) NSG mice with established OVMANA xenografts were treated daily with URML-3881 (10 mg/kg or 30 mg/kg) or control by oral gavage for 21 days, animal weight was measured weekly throughout treatment, and (C) tumor volume was measured weekly throughout treatment and for 5 weeks afterwards.
weekly, and all treatment categories were well tolerated with no significant changes between groups (Figure 5F). There was a nonsignificant trend towards weight loss at day 21 in the URML-3881 + cisplatin group that improved weekly thereafter. Animals in all treatment groups retained baseline activity levels and showed no outward signs of distress.

Discussion

The work detailed here highlights MEK inhibition as a novel treatment strategy in clear cell ovarian cancer. While MEK inhibition with URML-3881 showed promising in vitro activity as a single agent, this failed to translate into in vivo antitumoral efficacy. However, when combined with cisplatin, URML-3881 was shown to inhibit prosurvival MAPK signaling and result in significant and long-lasting tumor regression, while cisplatin alone appeared to have no effect. Therefore, we propose MEK inhibition with URML-3881 as a chemotherapy sensitizing adjunct to clear cell ovarian cancer treatment.

Due to incredibly poor chemotherapy response rates and the lack of treatment alternatives [30], advanced clear cell ovarian cancer has carried a significantly worse prognosis when compared to other EOC subtypes. An agent that can increase chemotherapy susceptibility in this resistant disease has the potential to substantially prolong patients’ lives and enhance cure rates. The combination of MEK inhibition with URML-3881 and cisplatin was well tolerated by animals, mirroring early phase clinical trials in patients that have reported an acceptable side effect profile for combined MEK inhibition and chemotherapy [20,31].

The MAPK and PI3K/mTOR pathways are known to extensively cross talk and co-regulate one another [29]. Additionally, CCOCs typically possess heightened baseline activity of the PI3K/mTOR pathway due to a high frequency of ARID1a and PTEN mutations [4,32]. One possible reason for the poor efficacy of single-agent URML-3881 in vivo is compensatory upregulation of PI3K activity. We did witness an increase in PI3K activity after MAPK inhibition with URML-3881 in vitro, and in our chemotherapy combination group, the addition of high-dose cisplatin also caused PI3K upregulation. These findings provide the rationale for future testing of alternative combinatorial treatment strategies in CCOC, such as...
MEK inhibition with PI3K/mTOR inhibition, platinum chemotherapy with PI3K/mTOR inhibition, or all three agents together.

In conclusion, this study provides evidence that combination of MEK inhibition and cisplatin is effective against chemotherapy-resistant clear cell ovarian cancer. This treatment strategy warrants further research in a disease that has limited therapeutic options and carries very poor survival rates in the advanced or recurrent setting. These findings are potentially applicable to the treatment of thousands of patients with many other cancer subtypes since MAPK overactivity and chemotherapy resistance are prominent features among highly fatal malignancies.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.tranon.2019.04.009.

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Conflict of Interest

R. B. R., R. K. S., and R. G. M. are co-inventors on a filed patent for URML-3881. Authors have no other conflicts to report.

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