Mammalian Neuraminidase-1 is a Critical Regulator of Platelet-derived Thromboxane A2 (TXA2) T-cell Immunosuppression to Promote Tumor Progression and Metastasis

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Abstract

Aspirin or celecoxib in combination with chemotherapy significantly reduced the risk for disease recurrence and improved survival after colorectal cancer surgery in the approximately 20% of patients harboring PIK3CA pathway mutations [1,2]. Meta-analyses have shown that daily aspirin reduces metastasis in various cancers [3]. The biological mechanisms behind the anti-cancer effects of these drugs are an area of intense research interest. Recently, Yang et al found that platelet-derived thromboxane A2 (TXA2) inhibits T-cell activity through the expression of the guanine exchange factor ARHGEF1, and that aspirin was able to inhibit ARHGEF1 expression and preserve T-cell functionality and cancer immunity by blocking thromboxane production [4]. TXA2 production is inhibited by the effect of aspirin on cyclooxygenase-1 (COX-1). Celecoxib, a selective COX-2 inhibitor, does not inhibit platelet TXA2 production but has shown almost identical efficacy to aspirin in controlled clinical trials [2]. Here we show that the enzyme mammalian neuraminidase 1 (NEU-1) is involved in the activation and downstream signaling of the thromboxane receptor in response to TXA2. Both aspirin and celecoxib as well as oseltamivir phosphate (OP) significantly inhibited TXA2-induced NEU-1 activity with subsequently reducing ARHGEF1 expression. This regulation of the TXA2 receptor activation by NEU-1 may explain the contemporaneous results of why both aspirin and celecoxib demonstrated comparable clinical efficacy in the cited trials above despite only aspirin being able to inhibit thromboxane synthesis. Targeting NEU-1 may provide a novel therapeutic strategy against cancer progression and metastasis.

Clinical Perspective

After potentially curative surgical resection of a primary cancerous tumor, patients often receive chemotherapy to eradicate any occult micro-metastatic disease that may be present. The absolute curative benefit of treatment with chemotherapy after primary tumor removal for lung, breast, and colorectal cancer is approximately 5% to 15% [5]. Surgical removal of the primary tumor has been shown to increase the number of circulating tumor cells and can trigger an inflammatory

response that can impair the immune response and facilitate metastasis [6]. Recent clinical studies have shown that treatment with aspirin and celecoxib added to standard chemotherapy after surgical resection of PIK3CA mutated colorectal cancer can significantly reduce risk for disease recurrence and improve survival [1,2]. This has prompted further studies into the biological mechanism that could explain these impressive clinical results.

An exciting research discovery that has illuminated a possible anti-cancer mechanism of aspirin found that platelet derived thromboxane A_2 (TXA_2) suppressed T cell activity by increasing the expression of the guanine exchange factor ARHGEF1 [4]. ARHGEF1 was found to limit T cell effector function and disrupt the ability of T cells to control metastatic colonization in mice [4]. The report revealed that aspirin exerted its anti-cancer effects by inhibition of cyclo-oxygenase 1 (COX-1) and the production of TXA2 and ARHGEF1, preserving a healthy T cell response. Celecoxib inhibits cyclo-oxygenase 2 (COX-2) and has no effect on the production of thromboxane yet had almost identical efficacy to aspirin in preventing metastasis in the clinical trials cited above.

The TXA2 receptor is a G protein-coupled receptor (GPCR) that can transactivate receptor tyrosine kinases (RTK) like EGFR [7] or the insulin receptor [8] to induce specific cellular processes, such as cell proliferation and differentiation. A molecular organizational G protein-coupled receptor (GPCR)-signaling platform was previously discovered by us for the EGFR receptor that may be relevant to the control of the G protein-coupled thromboxane receptor [9] (Figure 1). In this molecular arrangement NEU-1 and MMP9 form a complex tethered at the ectodomain of the receptor tyrosine kinase (RTK) cell surface receptor. RTK agonists induce a conformational change in the receptor, resulting in the trans-activation of the specific NMBR GPCR tethered to the receptor. The NMBR GPCR can also be activated directly by agonists to the other GPCRs such as TXA2 through a process of heterodimerization as reported by Haxho et al. [8]. The activated GPCR initiates $G\alpha_i$-protein signaling which triggers the activation of MMP9 to subsequently induce the expression of NEU-1. Activated MMP9 is proposed to remove the elastin-binding protein (EBP) as part of the molecular multi-enzymatic complex that contains NEU-1 and protective protein cathepsin A (PPCA) [10]. Activated NEU-1 hydrolyzes the α-2,3-sialyl residues linked to β-galactosides of the RTK. This desialylation process by NEU-1 is predicted to remove steric hindrance of the RTK to facilitate receptor association, subsequent activation and downstream signaling. We have previously found that both aspirin and celecoxib can inhibit NEU-1 [11]. Building on our previous discoveries and the recent novel role of TXA2 in T cell regulation, we investigated whether NEU-1 regulates the thromboxane G protein-coupled receptor and the subsequent expression of ARHGEF1.

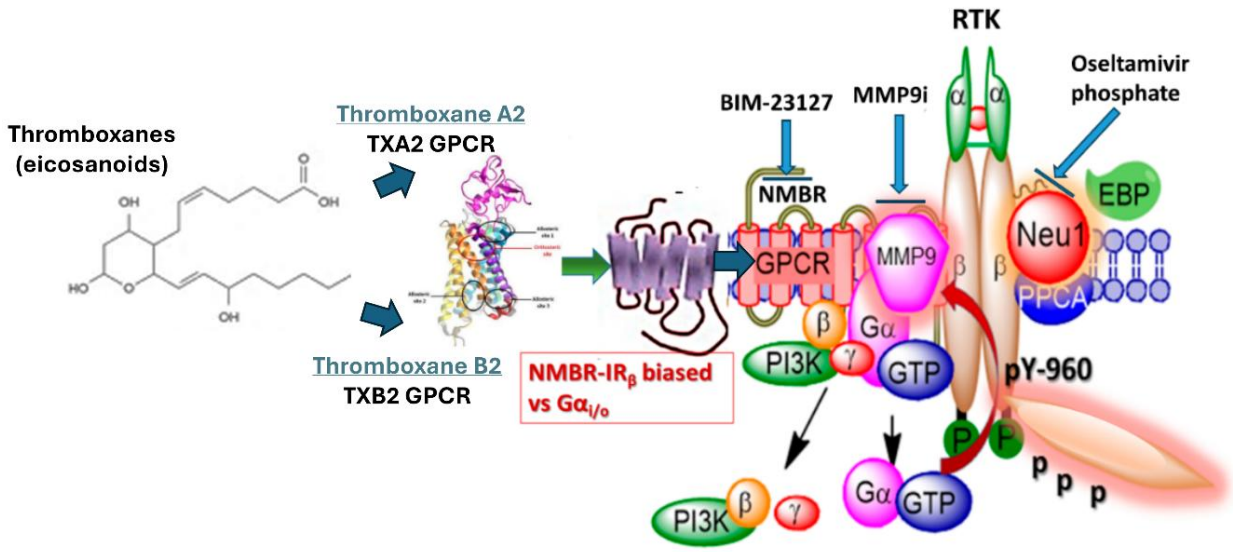


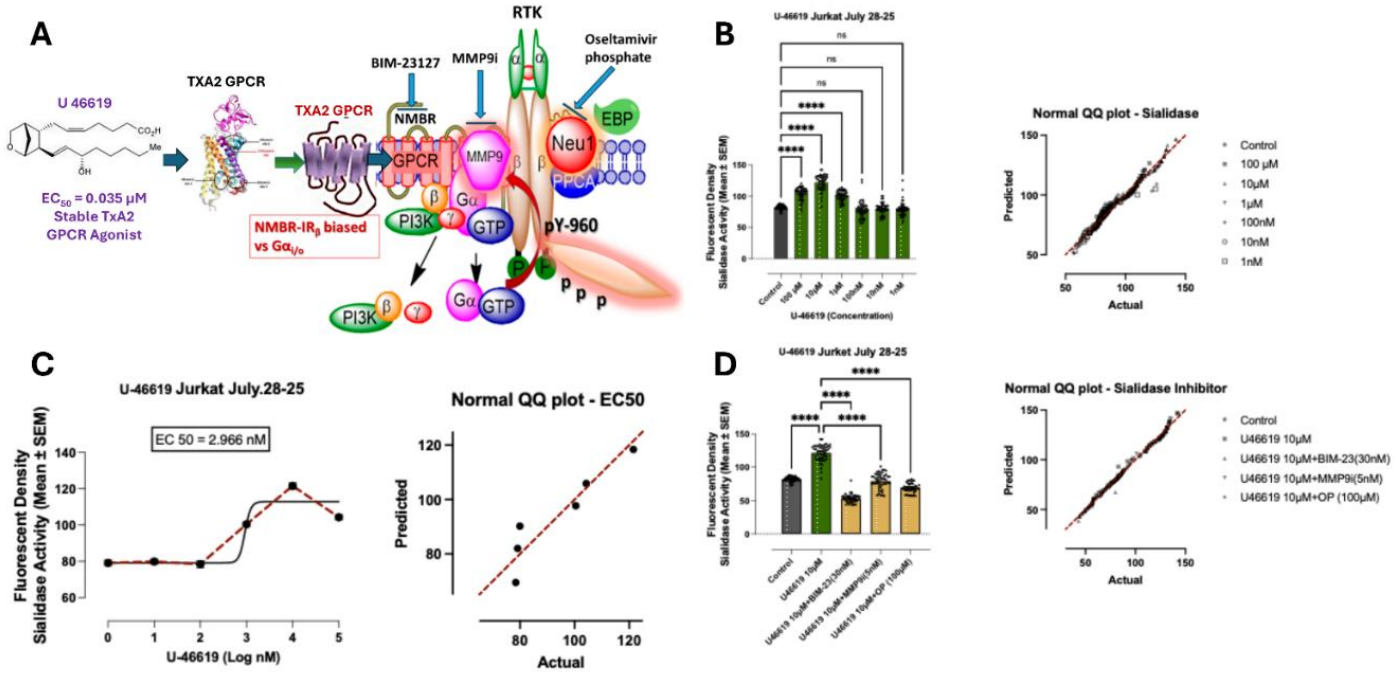
Figure 1. Thromboxane A2 (TXA2) and B2 (TXB2) exist in a multimeric receptor complex with NMBR, RTK, and NEU-1 in naïve (unstimulated) and stimulated immune cells. Here, a molecular link regulating the interaction and signaling mechanism(s) between these molecules on the cell surface uncover a biased GPCR agonist-induced RTK trans-activation signaling axis, mediated by Neu1 sialidase and the modification of RTK receptor glycosylation. The biased G-protein-coupled receptor (GPCR)-signaling platform potentiates neuraminidase-1 (Neu1) and matrix metalloproteinase-9 (MMP-9) crosstalk on the cell surface that is essential for the activation of the RTK tyrosine kinases. Notes: Activated MMP-9 is proposed to remove the elastin-binding protein (EBP) as part of the molecular multi-enzymatic complex that contains β-galactosidase/Neu1 and protective protein cathepsin A (PPCA). Activated NEU-1 hydrolyzes α-2,3 sialyl residues of RTK at the ectodomain to remove steric hindrance to facilitate RTK subunits association and tyrosine kinase activation. Abbreviations: NMBR, neuromedin-B receptor; phosphatidylinositol 3-kinase; GTP: guanine triphosphate; p: phosphorylation. Citation: Taken in part from Cellular Signaling, Volume 26, Issue 6, June 2014, Pages 1355–1368. © 2014 Alghamdi et al., Published by Elsevier Inc., Open access under CC BY-NC-ND license. This is an Open Access article which permits unrestricted non-commercial use, provided the original work be properly cited.

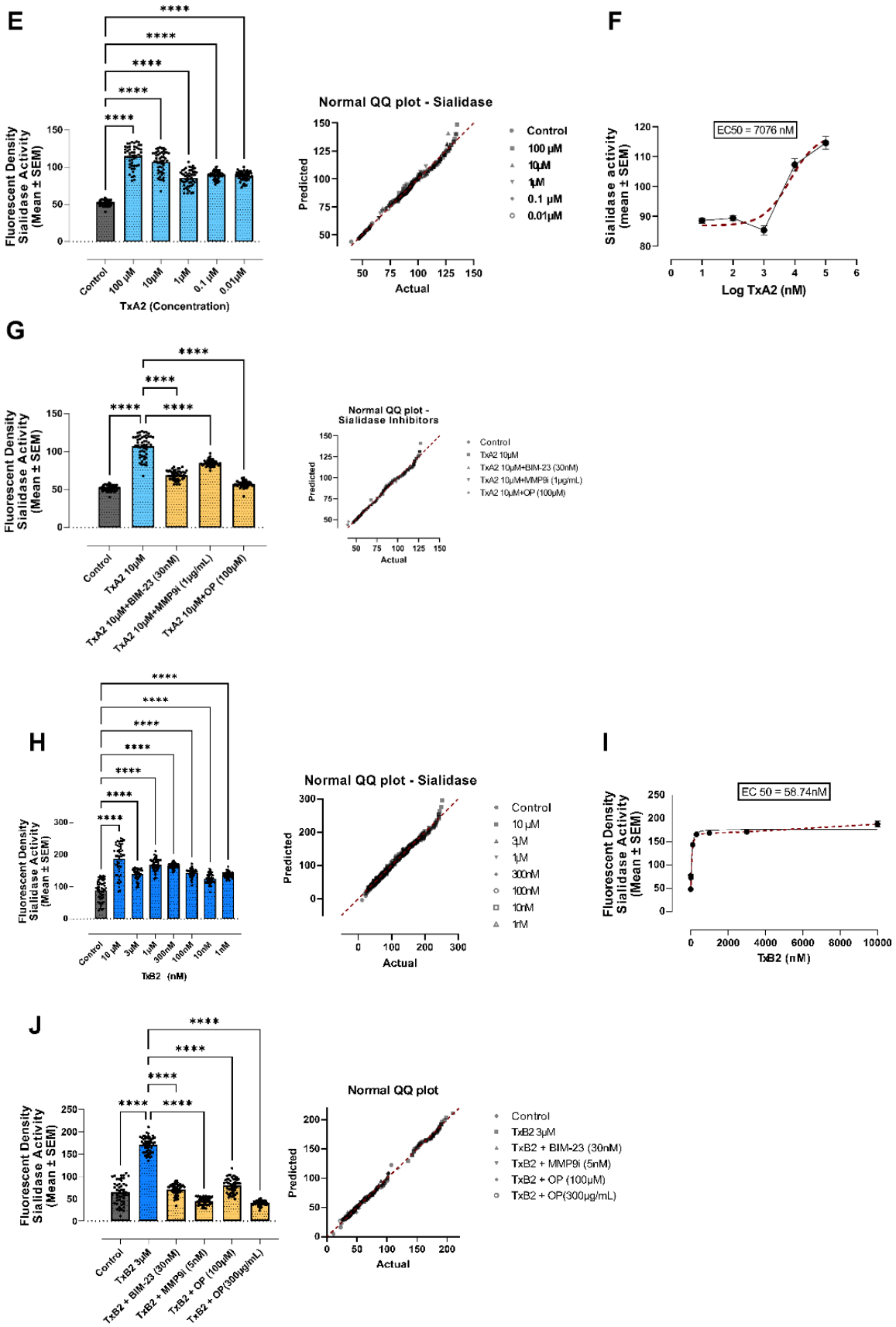
Thromboxane TXA2, TXB2, and U46619 induce NEU-1 sialidase activation in Jurkat T-cell line

Jurkat T-cells are a well-established human T-cell leukemia cell line that possess inherent anti-tumor properties and can be genetically modified to augment its effectiveness in cancer immunotherapy [12]. These cells can recognize and attack cancer cells, and to specifically target tumor sites. Jurkat T-cells have a multifaceted targeting approach to kill cancer cells, involving direct cytotoxicity, secretion of cytokines and chemokines, antibody-dependent cell-mediated cytotoxicity, and antibody-dependent cell-mediated phagocytosis [13]. Jurkat T-cells can be activated via specific antigens such as proteins or peptides on the surfaces of cancer cells [14].

Interestingly, Jurkat cells have been shown to express various growth factor receptors, including the insulin-like growth factor-I receptor (IGF-IR) [15,16]. Notably, several signaling molecules have been identified to play a critical role in the regulation of insulin-induced IR activation. Among these, the G protein-coupled receptors (GPCR) have been implicated in intracellular crosstalk pathways with IR [8,17,18]. Since thromboxane TXA2 binds to its GPCR that can transactivate EGFR[7] or the insulin receptor [8], we asked if thromboxane agonists like TXA2 binding to its GPCR can induce the activation of NEU-1 sialidase in the Jurkat T cell line, which expresses the IGF-IR.

The data depicted in Figure 2 predictably upregulated NEU-1 activity after exposure to a stable TXA2 GPCR agonist U46619 dose-dependently with an EC50 of 2.96 nM (**Figure 2 A-D**). We also found that specific inhibitors of MMP9, NEU-1, and NMBR GPCR depicted in Figure 1, all reduced NEU-1 activation in response to U46619 stimulation (**Figure 2 D**). We also tested this signaling paradigm as depicted in Figure 1 using thromboxane agonists, TXA2 (Figure 2 E-G) and TXB2 (Figure H-J). These data support the molecular heterodimerization of the thromboxane GPCR with NMBR to activate MMP9 and NEU-1 sialidase activity to transactivate growth factor receptors like IGF-IR expressed on Jurkat cells. These results also confirm that NEU-1 is activated in this cell line after exposure to U46619, TXA2 or TXB2, and that inhibition of these agonist-induced NEU-1 is specifically inhibited by OP, MMP-9i and BIM-23127.




E
Fluorescent Density Sialidase Activity (Mean ± SEM)
Control
100 µM
10µM
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TxA2 (Concentration)
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EC50 = 7076 nM
Log TxA2 (nM)
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TxA2 10µM+BIM-23 (30nM)
TxA2 10µM+MMP9i (1µg/mL)
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I
EC 50 = 58.74nM
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J
TxB2 3µM
TxB2 + BIM-23 (30nM)
TxB2 + MMP9i (5nM)
TxB2 + OP (100µM)
TxB2 + OP(300µg/mL)
Normal QQ plot

Figure 2. Thromboxane agonists U46619 (A-D), TXA2 (E-G), and TXB2 (H-J) increase NEU-1 sialidase activity in Jurkat T-cells dose-dependently, while OP, MMP9i, and BIM23 inhibitors of signaling paradigm (A) reverse thromboxane agonists NEU-1 activity. Jurkat cells were treated with indicated agonist doses and further treated in combination with the inhibitors for one minute. 4-MUNANA substrate was added to live cells to measure sialidase activity (emission 450 nm, excitation 365 nm). The mean ± SEM (biological replicates: 5, technical replicates: 50) fluorescence density of sialidase activity demonstrates a highly significant ($p < 0.0001$) downregulation of sialidase activity with all three inhibitors. A normal QQ plot depicts the normality of each of the datasets, modeled by a normal distribution. Abbreviations: TGFβ-1: transforming growth factor beta-1; IL-6: in-terleukin-6; HGF: hepatocyte growth factor; OP: oseltamivir phosphate; MMP9i: MMP9 inhibitor; BIM23: BIM-23127. **** $p < 0.0001$.

Thromboxane TXA2 GPCR Colocalizes with NEU-1 on the Cell Surface of Naïve Unstimulated Jurkat T-cells

The data depicted in Figures 2 demonstrate that U46619 (A-D), TXA2 (E-G), and TXB2 (H-J) binding to their respective GPCRs induced NEU-1 sialidase activity. Here, we investigated whether the thromboxane GPCR forms a complex with the signaling platform of NEU-1 and MMP-9 crosstalk in alliance with the NMBR GPCR on the cell surface Jurkat cells. As depicted in Figure 3, the cells were treated with equal amounts of Alexa Fluor™ 594-conjugated monoclonal antibody for the thromboxane GPCR TXA2 and the Alexa Fluor™ 488-conjugated monoclonal anti-NEU-1 antibody. The colocalization was quantified using a Pearson correlation coefficient (R-value; $0.5 < r < 0.59$ = moderate positive correlation; $0.6 < r < 0.89$ = strong positive correlation; $r > 0.9$ = near perfect correlation).

For naïve Jurkat T-cells, the TXA2 GPCR and NEU-1 had a moderate positive correlation (TXA2: $r = 0.41 \pm 0.06$ (Figure 3). These data confirm that TXA2 GPCR forms a moderate complex with NEU-1 expressed on the Jurkat T-cell line.

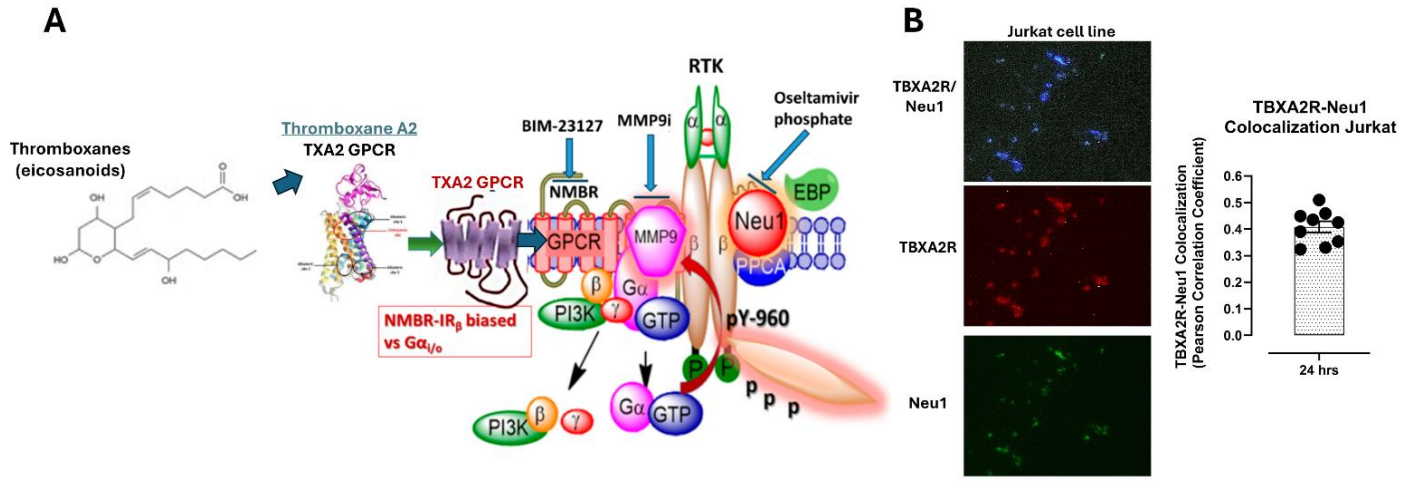

Figure 3. (A) Thromboxane TXA2 GPCR colocalizes with NEU-1 in Jurkat T-cells. (B) Cells were fixed, permeabilized, blocked, and immunostained with the specific antibodies. Cells were treated with Alexa Fluor™ 594-conjugated antibody for the TXA2 GPCR and Alexa Fluor™ 488-conjugated NEU-1 monoclonal antibody. (A) Merging of fluorescent images acquired from Zeiss M2 epi-fluorescent microscope (20× objective magnification) shows areas of colocalization through yellow fluorescence. Pearson correlation coefficient (R-value) measured a moderate positive correlation ($0.4 < r < 0.5$) between the TXA2 GPCR and NEU-1 (biological replicates: 4, technical replicates).

Celecoxib, aspirin and oseltamivir phosphate inhibition of NEU-1 impede thromboxane TXA2 induced ARHGEF1 expression in Jurkat T-cells.

TXA2 is produced by platelets under the control of cyclooxygenase 1 (Cox-1) but not cyclooxygenase-2 (COX-2). Aspirin blocks both COX-1 and COX-2, but celecoxib specifically blocks COX-2 and does not affect TXA2 production. Both celecoxib and aspirin have been reported to reduce significantly disease recurrence in patients with colorectal cancer harboring the PI3K3CA mutation[1,2]. These findings reveal a novel immunosuppressive pathway that limits T cell immunity to cancer metastasis. Qorri et al.[11] have reported on a novel multimodality mechanism(s) of action for aspirin and celecoxib, specifically targeting and inhibiting NEU-1 activity, as depicted in Figure 1.

Here, we investigated whether NEU-1 inhibitors, oseltamivir phosphate (OP), aspirin (ASP), and celecoxib (celec), would reduce the expression of the guanine exchange factor ARCGH1 after exposure to TXA2. As shown in Figure 4, all inhibitors of NEU-1 significantly reduced the expression of ARCGH1 in Jurkat T-cells after exposure to TXA2. Notably, we did not observe a significant difference in the baseline level of expression of ARCGH1 in the control or TXA2 stimulated cells, but we did note a significant reduction in the expression of ARCGH1 from TXA2 in combination with aspirin, celecoxib and OP.

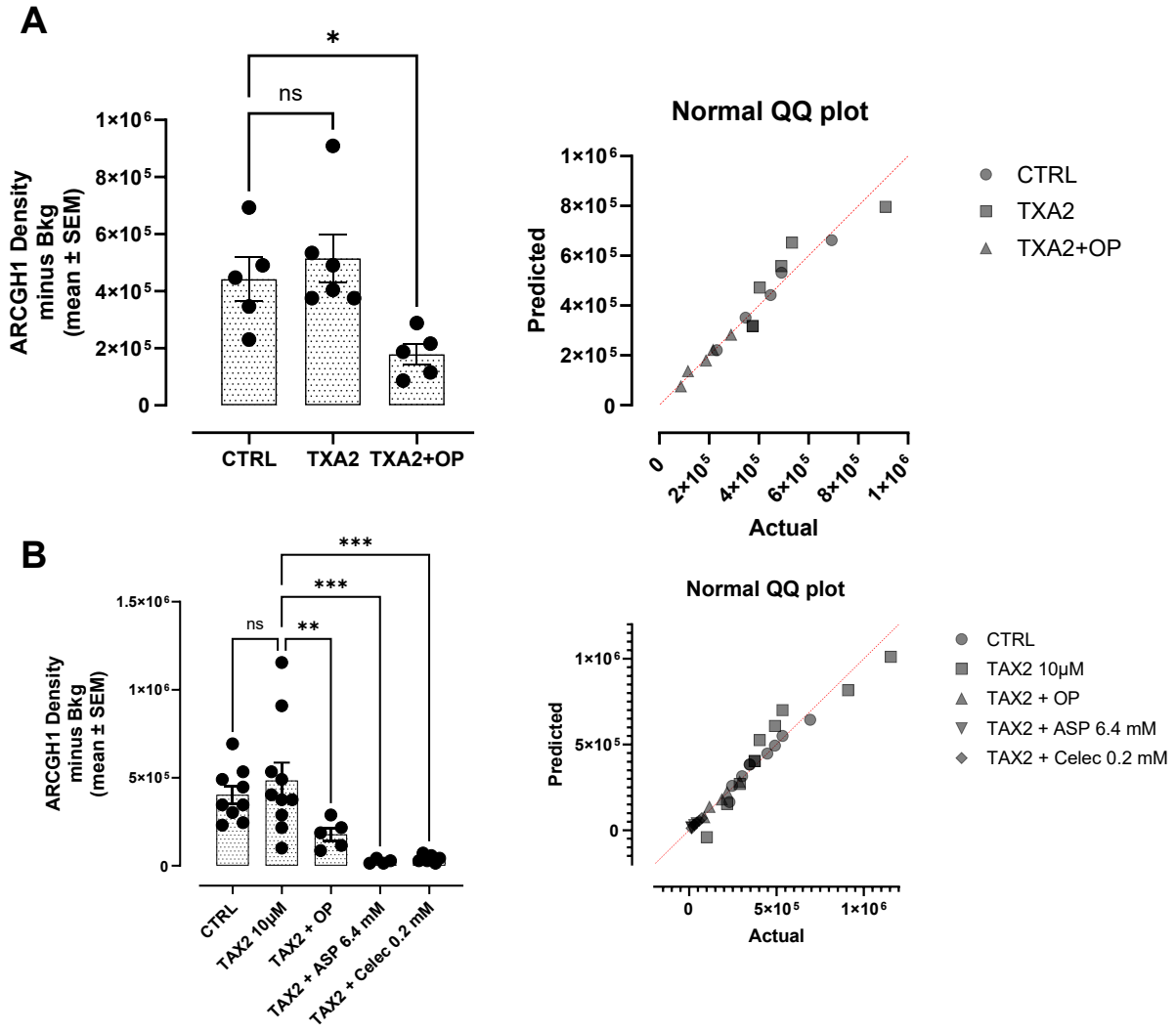

Figure 4. Analyses of Jurkat T- cells for ARCGH1 expression following 24-hour exposure to TXA2 in a combination of ASA, celecoxib and OP cocktail using immunocytochemistry. (A) Jurkat T-cells were plated at 150,000 cell/mL and left untreated, treated with TXA2 (10 μM) in combination with OP (300 μg/mL), ASP (6.4 mM) or celecoxib (0.2 mM) for 24 hours. Cells were immunostained for ARCGH1 expression using rabbit anti-ARHGEF1 and secondary goat anti-rabbit -Alexa 594. The results are depicted as a scatter plot of data visualization using dots to represent the indicated ARCGH1 expression values obtained from 2 independent experiments of multiple images (n = 4–10). The mean density staining corrected for background (Bkg) ± S.E.M. for indicated ARCGH1 expression values is indicated for each group. The density staining values of each group were compared to the untreated control as well as TXA2 in combination with inhibitor cocktail by ANOVA using the uncorrected Fisher's LSD multiple comparisons test with 95% confidence with indicated asterisks for statistical significance. (B) Immunocytochemistry of untreated and TXA2 (10 μM) treated Jurkat cells for 24 hrs in combination with ASP, celecoxib and OP. Data are presented as the percentage of cells expressing ARCGH1corrected for

background autofluorescence. ns, not significant, *p ≤ 0.01, **p ≤ 0.001, ***p ≤ 0.0001. Abbreviations: ASP, aspirin (acetylsalicylic acid); celec, celecoxib; OP, oseltamivir phosphate.

Discussion

Platelet derived TXA2 has been reported to act on T cells to trigger an immunosuppressive pathway dependent on the guanine exchange factor ARHGEF1 [4], suppressing T cell receptor-driven kinase signalling, proliferation and effector functions. T cell-specific conditional deletion of the *Arhgef1* gene in mice increased T cell activation at the metastatic site, thereby provoking an immune-mediated rejection of lung and liver metastases [4]. TXA2 is produced by platelets under the control of cyclooxygenase 1 (COX-1). Celecoxib specifically blocks COX-2 and does not affect TXA2 production. Both celecoxib and aspirin dramatically reduced disease recurrence in patients with colorectal cancer harboring the *PIK3CA* mutation [1,2]. These benefits were surprising and staggering: Low-dose aspirin for three years after surgical removal of the primary tumor reduced the risk of colorectal cancer recurrence by around 50% in patients with *PIK3CA* mutations (hazard ratio ~0.49) [1]. Similarly, patients with *PIK3CA* mutated colorectal tumors receiving celecoxib after surgery had their risk of dying reduced by about 50% to 60% [2].

Here we show that the TXA2 G-protein coupled receptor (GPCR) on the Jurkat T-cell line is regulated by mammalian neuraminidase-1 (NEU-1), and that blocking NEU-1 with aspirin, celecoxib and oseltamivir phosphate reduced significantly the expression of ARHGEF1. The reduction in the expression of ARHGEF1 triggered by TXA2 binding to the GPCR on the T-cell would be predicted to be important in the preservation of a T cell mediated immune response as previously revealed[4]. Our discovery that aspirin, celecoxib and oseltamivir phosphate can inhibit ARHGEF1 expression on T-cells via the inhibition of NEU-1 may explain the contemporaneous results of why these drugs demonstrated comparable clinical efficacy in the cited human clinical trials. This discovery may solve the puzzle of why both aspirin and celecoxib had almost identical clinical efficacy in human clinical trials despite only aspirin being able to inhibit TXA2 expression via its effect on COX-1 expression.

Surgical removal of a primary cancerous tumor has been shown to increase the number of circulating tumor cells and can trigger an inflammatory response that can promote tumor cell growth, impair the immune response, and facilitate metastasis [6]. Thromboxane A2 is significantly upregulated after cancer surgery, particularly in GI malignancies, and the use of aspirin to target this upregulation after surgical removal of a primary tumor has previously been proposed [19]. The recent clinical study results using aspirin or celecoxib after surgical removal of *PIK3CA* mutated colorectal cancer

showing almost identical clinical benefit suggest a similar mechanism of action against this cancer.

Why patients with the *PIK3CA* mutation received such curative benefit from treatment with these simple oral medications was unclear until now. *PIK3CA* mutations have previously been shown to recruit myeloid-derived suppressor cells (MDSCs) that could suppress the activity of cytotoxic T cells via the arachidonic acid (AA) metabolism pathway [20]. *PIK3CA* expression in tumors was found to decrease the number of $CD8^+$ T cells but to increase the number of inhibitory myeloid cells following immunotherapy [21]. Myeloid derived suppressor T cells have been hypothesized to hide or cloak circulating tumor cells from immune surveillance [22] and their presence in the circulation is predictive of disease recurrence after cancer surgery [23].

Whether the impressive clinical results using aspirin and celecoxib after surgery in the *PIK3CA* mutated colorectal cancer patients could be extended to other *PIK3CA* mutated cancers is currently unknown. This is not a trivial matter as the results in the colorectal cancer patients using inexpensive and relatively safe oral drugs were dramatic and offered a curative treatment with minimal side effects. *PIK3CA* mutations/amplifications occur at varying frequencies in different forms of cancer. Notably, these genetic alterations are present in approximately 30 to 35 percent of breast, endometrial, and cervical cancers [24]. In addition, *PIK3CA* mutations have also been identified in cancers such as colorectal, gastric, ovarian, and lung cancer, among others [24]. This broad distribution highlights the significance of *PIK3CA* mutations in the molecular landscape of various malignancies. The therapeutic targeting of NEU-1 as an anti-cancer treatment should be further explored in clinical trials, particularly in patients harboring *PIK3CA* mutations. This treatment would be predicted to be particularly important after surgical removal of the primary tumor and the time when effective treatment can make the difference between disease eradication or metastatic spread of the cancer [25,26].

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Methods

Cell lines

Human Jurkat leukemia T cells (Clone E6-1, TIB-152 ™) obtained from the American Type Culture Collection, Manassas, VA 20110-2209, USA, are an immortalized human T-lymphocyte cell line derived from a patient with T-cell acute lymphoblastic leukemia (T-ALL). Cells were grown in culture media containing Dulbecco's Modified Eagle's Medium (DMEM) (Gibco, Rockville, MD, U.S.A.) supplemented with 10% fetal bovine serum (FBS) (HyClone, Logan, UT, U.S.A.) and 5 μg/mL plasmocin (InvivoGen, San Diego, CA, U.S.A.) as a prophylactic inhibitor of mycoplasma. Cells with single passage were maintained in an incubator with 5% CO_2 at 37°C until 75% confluence was reached.

Reagents

U-46619 (Item No. 16450, Formal Name, (5Z)-7-[(1R,4S,5S,6R)-6-[(1E,3S)-3-hydroxy-1-octen-1-yl]-2-oxabicyclo[2.2.1]hept-5-yl]-5-heptenoic acid) (Cayman Chemical, Ann Arbor, Michigan 48108 USA) is a stable analog of the endoperoxide prostaglandin H2, and a TP receptor agonist.1 It exhibits properties similar to thromboxane A2 (TXA2), causing platelet shape change and aggregation, and contraction of vascular smooth muscle. 15(S)-Pinane Thromboxane A2 (Item No. 19020, Formal Name, (5Z)-7-[(1S,2R,3R,5S)-3-[(1E,3S)-3-hydroxy-1-octen-1-yl]-6,6-dimethylbicyclo[3.1.1]hept-2-yl]-5-heptenoic acid) (Cayman Chemical, Ann Arbor, Michigan 48108 USA) is a stable analog of TXA2. Thromboxane B2 (Item No. 19030, Formal Name, 9α,11,15S-trihydroxythromba-5Z,13E-dien-1-oic acid) (Cayman Chemical, Ann Arbor, Michigan 48108 USA) is a non-enzymatically derived, stable, inactive metabolite of TXA2, and positively correlates with platelet COX-1 activation.

Rabbit anti-ARHGEF1 antibody (Cat # PA578821, Life Technologies, Inc.) was obtained from Thermo Fisher, Canada at a dilution of 1 μg (per $1x10^6$ cells), followed by Alexa-594 conjugated goat anti-rabbit IgG for 30 minutes at 4°C using 5-10 μg (per $1x10^6$ cells) dilution. Rabbit monoclonal anti-TBXA2 receptor (Cat # MA557283, Life Technologies, Inc.) was obtained from Thermo Fisher, Canada, at a dilution of 1:100, followed by Alexa-594 conjugated goat anti-rabbit IgG for 30 minutes at 4°C using 5-10 μg (per $1x10^6$ cells) dilution.

Inhibitors

Oseltamivir phosphate (OP) (>99% pure OP, batch No. MBAS20014A, Solara Active Pharma Sciences Ltd., New Mangalore-575011, Karnataka, India), a broad-spectrum sialidase inhibitor that inhibits NEU-1, was used at a concentration of 300 μg/mL, as previously reported by Baghaie et al. [26]. Acetylsalicylic acid (>99% pure, Sigma-Aldrich, Steinheim, Germany) was dissolved in dimethyl sulfoxide (DMSO) to prepare a 5000 mM stock solution and stored in aliquots at −20°C. The highest used concentration of aspirin contains less than 0.5% v/v of DMSO in 1× PBS at a pH of 7. Celecoxib (Marcan Pharmaceuticals Inc., Ottawa, ON, Canada) was dissolved in DMSO to prepare a stock solution of 52.44 mM and stored in aliquots at −20°C.

Live Cell Sialidase Assay

Jurkat T- cells at a density of 100,000 cells/well were centrifuged at 900 rpms on 12 mm circular glass slides in sterile 24-well plates. 2 μL of 0.318 mM 2'-(4-methylumbelliferyl)-*α*- D-N-acetylneuraminic acid sodium salt hydrate (4-MUNANA; ≥95% pure, M8639, Sigma-Aldrich) substrate in Tris-buffered saline (TBS, pH 7.4) was added to a coverslip alone as a control, with 2 μL of 30 ng/mL TXA2 (, Cedarlane), or with 2 μL of TXA2 and 2 μL of aspirin or celecoxib at the indicated concentrations. 4-MUNANA is hydrolyzed by sialidase activity on the cell surface, releasing 4-methyl-lumbelliferone (4-MU), which has a fluorescence emission at 450 nm (blue color) following excitation at 365 nm. Fluorescent images were taken at 20× after 1–2 minutes of treatment using the Zeiss M2 Imager epi-fluorescent microscope. The mean fluorescence of 50 multi-point replicates surrounding the cells was quantified using the ImageJ software.

Co-Localization

Jurkat T- cells at a density of 100,000 cells/well were centrifuged at 900 rpms on 12 mm circular glass slides in sterile 24-well plates. Cells were fixed with 4 μg/mL paraformaldehyde (PFA) for 24 h at a 5 ◦C refrigerator. Subsequently, cells were permeabilized with 0.1% TritonX in PBS (PBST) for 5 min and then blocked with 4% BSA in 0.1% Tween-TBS in the fridge for 1 h. Cells were treated with monoclonal rabbit anti-TXA2 receptor at 4°C for 24 hrs, washed 3x with PBS and followed with Alexa-488 conjugated mouse anti-NEU-1 antibodies at 4°C for 24 hrs. Coverslips with stained cells were mounted on microscope slides onto 3 μL of DAPI fluorescent mounting medium. Slide images were observed using ZeissM2 epi-fluorescent microscopy (40× magnification, Carl Zeiss Canada Ltd., M3B 2S6 Toronto, Canada), capturing images under green (488 nm) and red (594 nm) channels. The Pearson correlation coefficient quantifies protein co-localization in the acquired images, and the results were expressed as a percentage determined using AxioVision software, Rel. version 4.6. Pearson's correlation coefficient measures the linear

proximity and association between two variables. A correlation value of 0.5 to 0.7 between the two variables would indicate that a significant and positive relationship exists between the two.

Immunocytochemistry

Jurkat T-cells plated at 150,000 cell/mL were centrifuged at 900 rpms on 12 mm circular glass slides in sterile 24-well plates. Cells were left untreated, treated with TXA2 (10 μM) or in combination with OP (300 μg/mL), ASP (6.4 mM) or celecoxib (0.2 mM) for 24 hours at 37°C. Cells were washed and fixed with 4% paraformaldehyde (PFA) for 30 minutes, followed by permeabilization with 0.1% TritonX in PBS (PBST) for 10 minutes. Cells were blocked with 4% BSA in PBST for 1 hour at 4°C. Following blocking, cells were washed with 1× PBS 3× for 10 minutes, followed by the addition of rabbit monoclonal anti-ARCGH1 diluted in 4% BSA in PBST in a 1:20 ratio overnight at 4°C. Rabbit serum were used and diluted in the same ratio as primary antibodies for isotype controls. Cells were washed 3× for 5 minutes with 1× PBS and incubated for 1 hour with Goat anti-rabbit AlexaFluor Plus 594 (A32740, Invitrogen). Cells were then washed 3× for 10 minutes. Coverslips with attached cells were inverted on a droplet of mounting media containing 4′,6-diamidino-2-phenylindole (DAPI; VECTH1200, MJS BioLynx Inc., P.O. Box 1150, 300 Laurier Blvd., Brockville, ON K6V 5W1, Canada) to visualize the nuclei and sealed. The stained cells were visualized by epifluorescence microscopy at 20× mag.

Statistical Analysis

All statistical analyses were performed using GraphPad Prism 10 software and are presented as the mean ± standard error of the mean (SEM). Comparisons between groups from two to three independent experiments were made by one-way analysis of variance (ANOVA) at 95% confidence using the uncorrected Fisher's LSD multiple comparisons test with 95% confidence. Asterisks denote statistical significance.

Patents. M.R.S. reports patents for the use of NEU-1 sialidase inhibitors in cancer treatment (Canadian patent no. 2858,246; United States patent no. US2015/0064282 A1; European patent no. 1874886.2; Chinese patent no. ZL201180076213.7; German patent no. 602011064575.7; Italian patent no. 502020000014650; UK patent no. 2773340; Swedish patent no. 2773340; Spanish patent no. 773340; Switzerland patent no. 2773340; and French patent no. 2773340). M.R.S. reports a patent for using oseltamivir phosphate and analogs thereof to treat cancer (International PCT patent no. PCT/CA2011/050690). W.W.H. and M.R.S. report a patent on a method to improve the effectiveness of anti-cancer therapies by exposing them to an inflammatory stimulus prior to treatment (Canadian patent no. PCT/CA2017/050765, pending). W.W.H. and M.R.S. report a patent on the compositions and methods for cancer treatment (Cana-dian patent no. PCT/CA2017/050768, pending). W.W.H. and M.R.S. report patents 55983477-9CN and CAN_DMS_150056368.1 on the COMPOSITIONS AND METHODS FOR THE TREATMENT OF CORONAVIRUS INFECTION AND RESPIRATORY COM-PROMISE.

Clinical Trials USFDA. W.W.H. and M.R.S. have US FDA clinical trial approval to test OP in patients with pancreatic cancer (clinical trial number #173874).

Abbreviations: PFA: paraformaldehyde, BSA: Bovine Serum Albumin, SEM: mean ± standard error of the mean, ANOVA: one-way analysis of variance, FBS: Fetal Bovine Serum, MMP9i: metalloproteinase inhibitor, MET: mesenchymal-epithelial transition, LPS: lipopolysaccharide.

Author Contributions: W.W.H., YL, and M.R.S. analyzed the literature and contributed to writing and revising the manuscript. All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or all these areas; took part in drafting, revising, or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work. All authors have read and agreed to the published version of the manuscript.

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Informed Consent Statement: Not applicable.

Data Availability Statement: All data needed to evaluate the paper's conclusions are present. The preclinical data sets generated and analyzed during the current study are not publicly available but can be obtained from the corresponding author upon reasonable request. The data will be provided following the review and approval of a research proposal, Statistical Analysis Plan, and execution of a Data Sharing Agreement. The data will be accessible for 12 months for approved requests, subject to possible extensions; contact szewczuk@queensu.ca for more information on the process or to submit a request.

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Conflicts of Interest: William W. Harless owns shares in ENCYT Technologies Inc. and has a commercial interest and/or patents in the work under consideration**.**

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