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Suramin potently inhibits cGAMP synthase, cGAS, in THP1 cells to modulate IFN- β levels

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Aim: Persistent activation of STING pathway is the basis for several autoimmune diseases. STING is activated by cGAMP, which is produced by cGAS in the presence of DNA. **Results/methodology:** HPLC-based medium throughput screening for inhibitors of cGAS identified suramin as a potent inhibitor. Unlike other reported cGAS inhibitors, which bind to the ATP/GTP binding site, suramin displaced the bound DNA from cGAS. Addition of suramin to THP1 cells reduced the levels of IFN- β mRNA and protein. Suramin did not inhibit lipopolysaccharide- or Pam3CSK4-induced IL-6 mRNA expression. **Conclusion:** Suramin inhibits STING pathway via the inhibition of cGAS enzymatic activity. Suramin or analogs thereof that displace DNA from cGAS could be used as anti-inflammatory drugs.

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The presence of nucleic acid in the cytoplasm induces a strong immune response via activation of the cGAS-STING pathway [1]. DNA in the cytoplasm is recognized by cyclic GMP-AMP synthase (cGAS), which catalyzes the synthesis of the signaling molecule 2',3'-cGAMP (cGAMP) from ATP and GTP [1]. cGAMP binds to the stimulator of interferon (IFN) genes (STING) and triggers a type I IFN response (Figure 1) [2]. Although activation of the cGAS-STING pathway plays a key role in host-pathogen interaction [3,4], persistent activation of cGAS by endogenous DNA can result in autoinflammatory disorders [5]. cGAS has been reported to stimulate cytokine production in *Trex1*^{-/-} deficient mice, which results in inflammation and death. Furthermore, cGAS deletion in *Trex1*^{-/-} mice can rescue mice from lethal autoimmune phenotypes (Figure 1) [5,6].

When DNA is present in the cytosol, it is an indication of damage (such as DNA leakage from the nucleus or mitochondria) [12] or the DNA could be a danger signal from an invading virus or bacteria [13]. Cytosolic DNA may be degraded by the exonuclease TREX1 [13–15] or it can interact with cGAS, leading to the activation of cGAS to produce 2',3'-cGAMP, which binds to and activates STING [4,16]. STING, an endoplasmic reticulum adaptor protein [17], activates TBK1, which phosphorylates IRF3 (Figure 1) [18]. Phosphorylated IRF3 dimerizes and crosses into the nucleus, where it associates with the *IFN- β* gene and, with c-Jun and ATF-2, facilitates the expression of the IFN- β mRNA, which ultimately leads to increased levels of IFN- β protein [19–21]. This results in a type I IFN immune response (Figure 1) [13]. If DNA is not cleared from the cytoplasm, for example, in instances whereby TREX1 is inactive due to mutation, the type I IFN immune response may be persistently activated and lead to autoimmune diseases such as Aicardi-Goutières syndrome (AGS) and lupus [14–15,22].

Efforts are underway by a number of research groups to develop new inhibitors of cGAS or to repurpose current drugs for cGAS inhibition. Recently, several antimalarial drugs were screened *in silico* using mouse cGAS/DNA target (PDB 4LEZ) to identify compounds that inhibit cGAMP synthesis. The identified cGAMP synthesis inhibitors, hydroxychloroquine, quinacrine (QC) and 9-amino-6-chloro-2-methoxyacridine (ACMA), which inhibited cGAMP synthesis with micromolar IC₅₀ values, did not bind to the active site of cGAS but were instead found to localize to the minor groove of DNA between the cGAS/DNA interface [23].

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Figure 1. Schematic representation of cGAS-STING pathway and its role in autoimmune disease. Also shown are condensed pathways for activation of TLR pathways by LPS [7] and Pam3CSK4 [8] to activate IL-6 gene expression via NF-κβ [9] and the inflammasome-mediated activation of IL-1β [10] via caspase-1 [11]. LPS: Lipopolysaccharide; TLR: Toll-like receptor.

Recently, other small molecules, such as RU.521 and RU.365, were found to be inhibitors of cGAS [24]. Unlike the antimalarial compounds that bound to the DNA in the cGAS/DNA complex, the crystal structure of RU.365 in complex with mouse cGAS/dsDNA complex indicated that these new cGAS inhibitors acted at the cGAS active site [24]. RU.521 displayed activity in a cellular assay. Using a novel fluorescence polarization assay, a Pfizer group identified PF-06928215 as a potent inhibitor of human cGAS activity ($IC_{50} = 4.9 \mu M$). PF-06928215, however, displayed no activity in cellular cGAS assays measuring dsDNA-induced IFN-β expression [25].

In our ongoing efforts to identify small molecule inhibitors of cyclic dinucleotide metabolism enzymes [26–28], we have also been trying to identify inhibitors of cGAMP synthesis by cGAS. Here, we disclose that suramin, which is

on the WHO list of essential drugs for the treatment of river blindness and African sleeping sickness [27], potently inhibits the formation of 2',3'-cGAMP by human cGAS/DNA complex.

Materials & methods

The fluorescently labeled and unlabeled 100-bp dsDNA (HPLC-purified) were obtained from Midland Certified Reagent Company, Inc. (TX, USA). DNA gel electrophoresis indicated that the purchased DNA was pure. Primers for reverse transcription polymerase chain reaction (RT-PCR) were purchased from Integrated DNA Technologies, Inc. (IL, USA). Suramin sodium salt ($\geq 99\%$), Deoxyribonucleic acid sodium salt from herring testes, lipopolysaccharides (phenol extraction), Poly(I:C) ($\geq 99\%$), proflavine hemisulfate salt hydrate and diminazene aceturate (analytical standard) were purchased from Sigma-Aldrich (MO, USA). Pam3CSK4 was purchased from Invivogen (CA, USA). Lipofectamine[®] LTX with Plus[™] Reagent, SuperScript[™] II reverse transcriptase, random hexamers, DAPI were purchased from Thermo Fisher Scientific (MA, USA). Aurum[™] Total RNA Mini Kit was purchased from Bio-Rad (CA, USA). Deoxynucleotide (dNTP) Solution Mix was purchased from New England Biolabs (MA, USA). QuantiTect SYBR[®] Green PCR Kit was purchased from QIAGEN (MD, USA). All reagents were used as purchased without further purification unless specified.

Cell culture methods & maintenance

THP1-Dual[™] cells and THP1-Dual KO-cGAS cells were purchased from InvivoGen. Cells were cultured in RPMI 1640 containing 10% heat inactivated FBS, 10 mM HEPES, 2 mM glutamine, 100 U/mL penicillin and 100 mg/mL streptomycin, and maintained at 37°C under 5% CO₂. All reagents and medium were purchased from Fisher Scientific, (PA, USA) unless otherwise noted. Cells were cultured in EMEM containing 10% FBS and 10 mM HEPES. EMEM was purchased from ATCC (VA, USA).

Expression & purification of hcGAS

Full-length human 6xHis-MBP-tagged plasmid was donated by the Doudna lab [29]. cGAS was expressed in BL21-RIL DE3 along with pRARE2 human tRNA plasmid. hcGAS was purified as previously described with slight modification. Briefly, bacteria were grown in 2XYT media at 37°C to an OD (optical density at 600 nm) of approximately 0.6 and induced with 0.5 mM IPTG for 20 h at 16°C. Cells were sonicated in lysis buffer containing 20 mM Tris-HCl (pH 7.5), 400 mM NaCl, 10% glycerol, 30 mM imidazole and 1 mM PMSF. The protein was passed through a HisTrap HP 1 mL column and washed with lysis buffer. hcGAS was eluted with lysis buffer supplemented with 300 mM imidazole [29]. TEV protease was added to the eluted cGAS to remove the His-MBP tag. The mixture was passed through a HisTrap HP 1 mL column to purify the cGAS from the His-MBP tag [30]. cGAS protein purity is shown in Supplementary Figure 1.

cGAS HPLC activity assay

Assay was performed as described previously with slight modifications [29,31]. The reaction mixture included 20 μ M library compound, 3 μ M enzyme, 200 μ M ATP, 200 μ M GTP, 10 mM MgCl₂, 5 mM CoCl₂ and 0.1 mg/mL herring testes-DNA (HT-DNA) in 20 mM Tris buffer (pH 7.5) and was incubated at 37°C for 2 h. HPLC analysis was used to detect cGAMP, the product of cGAS activity [29]. To identify potential inhibitors of cGAS, we performed an initial screening with suramin and kinase inhibitors (see Supplementary Table 2 for commercial kinase inhibitors screened) utilizing HPLC. The Sigma Kinase and Sigma PDE compounds were from Sigma-Aldrich.

Thin layer chromatography

To verify cGAMP inhibition, we used a modified radioactive assay [29]. Briefly, the assay mixture contained 20 mM Tris (pH 7.5), 200 μ M ATP, 200 μ M GTP, 11 nM ³²P-ATP, 10 mM MgCl₂, 5 mM CoCl₂, 0.1 mg/mL HT-DNA and 20 μ M suramin (or an equivalent volume of water as control). The reaction was initiated by the addition of 3 μ M enzyme and was incubated for 2 h at 37°C. The reaction was terminated by holding at 95°C for 5 min. A 0.5 μ L aliquot of the reaction mixture was spotted onto a cellulose plate. Plates were developed using saturated (NH₄)₂SO₄ and 1.5 M KH₂PO₄ buffer (1:1.5, v:v). The plates were dried and exposed to Molecular Dynamics Phosphor Imager Screen overnight.

Electrophoretic mobility shift assay

Electrophoretic mobility shift assay (EMSA) was performed to interrogate the dsDNA-binding of cGAS, in the presence or absence of suramin *in vitro*. For the dsDNA-binding studies, 1 μM 100-bp IFN-stimulatory dsDNA labeled with FAM was incubated with 5 μM cGAS and incubated at room temperature for 1 h [23]. Suramin was added at concentrations of 0, 1, 10, 100 and 200 μM and incubated for an additional 2 h at room temperature. The mixtures were resolved on a 6% polyacrylamide gel by electrophoresis and visualized using a FluorChem R system (Protein Simple, CA, USA).

Effects of suramin on IFN- β expression

Cellular levels of IFN- β were detected via a luminescence assay as previously described [24]. Briefly, 1.0×10^5 THP1-Dual cells were seeded in RPMI 1640 according to the manufacturer's protocol in 24-well plates. THP1-Dual cells were transfected with 0.5 μg of HT-DNA using Lipofectamine LTX with Plus Reagent (Thermo Fisher Scientific) in the presence of 200 nM, 1 μM or 5 μM suramin. Each condition was replicated at least in triplicate. The cells were harvested after 24 h and lysed with PierceTM Luciferase Cell Lysis Buffer (Thermo Fisher Scientific). The QUANTI-LUCTM luminescence assay (Invivogen) was performed to detect changes in IFN- β expression according to the manufacturer's procedure. Plates were read using a CytationTM5 Multi-Mode Microplate Reader.

For mRNA analysis, 5.0×10^5 THP1-Dual cells were seeded in RPMI 1640 according to the manufacturer's protocol in 6-well plates. THP1-Dual cells were transfected with 2.5 μg of HT-DNA using Lipofectamine LTX with Plus Reagent in the presence of 0.2, 1 or 5 μM suramin. Each condition was replicated in triplicate. Cells were harvested 24 h post-transfection, and RNA was extracted by Aurum Total RNA Mini Kit. Total RNA was reverse transcribed using random hexamer primer and SuperScript II reverse transcriptase into cDNA. Real-time PCR was carried out with QuantiTect SYBR Green PCR Kits and run on a BIORAD C1000 Touch Thermal Cycler. The IFN- β primer [32] used is listed in Supplementary Table 1. Target C_q values were normalized to GAPDH C_q values and used to calculate ΔC_q ($C_{q(\text{target})} - C_{q(\text{ref})}$). The relative gene expression was obtained by $\Delta\Delta C_q$ calculation method. This is accomplished by normalization of a gene target with experimental treatment to an endogenous reference gene (GAPDH).

For the experiment of dsDNA transfection for 30 min, 5.0×10^5 THP1-Dual cells were seeded and transfected with HT-DNA for 30 min using the same protocol. The media containing DNA was then removed and cells were washed with fresh media. Cells were then cultured in fresh media for 24 h in the presence of 0 or 5 μM suramin. mRNA expression values were analyzed by the previously mentioned method.

Effects of suramin on cellular cytokine expression

5 μM suramin was co-incubated with 200 ng Pam3CSK4 (Invivogen), 25 μg Poly(I:C) or 100 ng lipopolysaccharide (Sigma-Aldrich) at the cell counts and conditions previously described for the IFN- β expression. Each condition was replicated in triplicate. Cells were harvested 24 h post-transfection, and RNA was extracted by Aurum Total RNA Mini Kit. RNA reverse transcription and RT-PCR were carried out using the same method as IFN- β expression. The IL-6 primer [33] used for RT-PCR is listed in Supplementary Table 1.

Microscale thermophoresis

Microscale thermophoresis (MST) experiments were performed, using a NanoTemper Monolith NT.155. Briefly, 50 nM FAM labeled 100 bp dsDNA (sequence in Supplementary Table 1) in HEPES buffer (10 mM HEPES, 140 mM NaCl and 5 mM MgCl_2 ; pH=7.5) was incubated with a dilution series of suramin, diminazene aceturate or propidium iodide. Samples were loaded into MST Premium Coated Monolith NT.115 Capillaries. MST was performed using 20% Blue Excitation Power and Medium MST power. The On Time 1.5 s Evaluation Strategy was used by the NT Analysis Software to generate the MST response. Samples were prepared in triplicate.

Fluorescent DNA displacement assay

100-bp annealed dsDNA (0.1 μM for proflavine assay or 0.2 μM for DAPI assay) was prepared in Tris-HCl buffer (20 mM Tris, 100 mM KCl, pH 7.2). Two micromolar proflavine or DAPI was added and the mixture was incubated for 5 min. Emission measurement was taken after addition of each indicated concentration of suramin followed by an equilibration time for 5 min. The fluorescence intensity (F) of DAPI (Ex: 340 nm, Em: 460 nm) and proflavine

(Ex: 444 nm, Em: 510 nm) was converted into percentage of displacement (PD) by using the following equation.

$$\text{DAPI : PD} = 100 - \left[(F_s / F_0) \times 100 \right]$$

(F_0 = fluorescence intensity of DNA–DAPI complex in the absence of suramin; F_s = fluorescence intensity in the presence of suramin).

$$\text{Proflavine : PD} = (F_s - F_0) / (F_{\text{max}} - F_0) \times 100$$

(F_0 = fluorescence intensity of DNA–proflavine complex in the absence of suramin; F_s = fluorescence intensity in the presence of suramin; F_{max} = fluorescence intensity of proflavine in the absence of DNA).

Results

Discovery of suramin as cGAS inhibitor from compound library

To identify potential cGAS inhibitors we screened a small library of 268 compounds to evaluate inhibition of human cGAS activity via HPLC analysis of the reaction mixture (after 2 h of incubating cGAS with ATP, GTP and the putative inhibitors). Library compounds were selected based on structural similarities to ATP and the potential for inhibition of dsDNA binding to cGAS [27]. For the initial screening (using 3 μM cGAS, 200 μM ATP/GTP and 20 μM of putative inhibitors), a cut-off of 30% enzymatic inhibition was considered significant and suramin was identified as an inhibitor of cGAS activity (Figure 2A).

A panel of suramin analogs (Figure 2A) [34] was then examined to evaluate the suramin structure–activity relationship, however, no inhibition activity was observed. Although the HPLC analysis of the cGAS reaction mixture had indicated inhibition by suramin (Figure 2B), we proceeded to use a second assay to confirm the inhibition of cGAS by suramin by using radioactive α - ^{32}P -ATP as a substrate for cGAS and visualization using TLC with autoradiography imaging to analyze the reaction mixture (Figure 2C). In line with the HPLC results, the TLC analysis also indicated that the inhibition of cGAMP synthesis by suramin was dose-dependent.

Suramin facilitated displacement of DNA from hcGAS

DNA binding induces conformational changes and crosslinks cGAS dimers to restrict the structural flexibility of cGAS and stabilize the active conformation, which catalyzes the formation of 2',5'-cGAMP [16]. cGAS activity is known to be stimulated by DNA of varied sequences, including poly(deoxyadenosine-deoxythymidine), poly(deoxyguanosine-deoxycytidine) and 45-base pair immune stimulatory DNA [1]. Inhibitors of cGAMP synthesis can either inhibit dinucleotide synthesis by binding to the active site of cGAS [24,25] or the DNA [23]. An alternative mode of inhibition, which has yet to be demonstrated, is for an inhibitor to compete with the duplex DNA for cGAS binding. This could occur via inhibitor binding directly to the DNA binding site or binding to an alternative site, which results in a conformational change to reduce cGAS affinity for DNA. Suramin is polyanionic and is not a potent DNA binder, *vide infra*, so we rationalized that the inhibition of cGAS by suramin could be via the displacement of DNA from cGAS. In other words, suramin could act as a DNA mimic but unable to activate cGAS for cGAMP synthesis. To investigate this possibility, an EMSA was performed using FAM labeled DNA to assess the effect of suramin on DNA binding to human cGAS (Figure 3). The suitability of 100-bp dsDNA in a cGAS inhibition assay was previously demonstrated, which guided our choice for its use in this experiment [23]. cGAS addition to the DNA resulted in a sharp decrease in free 100 bp DNA (Figure 3, lane 2). Addition of QC, a known cGAS inhibitor, which perturbs cGAS/dsDNA binding, resulted in recovery of the 100-bp DNA band (Figure 3, lane 7). Addition of increasing concentration of suramin resulted in an increase in the 100-bp dsDNA band density in a dose-dependent manner (Figure 3, lanes 3–6). This indicates that suramin could inhibit cGAS activity in a dose-dependent manner through disrupting dsDNA/cGAS binding. Suramin was able to disrupt dsDNA/cGAS binding even when DNA and cGAS were preincubated to form the DNA/cGAS complex prior to suramin addition as was the case here.

To ensure that the displacement of DNA observed was not due to an interaction with DNA itself, which is the mode of cGAS inhibition by QC [23], MST experiment was performed in which the response of the FAM labeled dsDNA was measured in the presence of suramin, DNA minor groove binder diminazine acetate or

Figure 2. Screening and identification of suramin as potential inhibitor of cGAMP synthesis by cGAS. (A) Structures of suramin and analogs. **(B)** HPLC analysis of cGAS synthesis of cGAMP in the absence (blue) and presence (red) of 20 μ M suramin. Inset shows retention time 0–20 min. **(C)** TLC of cGAS inhibition experiments, using indicated concentrations of suramin.

DNA intercalator propidium iodide. The data is shown in the Supplementary Data (Supplementary Figure 3) and, as expected for a polyanionic species, suramin does not show evidence of an interaction with DNA, even at concentrations of suramin as high as 5 mM. In addition to the MST experiments, several indirect displacement experiments (using fluorogenic DNA intercalators or minor groove binders) indicated that suramin does not bind to DNA (Supplementary Figure 4). DAPI, a fluorescent minor groove binding probe for DNA, was used to stain dsDNA. Displacement of DAPI by a minor groove binder such as diminazene will result in a decrease in the fluorescence of DAPI. Diminazene was found to displace 50% of DAPI at a concentration of 2 μ M while suramin was found to exhibit only approximately 26% displacement of DAPI at concentrations as high as 20 μ M suramin. This low level of DAPI displacement by suramin could be due to the interactions of anionic suramin with DAPI. No significant displacement of DNA intercalator proflavine was observed upon addition of up to 50 μ M of suramin, contrasted to the obvious displacement by propidium iodide under the same conditions. These results support the notion that DNA and suramin do not significantly interact.

Figure 3. Inhibition on DNA–cGAS binding by suramin was investigated using electrophoretic mobility shift assay. Fluorescently labeled dsDNA (1 μ M) and cGAS (5 μ M) were incubated for 1 h at room temperature. Suramin was then added for an additional 2 h and subjected to electrophoresis. Lane 1: 1 μ M fluorescently labeled dsDNA. Lane 2–6: Fluorescently labeled dsDNA and cGAS with 0, 1, 10, 100 and 200 μ M suramin. Lane 7: positive control – QC (250 μ M). QC: Quinacrine.

Functional modulation of hcGAS activity by suramin

Having observed *in vitro* that suramin inhibits cGAS (Figure 2B & C) and also inhibits dsDNA binding to cGAS (Figure 3), we sought to evaluate the activity of suramin in suppressing dsDNA-dependent activation of cGAS and therefore downregulation of type I IFN genes in cells (using THP1-Dual cells [25]). Upon IRF pathway stimulation with 2'3'-cGAMP in THP1-Dual cells, a luciferase reporter protein, which is readily measurable in plate format via a commercially available kit, is produced [35]. As expected, when THP1-Dual cells were stimulated with dsDNA to produce cGAMP, the reporter protein indicated that the IRF pathway was stimulated and therefore IFN- β was expressed. When increasing concentrations of suramin were added to the stimulated THP1-Dual cells, the level of IFN- β expression decreased (Figure 4B). At 5 μ M of suramin, the level of IFN- β expression was similar to that seen in unstimulated cells, indicating that the suramin could suppress cGAMP production in the cells. To eliminate the possibility that suramin exhibits toxicity to cells, which reduced the IFN- β production, we investigated the toxicity of suramin against the THP1-Dual cells and no significant change of cell viability was observed in the presence of 5 μ M suramin, which is the maximum dosage used throughout this study (Figure 4A). This result eliminates the possibility that suramin may be exhibiting toxicity to the cells resulting in a reduction of the IFN- β production.

If suramin affects IFN- β protein expression levels via inhibition of cGAMP synthesis, the levels of IFN- β mRNA would be expected to show a decrease. To test this hypothesis, we transfected the THP1-Dual cells with dsDNA and investigated the mRNA expression of IFN- β . As observed in Figure 4C, type I IFN response was enhanced significantly upon transfection of dsDNA while the response was inhibited in the dose-dependent manner of suramin. It indicates the ability of suramin to inhibit dsDNA-dependent cGAS-mediated signaling *in cellulo*.

To demonstrate that suramin could enter the cell and attenuate cGAS activity, but not simply affect DNA transfection efficiency, we incubated the transfection cocktail (DNA/Lipofectamine complex) with cells for 30 min, then washed the cell and cultured the cell in fresh media with and without suramin for 24 h. The rationale for the aforementioned protocol is that the DNA will enter the cells in the first 30 min and the remaining DNA will be washed out before suramin was added. Preliminary investigations had shown that the IFN- β mRNA level as well as the level of the luciferase reporter in THP1-Dual cells increased upon a brief DNA transfection time of 30 min (although 24 h transfection time improved response). Using this 'washout' protocol, we observed that the expression of IFN- β (the gene product of cGAS mediated signaling) as well as the level of the luciferase reporter (determined via the enzymatic activity of the reporter) were increased upon brief DNA transfection (30 min) and that suramin (added after the DNA transfection step) could still reduce the IFN- β expression (Figure 5A) and the level of the luciferase reporter (Figure 5B). Additionally we also determined that suramin did not affect the transfection of GFP plasmid into HEK293 cells (Supplementary Figure 2). These results demonstrate that the effect of suramin on IFN- β expression is not via the disruption of DNA transfection but rather it is due to the ability of suramin to enter the cells and attenuate the activity of cGAS.

Figure 4. Suramin decreases the expression of the dsDNA-stimulated IRF pathway at concentrations that do not exhibit cell lethality. (A) Viability of THP1-Dual™ cells in the presence of Suramin. Suramin does not affect the viability of THP1-Dual cells up to a concentration of 5000 nM. (n.s.₁, $p = 0.0845$, not significantly different; n.s.₂, $p = 0.2696$, not significantly different; n.s.₃, $p = 0.2377$, not significantly different). (B) Change in luciferase reporter expression (luminescence signal) in response to IRF pathway stimulation indicating IFN- β expression in THP1-Dual cells upon transfection of dsDNA in the presence and absence of suramin. (C) Relative mRNA level of IFN- β in THP1-Dual cells upon transfection of dsDNA in the presence and absence of suramin. Error bars shown as standard error of the mean from three independent trial.

* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

n.s: Not significant.

To demonstrate that suramin inhibits cGAS activity but not the downstream target of cGAS mediated signaling pathway, we used THP1-Dual KO-cGAS cells that are deficient in the cGAS protein and stimulated them with dsDNA or cGAMP. When these cells are exposed to cGAMP, the STING pathway is activated but suramin was not found to significantly decrease the IFN stimulation (Figure 6). This emphasizes that suramin selectively inhibits the cGAS activity but not the downstream target of cGAS mediated signaling.

We also examined the ability of suramin to inhibit other innate immune signaling pathways such as TLR2/TLR1 and TLR4. We performed RT-PCR to investigate the expression level of IL-6, which is a proinflammatory cytokine triggered by toll like receptors (TLRs). Suramin is known to have anti-inflammatory activity upon stimulation of immune cells by other stimuli [36]. To test whether in addition to inhibiting the DNA sensing pathway, suramin also affected additional signaling pathways that are activated by other pathogen-associated molecular patterns, we determined the level of IL-6 mRNA following stimulation by other non-DNA stimuli (lipopolysaccharide [LPS], Pam3CSK4 lipopeptide and Poly(I:C)). Upon stimulation of the cognate ligands, Pam3CSK4 (Tlr2/1) and

Figure 5. Suramin-mediated inhibition of IFN- β stimulation is not the result of DNA transfection disruption. The relative mRNA level normalized to GAPDH (A) and the relative luminescence from luciferase reporter (B) indicate the level of IFN- β expression of THP1-Dual™ cells upon 30 min pretransfection of dsDNA and 23.5 h of incubation of suramin. Error bars shown as standard error of the mean from three independent trials. *p < 0.05; ***p < 0.001.

Figure 6. cGAMP rescues IFN- β stimulation in the presence of suramin. (A) Relative expression of IFN- β mRNA (normalized to GAPDH) in THP1-Dual™ KO-cGAS cells exposed to dsDNA or cGAMP and simultaneously treated with 5 μ M suramin. (n.s.₁, p = 0.2853, not significantly different) (B) Change in expression of luciferase reporter protein in response to type 1 IFN stimulation indicating IFN- β expression in THP1-Dual KO-cGAS cells. cGAS produces cGAMP which activates the STING pathway. KO-cGAS activity may be rescued by the addition of cGAMP. THP1-Dual KO-cGAS cells are insensitive to cGAS inhibition by suramin. Error bars shown as standard error of the mean from three independent trials. (n.s.₂, p = 0.0809, not significantly different). n.s.: Not significant.

lipopolysaccharide (Tlr4), an increase of IL-6 expression was recorded, while no significant change was observed in the presence of suramin (Figure 7A & B). In the case of Poly(I:C), the addition of 5000 nM suramin resulted in a suppression of IL-6 expression (Figure 7C).

Discussion

cGAS is indispensable for IFN- β induction by cytosolic DNA, as determined by transfection experiments of DNA into the lung fibroblasts of WT and cGas^{-/-} mice resulting in robust production of IFN- β , while induction was

Figure 7. Suramin does not significantly decrease all signaling pathways examined that are activated by non-DNA pathogen-associated molecular patterns. Relative expression of IL-6 mRNA (normalized to GAPDH) in THP1-Dual™ cells exposed to (A) Pam3CSK4, (B) LPS and (C) Poly(I:C) simultaneously treated with 5 μ M suramin. (n.s.₁, $p = 0.06$, not significantly different; n.s.₂, $p = 0.42$, not significantly different) Error bars shown as standard error of the mean from three independent trials.

* $p < 0.05$.

LPS: Lipopolysaccharide; n.s.: Not significant.

completely abolished in a cGAS^{-/-} mouse strain [37]. Though the pathway is essential for antiviral immune response, it must be tightly regulated to avoid an inappropriate response against self DNA, which may be present in cytoplasm, as chronic activation is implicated in autoimmune disease [5]. TREX1 is an exonuclease that degrades DNA in the cytoplasm [14]. Gray *et al.* utilized TREX1-deficient mice, which developed multi-organ inflammation, including severe inflammatory myocarditis that is dependent on type I IFNs and STING, and found that when cGAS was knocked out, the mice showed no signs of inflammation [5]. Their study demonstrated that cGAS is a key driver of autoimmune disease and suggested that cGAS inhibitors may be useful therapeutics for AGS patients with TREX1 mutations [5]. Gao *et al.* also suggested that inhibition of cGAS may be an effective therapy for autoimmune diseases such as AGS and systemic lupus erythematosus [6].

An *et al.* performed *in silico* screening of drug libraries which identified several antimalarial drugs that were predicted to interact with a cGAS•dsDNA complex [23]. Hydroxychloroquine and QC were found to disrupt the cGAS•dsDNA complex as determined by EMSA [23]. Dose-titration experiments with recombinant cGAS were performed and cGAMP production was quantified using TLC to determine the IC₅₀ for inhibition of cGAMP production with QC and ACMA revealed as the most potent inhibitors of cGAMP production examined (low μ M range) [23]. An *et al.* also tracked the expression of IFN- β in THP1 cells in response to DNA transfection and found QC and ACMA to have single digit micromolar IC₅₀ values for inhibition of IFN- β expression [23].

Suramin inhibits cGAS & has been shown to enter cells by an uncharacterized mechanism

Our screening for inhibitors of cGAS identified suramin. This screening hit was exciting because repurposing known drugs for novel indications has benefits including known toxicological profiles and a scaffold that is known to be compatible with dosing cellular targets, all of which may expedite translation into a new therapeutic. In addition to being an approved drug for trypanosomiasis, suramin has also been investigated in the clinic for a few disease states (autism [clinical trials number NCT02508259], breast cancer [clinical trials number NCT00054028]). Suramin, being polyanionic is expected to have poor cellular permeation properties but interestingly suramin has been shown to cross into cells to affect signaling processes [38]. Using autoradiographic techniques, Baghdiguian *et al.* showed that tritiated suramin enters colon (HT-29-D4) cells and therefore put to rest any doubts regarding suramin's ability to enter cells [39]. Additionally, several studies have shown that suramin is able to inhibit various cancer cell lines, presumably after entering those cells to affect various intracellular proteins [40,41]. Suramin has been demonstrated to accumulate in mammalian cell lines in the acidic intracellular compartments (trans-Golgi complex; exocytic vesicles) at concentrations in excess of 150 μ M [2]. The mechanism of suramin uptake is not well understood but appears to occur via receptor-mediated endocytosis by a non-LDL-mediated route in trypanosome cells [1].

Drugs that are unable to diffuse freely across membranes must engage in transport through a channel, transporter or the flagellar pocket in trypanosome cells [1]. The identity of the receptor on human cells that bind to suramin to facilitate uptake remains uncharacterized, though once internalized the charge of suramin may prevent an easy escape from the cell, which allows internal cellular concentrations to exceed that of the extracellular environment [1].

Suramin disrupts dsDNA/cGAS binding & downregulates the type-I IFN response

EMSA experimental results indicated that suramin disrupts dsDNA/cGAS binding. Suramin was able to disrupt dsDNA/cGAS binding even when DNA and cGAS were preincubated to form the DNA/cGAS complex prior to suramin addition and this disruption is a potential mechanism for the inhibition of dsDNA stimulated cGAS activity. We saw that suramin inhibited the expression of INF- β , likely by inhibition of cGAS, which limits cGAMP production and therefore activation of the STING pathway. This could be due to either binding to the active site of cGAS, or more likely by interfering with the ability of cGAS to bind DNA, with the latter mode being suggested by EMSA.

Having shown that suramin is able to disrupt the interaction of DNA with cGAS *in vitro*, we undertook *in cellulo* studies to examine the expression of IFN- β . IFN- β expression is stimulated by STING, which also controls proinflammatory cytokine production and chronic activation is a key factor in development of autoimmune diseases, such as rheumatoid arthritis, inflammatory bowel disease and others [42]. STING is stimulated by 2'3'-cGAMP, which is produced by cGAS when stimulated with dsDNA. Transfection experiments demonstrated a reduction in IFN- β expression in response to HT-DNA stimulation in the presence of increasing concentrations of suramin. As expected, the expression of IFN- β mRNA followed a similar trend to the protein expression experiment (determined via the luciferase reporter), with a decrease in STING mediated mRNA expression in response to the lower levels of cGAMP present due to cGAS inhibition. Experiments using a cGAS knockout cell line clearly illustrated the recovery of the STING pathway upon activation with cGAMP, even in the presence of high suramin concentrations (5 μ M) (Figure 6B).

Pam3CSK4 activates TLR2 [43], which leads to the recruitment of MyD88 and the IRAKs [8]. IRAK4 phosphorylates IRAK1 [8]. Phosphorylated IRAK1 activates TRAF6, which becomes ubiquitinated and triggers the IKK complex [8]. I κ B phosphorylation and ubiquitination by the IKK complex results in dissociation of I κ B from NF- κ B [8]. NF- κ B is then able to cross into the nucleus and activate the IL-6 producing gene [8]. LPS interacts with TLR4, which in the classical pathway phosphorylates I κ B, resulting in its dissociation from NF- κ B, which allows NF- κ B to cross the nuclear membrane and activate the IL-6 producing gene [7]. The lack of inhibition of IL-6 expression upon stimulation by LPS and Pam3CSK4 by suramin (Figure 7A & B) indicates that the effect of suramin on cell signaling is not promiscuous.

dsRNAs induce type-I IFN responses differently, depending on the source of the RNA used for experiments, be it endogenous, *in vitro* synthesized, synthetic (such as Poly(I:C)), or of viral origin, and mammalian cells detect RNA substrates using a multitude of pathways to elicit the type-I IFN response [44]. In response to dsRNA, PKR acts on I κ B via the IKK complex which activates NF- κ B and leads to IFN- β induction [45]. Stimulated PKR also phosphorylates eIF2 α , which then binds eIF2B and this complex inhibits translation initiation by the ribosomal complex that confers an antiviral state [44]. Other sensors for dsRNA have been discovered, including TLR3, with responds to viral dsRNA. Synthetic Poly(I:C) engages the TLR3 signaling pathway to activate a type-I IFN response via NF- κ B [44]. RIG-I is activated by cytoplasmic dsRNA, which leads to the activation of IRF3 and NF- κ B to elicit the type-I IFN response [44]. MDA5 is also a key mediator of virally induced type-I IFN signaling, which acts through IRF3 and NF- κ B [44]. LGP2 stimulation by Poly(I:C) acts as a negative regulator of IRF3, NF- κ B and IFN- β induction, potentially through the inhibition of RIB-I and MDA5 induction by the same substrate [46]. The effects of duplex RNA on immune response is further complicated because type-I IFN response is involved in the induction of cGAS [47]. The increased expression has the potential to increase activity of the STING pathway if DNA is present in the cytoplasm, and result in type-I IFN signaling [1]. Clearly the impact of Poly(I:C) stimulation has consequences on multiple pathways and cellular data would be very convoluted by the interplay of pathways. Despite these complications we still evaluated how suramin affected the effects of duplex RNA (Poly(I:C)).

Suramin may act as a nucleic acid mimic

The inhibition of Poly(I:C)-stimulated IL-6 expression by suramin, a polyanionic compound, is interesting and it could imply that suramin is capable of mimicking nucleic acids and competes with DNA or RNA for binding to positive patches found in DNA or RNA binding proteins. Perhaps the anionic sulfonates act as phosphate mimics.

This is an interesting proposal and in general could impact the design of molecules to inhibit other nucleic acid binding proteins. In fact others have also observed that suramin can bind to positive patches on other proteins. For example, the acidic C-terminal tail of Cdc34, ubiquitin-conjugating enzyme E2 R1, binds to a basic cleft of Cullin-Ring ubiquitin ligases, such as CUL1, via electrostatic interactions [34]. Wu *et al.* showed that suramin can interact with the basic patch cleft of CUL1 (a component of SCF E3 ubiquitin ligase complex) and prevent the binding of Cdc34 [34]. Figure 8 shows an electrostatic surface of DNA, RNA and several proteins (AIM2, CUL1 RIG-1 and cGAS) with electrostatic surfaces indicating where the nucleic acid or suramin is found to bind. A common theme of this figure is the overwhelmingly positive character of these binding regions, which interact readily with negatively charged substrates such as nucleic acids.

Recently, suramin was found to bind the positive region of fivefold axis of the EV-A71 capsid and interfere with capsid binding of attachment factors (heparin sulfate glycosaminoglycans) required for virus attachment and entry into a host cell and thereby inhibited multiple enterovirus A [51]. Mutations to the capsid greatly reduced suramin binding and also reduced binding of heparin, while mutations to nearby residues that restored the positive character facilitated heparin and suramin binding once more [51].

Additional evidence for suramin as a binder of positive patches on proteins comes from the binding of suramin to Moloney murine sarcoma virus reverse transcriptase at the primer/template binding site [52]. Suramin interacts with the template-primer binding site in a competitive manner, and was described by the author as belonging to a new class of reverse transcription inhibitors that are not primer or template DNA analogs, but interact with the template-primer region of DNA polymerase [52]. Suramin is known to have a protective effect in ATH8 cells against the cytopathic effect of human T-cell lymphotropic virus type III/lymphadenopathy-associated virus (HTLV-III/LAV) reverse transcriptase at 50 µg/ml, a dose that did not exhibit toxicity to uninfected cells [53]. The viral core (gag) p24 protein expression is completely suppressed at this concentration, which suggests that virus replicative cycle inhibition is the most likely cause of the protective effect [53,54]. The uptake of suramin by endocytosis for release into the cytoplasm would give suramin the opportunity to bind the viral reverse transcriptase before RNA is integrated into viral dsDNA for transport to the nucleus where cellular DNA polymerases would be sequestered [53].

It is therefore tantalizing to propose that suramin or other polysulfonated aromatics are general motifs to interrupt protein–nucleic acids or protein–protein interactions that occur via extensive electrostatic interactions. We, however, note that the immune activation by duplex RNA is complex and could involve regulation of cGAS, which is a DNA binder. It is therefore premature to suggest that suramin will also interrupt RNA–RIG-1 interactions without more data, which is beyond the scope of the current report. Others had also noted the inhibition of Poly(I:C)-stimulated induction of IL-6 expression by antimalarial drugs, also cGAS inhibitors, in THP-1 cells [23]. Plausibly, these antimalarial drugs, which bind to nucleic acids, interrupted the Poly(I:C)-stimulated induction of IL-6 by directly interacting with the Poly(I:C) or via other ill-defined mechanism(s). Suramin, on the other hand, does not strongly bind to nucleic acids (Supplementary Figures S4 & S5) so it is unlikely that the reduction of the IL-6 mRNA induction (via Poly(I:C) stimulation) is via binding to RNA. More detailed future work (beyond the scope of this report) is needed to clarify if suramin is indeed a general inhibitor of nucleic acid binding proteins.

Conclusion

Using HPLC analysis of cGAS reaction mixture, we uncovered that suramin is a potent inhibitor of cGAS. This was confirmed by using a radiolabelled α -³²P-ATP substrate for cGAS followed by TLC and autoradiography imaging. Both results indicated that the inhibition of cGAMP synthesis by suramin was dose-dependent. Reduced activity of TREX1 can lead to increased IFN- β production [55]. Persistent activation of cGAS by cytosolic dsDNA will lead to unregulated activation of STING by 3',5'-cGAMP, which can result in chronic inflammation. Inhibition of cGAS activity could be a potential therapeutic strategy for diseases caused by reduced TREX1 activity, such as lupus [56]. Here, we have identified suramin as a cGAS inhibitor with potential application in treating chronic inflammation due to unregulated cGAS-STING pathway activation. In addition to cGAS playing a major role in bacterial pathogen clearance, cGAS has also emerged as important player in macular degeneration [57], cellular senescence [58–60], inflammatory response to malaria infection [61], myocardial infarction-related inflammation [62] and macrophage transformation [63] among others. Inhibitors of cGAS-mediated production of cGAMP could have extensive utility in medicine.

Figure 8. Positive patches of DNA binding proteins could be targets for suramin interactions. Not drawn to scale. (A) DNA (PDB 3RN2 [48]); **(B)** RNA (PDB 5E3H [49]); **(C)** AIM2 (PDB 3RN2 [48]); **(D)** CUL1 (PDB 1LDJ [50]). The basic cleft where suramin was demonstrated to bind is shown as an electrostatic potential surface [34]. **(E)** RIG-1 (PDB 5E3H [49]); **(F)** human cGAS (PDB 4LEV [16]). Figures are shown with an electrostatic potential surface which is confined to the DNA binding region (C–F) or suramin binding region (D) of the protein models.

Future perspective

The cGAS-STING pathway plays important roles in various autoimmune diseases and the past year has witnessed increasing interest by research groups to identify cGAS inhibitors for treating autoimmune diseases. We predict that various groups will report new cGAS inhibitors with cellular and animal activity. Suramin is already an approved drug but has more room for improvement. Analogs of suramin that bind better to cGAS and also with enhanced

cell permeation properties could have applications in the management of various inflammatory diseases. We are actively working on such analogs and will report in due course.

Summary points

- Suramin inhibits cGAS, potentially via binding to its DNA binding site.
 - Discovery of suramin as cGAS inhibitor from compound library.
 - Suramin facilitated displacement of DNA from hcGAS.
 - Suramin disrupts dsDNA/cGAS binding.
- IFN- β levels produced by THP-1 cells are reduced by suramin.
 - Functional modulation of hcGAS activity by suramin.
- Suramin can enter the cell and attenuate cGAS activity rather than simply affect DNA transfection efficiency.
 - Functional modulation of hcGAS activity by suramin.
 - Suramin inhibits cGAS and has been shown to enter cells by an uncharacterized mechanism.
- Suramin may be a nucleic acid mimic.
 - Suramin disrupts dsDNA/cGAS binding and downregulates the type-I interferon response.
- Suramin and/or analogs have the potential to be used to treat chronic inflammation.
 - Suramin analogs with better cell permeability should be pursued as anti-inflammatory agents.

Supplementary data

To view the supplementary data that accompany this paper please visit the journal website at: www.future-science.com/doi/full/10.4155/fmc-2017-0322

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