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Inhibition of Wnt Signaling and Breast Tumor Growth by the Multi-purpose Drug Suramin through Suppression of Heterotrimeric G Proteins and Wnt Endocytosis

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Summary statement: Multi-purpose drug suramin is found to be active against cancer-related Wnt signaling. As a consequence of heterotrimeric G proteins suppression, suramin inhibits Wnt ligand internalization, which renders the drug active against triple-negative breast cancer.
Short title: Suramin counteracts Wnt endocytosis through inhibition of heterotrimeric G proteins
Keywords: Wnt pathway, triple-negative breast cancer, internalization, nucleotide analog, drug repositioning

Abbreviations:
TNBC – triple-negative breast cancer
FZD – Frizzled
GPCR – G protein-coupled receptor
GTPγS – Guanosine 5′-[γ-thio]triphosphate
GTP – Guanosine 5′-triphosphate
7-TM – seven-transmembrane
GSK3β - glycogen synthase kinase 3β
LRP5/6 – LDL (Low-density lipoprotein) receptor-related proteins 5 and 6
Gα – α-subunit (catalytic) of heterotrimeric G protein
TCF/LEF – T-cell factor/lymphoid enhancer factor
BODIPY – boron-dipyrromethene (4,4-difluoro-4-bora-3a,4a-diaza-s-indacene)
Kd – dissociation constant
GDI – guanine nucleotide dissociation inhibition
GEF – guanine nucleotide exchange factor
HA – hemagglutinin

1. ABSTRACT

Overactivation of the Wnt signaling pathway underlies oncogenic transformation and proliferation in many cancers, including the triple-negative breast cancer (TNBC) – the deadliest form of tumor in the breast, taking about a quarter of a million lives annually world-wide. No clinically approved targeted therapies attacking Wnt signaling currently exist. Repositioning of approved drugs is a promising approach in drug discovery. Here we show that a multi-purpose drug suramin inhibits Wnt signaling and proliferation of TNBC cells *in vitro* and in mouse models, inhibiting a component in the upper levels of the pathway. Through a set of investigations we identify heterotrimeric G proteins and regulation of Wnt endocytosis as the likely target of suramin in this pathway. G protein-dependent endocytosis of plasma membrane-located components of the Wnt pathway was previously shown to be important for amplification of the signal in this cascade. Our data identify endocytic regulation within Wnt signaling as a promising target for anti-Wnt and anti-cancer drug discovery. Suramin, as the first example of such drug, or its analogs might pave the way for appearance of first-in-class targeted therapies against TNBC and other Wnt-dependent cancers.

2. INTRODUCTION

Suramin is a rather large (M_r=1297) symmetric polysulphated polyaromatic compound. It was introduced in 1916 as a drug against the trypanosome *Trypanosoma brucei*, the cause of sleeping sickness [1]. Decades of application and investigations on this compound revealed its numerous off-target activities; moreover, it seems that even in its target organism trypanosome it acts through multiple mechanisms [2,3]. Normally, the off-target activities of drugs are highly undesirable. However, in the case of suramin the cheapness, high bioavailability, excellent solubility and absence of acute toxicity of this compound contributed to its popularity and numerous studies of the mechanisms of its action. Among the off-target activities of suramin are its activities as: antagonist of P2 receptors [4], agonist of ryanodine receptors [5], and inhibitor of growth factors and topoisomerases [6]. Suramin is also known to negatively affect cellular folate transport [7] and steroidogenesis [8]. Finally, it was shown to be an inhibitor of G protein activation [9,10].

Suramin was also proposed to affect the Wnt signaling – the developmentally and medically important pathway controlling multiple steps in embryogenesis and misactivated in many cancers [11–13]. Wnt signaling is initiated by the interaction of secreted lipoglycoproteins of the Wnt family with the cell surface receptors of the FZD family. Pathways initiated by Wnt ligands are diverse, and different co-receptors, such as LRP5/6 are thought to play directional role in choosing between them. In the present work we are focusing on the "canonical" Wnt pathway, widely considered to be the main one involved in carcinogenesis and cancer sustaining. Inside the cell, the key transducer of the "canonical" Wnt pathway is β-catenin, which in the absence of the pathway activation is sent to degradation through the action of the Axin-based complex of proteins, additionally containing APC and glycogen synthase kinase 3β (GSK3β) and casein kinase. When the signaling is activated, this destruction complex becomes inactivated, leading to stabilization of β-catenin and its translocation into the nucleus, leading to activation of LEF/TCF-dependent transcription [11,14].

In the context of the Wnt pathway, suramin was reported to induce release of the Wnt proteins from the cell surface by an undescribed mechanism, thus reducing Wnt/FZD interactions and activation of the pathway [15]. In the present work, we identify suramin as an inhibitor of the Wnt signaling pathway, acting through suppression of the Wnt endocytosis by the Wnt-responding cells. We provide evidence showing that this effect is achieved through inhibition of heterotrimeric G proteins by suramin rather than by effects of this drug on the surface adhesion of Wnts as was suggested before. These findings therefore shed light on the participation of the heterotrimeric G proteins in Wnt signaling in mammals as well as indicating G protein-dependent endocytosis as a potential drug target in this pathway. We further demonstrate the ability of suramin to arrest proliferation of triple-negative breast cancer cells *in vitro* and

in mouse models.

3. MATERIALS AND METHODS

.3.1. Luciferase-based assay of the Wnt-dependent transcriptional activity

HEK293T and BT-20 cells stably transfected with M50 Super 8x TOPFlash plasmid [16] were used to analyze the Wnt inhibitory activity of suramin (Sigma, St. Gallen, Switzerland, cat.#S2671). The assay was performed in white tissue-culture treated 96-well plates (Greiner, cat.# 655073). The HEK293-Tf or BT-20-Tf cells were seeded in 100µl DMEM medium containing 10% FCS at ~10000 cells/well and subsequently stimulated by 0.5µg/ml mouse Wnt3a purified as described [17] or 20mM LiCl in the medium in presence or absence of the drug for 12 hours. 1µg/ml pertussis toxin (PTX, Enzo) and gallein (Tocris) pretreatments were done overnight before the assay. For investigation of suramin effects on Wnt3a, purified ligand was incubated with 1mM suramin overnight and then suramin was removed by 100-fold dilution by the Wnt3a buffer (20mM HEPES pH 7.4, 150mM NaCl, 1% CHAPS) followed by concentration using Amicon 10K centrifugal concentrators. To account for effects of increased CHAPS concentrations and residual suramin (~1µM in the medium during assays), identical procedure was performed with the Wnt3a buffer only.

If indicated, the cells were additionally transfected by the pCMV-RL plasmid for constitutive expression of *Renilla* luciferase (kindly provided by Konrad Basler [18]) using X-tremeGENE 9 reagent (Roche, Basel, Switzerland) according to the manufacturer's protocol. The medium was subsequently removed, and 15µl of the lysis buffer (25mM Glycylglycine pH 7.8, 1% Triton X-100, 15mM $MgSO_4$, 4mM EGTA, 1mM DTT) were pipetted into each well. After incubation for 5 minutes at room temperature the 96-well plate was analyzed using the Victor^3 Multilabel Counter (PerkinElmer, Waltham, USA) with the two-channel dispensing unit primed with the buffer solutions for activity measurements of firefly and *Renilla* luciferase (if necessary) (prepared as described in [19]). The final volumes dispensed per well were 50µl of firefly and 50µl of *Renilla* solutions.

.3.2. β-catenin stabilization assay

Analysis of β-catenin stabilization in response to cell activation by Wnt3a was modified from [20]. The following densities were used for different cell types: L-cells – ~50000 cell/well in 48-well plates; HEK293T, BT-20 and HeLa – ~70000/well in 24-well plates. The next day the old medium was removed and replaced by either the vehicle or suramin-containing fresh one. 1 hour pre-incubation was used in all experiments involving suramin; PTX and gallein pretreatment were done overnight, the HeLa cells were additionally transfected by FZD_7 subcloned in pcDNA3.1 plasmid as described. Subsequently, purified Wnt3a or vehicle buffer were directly added into the wells to the final concentration of 1µg/ml and incubated at 37ºC for 2 hours in case of L-cells or 12 hours in case of BT-20, HEK293 or HeLa cells. Subsequently the medium was removed, the cells in each well washed once with 500µl of 1x PBS (Biochrom AG, Berlin, Germany) and lysed directly in the well by addition of 50µl of ice-cold RIPA buffer for 10 minutes on ice. The cells were then resuspended, the debris was removed by 10 min centrifugation at 16000g, 4ºC, the probes were further analyzed by Western blot with antibodies against β-catenin (BD, Allschwil, Switzerland, cat.# 610153) and α-tubulin (Sigma, St. Gallen, Switzerland, cat.# T9026).

.3.3. Wnt internalization and surface binding

For internalization or surface adhesion of exogenous Wnt3a, wild-type HeLa cells were seeded at $5x10^5$ cells/well in 6-well plates. Next day, Wnt3a-HA conditioned medium or purified Wnt3a was added to the cells and incubated either at 37ºC (internalization) or 4ºC (surface adhesion; cells were preincubated on ice for 1h prior to addition) for indicated amounts of time in presence or absence of 1mM suramin.

Subsequently the medium was removed and cells were washed 2x with ice-cold 1x PBS and then lysed in 1x RIPA buffer and analyzed by Western blotting.

For quantification of endogenous Wnt3a-HA, the monoclonal HeLa cell line stably transfected with Wnt3a-HA subcloned into the pcDNA3.1 vector (Invitrogen, Lucerne, Switzerland) was seeded at 5×10^5 cells/well. Next day, the cells were preincubated on ice for 30min, then washed 2x by ice-cold 1xPBS and then incubated with 0.5mg/ml of NHS-SS-Biotin (Pierce, Zug, Switzerland) solution in 1x PBS for 1h, afterwards the NHS-SS-Biotin was quenched by 2x wash with 1xTBS solution and further incubation of cells for 30min in 1xTBS. The cells were subsequently lysed in 200µl RIPA buffer without reducing agents, lysates were cleared by centrifugation for 10min at 16000g. Biotinylated membrane proteins were isolated by incubation of lysates with 30µl/sample of Streptavidin beads (Pierce, Zug, Switzerland) for 1h at 4ºC and subsequent 4x wash with 30x beads volume of 1xTBS/1%Triton X-100. Membrane proteins were eluted by addition of the sample buffer and boiling for 10min. These samples were subsequently separated and analyzed by SDS-PAGE/Western blot.

.3.4. BODIPY-GTPγS binding assay

The assay was performed essentially as described [21]. 1µM His_6-Gαo or His_6-Gαo[Q205L] was mixed with indicated amounts of suramin. BODIPY-FL-GTPγS (both from Invitrogen, Lucerne, Switzerland) was added to the mixture after 25min to a final concentration of 1µM. The kinetics of *in vitro* G-protein activation was measured by the VICTOR^3 multiwell reader (PerkinElmer, Waltham, USA). For suramin preloading of Gαo, 10µM protein in 1xTBS buffer was adjusted to 20mM $MgCl_2$ and supplemented with 1mM of suramin and incubated for 1h at 37ºC. The unbound suramin was removed at 4ºC by the 10000-fold buffer exchange into 1xTBS on Amicon 10K ultracentrifugation concentrators and the proteins were used in the fluorescent measurements as described.

.3.5. Transferrin and Dextran uptake analysis.

For quantitative analysis of suramin influence on endocytosis, we used fluorescent labeled transferrin - DyLight 488 (Jackson Immunoresearch, West Grove, PA, USA) and TexasRed-Dextran 3000 (Invitrogen, Zug, Switzerland) in the medium at final concentrations of 1µg/ml and 1mM essentially as described before [22,23] with the following modifications. After incubation of HeLa cells with the labels for 30min at 37ºC they were detached by collagenase solution Accutase (BD, Franklin Lakes, NJ, USA), resuspended in 1xPBS/1%FCS and subsequently analyzed on Gallios flow cytometer (Beckman, Nyon, Switzerland). At least 10000 cells were counted for a single assay point. For data representation, the mean fluorescence of cell was used.

.3.6. Proliferation, scratch-wound and colony formation assays with BT-20 TNBC cells

For proliferation assay, BT-20 cells were seeded at the initial concentration of 7000 cells/well in 96-well plates. BT-20 were grown in DMEM (Invitrogen, Lucerne, Switzerland) supplemented with 10% FCS (PAA, Cölbe, Germany). Next day after seeding the medium was replaced with a fresh batch containing indicated concentrations of suramin or vehicle. Every 48h the medium was replaced with a fresh batch containing the same concentration of suramin. The cell numbers were quantified by incubation of cells for 2h in 1mg/ml thiazolyl blue solution in 1x PBS followed by lysis in 50µl DMSO and reading of the absorbance at 570nm.

For migration analysis, the scratch-wound assay was used. BT-20 cells were seeded at 30 000 cells/well in 96-well flat-bottom plates. Next day, monolayer in each well was wounded by a single strike of 10µl pipette tip. The detached cells were removed by 2x wash with 1x PBS. For each experimental well, a random area of the scratch was labeled and its phase-contrast picture was taken. The cells were left for 12h in presence of indicated amounts of suramin. Afterwards, the pictures of the same area were taken and the migration of the cell front was analyzed in ImageJ.

For colony formation assay, BT-20 cells were seeded at 1000 cells/well in 6-well plates. Next day the indicated amount of suramin was added. Colonies of 70-100 cells were formed after 8-9 days, were fixed by incubation in 4% PFA in 1x PBS, pH 7.4 and visualized by staining with crystaline violet solution and the number of colonies was counted.

.3.7. Mouse xenograft experiments

The experiments were approved by the Swiss Federal Veterinary Office and carried out in accordance with the local animal welfare act. 7 NOD-SCID-gamma (NSG) mice were each injected intramammary with 50µl suspension 1×10^6 BT-20 cells stably transfected with pcDNA3-Luciferase construct (Addgene, Cambridge, MA, USA). For injection, the cells were detached by trypsin, washed 2x with ice-cold PBS and resuspended in ice-cold Matrigel (BD, Franklin Lakes, NJ, USA, cat.#356237). Tumor volume (mm^3) was determined using the following formula: tumor volume = length×$(width)^2\times\pi/6$. The drug treatment was started as soon as the tumor reached the volume of ~$100mm^3$. Mice were separated in two groups. The drug treatment group received 300mg/kg dose of suramin solution in water weekly. *In vivo* imaging was performed using IVIS Lumina II (Xenogen) system. Mice were injected with 50mg/kg D-luciferin solution (Goldbio St. Louis, MO, USA) and the luminescence was measured 10min post-injection. Intensity (expressed as photon flow) was quantified using Living Image (PerkinElmer, Waltham, USA) software.

4. RESULTS

.4.1. Suramin inhibits at least two targets in the Wnt pathway

Existing reports on suramin effects in the Wnt signaling pathway [15,24,25] presume that this substance might interfere with Wnt ligand adhesion and receptor interaction. However, it was unclear to which extent this would impose on the downstream signaling in the Wnt pathway. We first addressed this question by analysis of the Wnt3a-induced activation of the pathway using the HEK293T cell line stably transfected with the TOPFlash reporter plasmid and additionally transiently co-transfected with the constitutive *Renilla* luciferase construct [16]. According to our expectations, we found that suramin inhibited Wnt3a-induced signal transduction in a dose-dependent manner (green curve, Fig. 1A). This activity did not correspond to any significant decrease of *Renilla* luciferease levels (data not shown), indicating that suramin does not affect transcription/translation or general cell well-being unspecifically during the time required for the assay.

To roughly estimate the level at which suramin inhibited Wnt signaling, we also analyzed the transcriptional response to 20mM LiCl in the same setting. LiCl directly inhibits GSK3β, a negative downstream component of the Wnt pathway, and thus stimulates downstream elements of the pathway independently of the Wnt protein. To our surprise, suramin also efficiently inhibited the LiCl-induced activation of the Wnt pathway; moreover, the IC_{50} of this effect was identical to that of the Wnt3a-stimulated activity (Fig. 1B). These data point to the existence of a suramin target downstream in the Wnt pathway, i.e. at the level below GSK3β, and speak against the mode of action of suramin at the level of the interaction of Wnt ligands with cell surface receptors or cell surface in general.

To complement our analysis of the action of suramin on Wnt signaling, we used the β-catenin stabilization assay as a secondary readout monitoring pathway activation upon treatment of cells with Wnt3a and LiCl (Fig. 1C). These experiments were performed on two cell types: L-cells and HEK293T cells. The former have virtually no β-catenin in the non-stimulated state and therefore provide much better signal-to-background ratio in this assay. Surprisingly, in this assay we found that suramin blocked β-catenin stabilization induced by Wnt3a, but not that induced by LiCl, in both cell types (Fig. 1C). This observation argues for existence of two different components of the Wnt pathway being the molecular targets of suramin – one acting above β-catenin, and the other – below β-catenin. This idea is further

supported by the observation that the effects of suramin on the two proposed targets occur at different concentrations. Indeed, while the IC_{50} for suramin inhibition of Wnt3a- and LiCl-induced transcriptional response is close to 15μM (green curve on the Fig. 1A), inhibition of the Wnt3a-induced β-catenin stabilization occurs with the IC_{50} of about 150μM (Fig. 1D, see red curve on the Fig. 1A for quantification).

Cumulatively, these data suggest that one molecular target of suramin, playing at 'upstream' levels of the pathway between Wnt3a and GSK3β, is inhibited by suramin with the IC_{50} of about 150μM, while the other molecular target, playing at 'downstream' levels between GSK3β and the transcriptional induction, is inhibited with the IC_{50} of about 15μM (Fig. 1E).

.4.2. Wnt3a ligand endocytosis but not surface adhesion are affected by suramin

We next decided to address in details by which mechanism suramin inhibits the Wnt signal transduction chain between the step of ligand-receptor interaction and the step of β-catenin accumulation. Previous studies suggested that suramin could increase accumulation of the Wnt protein in the extracellular medium through its solubilization from the cell surface [24]. We thus suspected that the ability of the drug to inhibit the Wnt pathway might result from the interference of the drug with the surface adhesion of the Wnt ligand. We decided to verify this proposition directly by assessing the levels of the HA-tagged and untagged Wnt3a ligand remaining on the cell surface after treatment in presence and absence of suramin at 4°C, when the endocytic uptake is inhibited. We first analyzed the binding of untagged Wnt3a to the HEK293 cells; however, due to instability of the HEK293 monolayer at 4°C, we used a suspension of these cells produced by gentle detachment using commercially available collagenase. To achieve more physiologically relevant conditions in presence of the intact extracellular matrix, we also employed HeLa cells for this assay due to their superior adhesive properties during prolonged incubations on cold. Due to lower sensitivity of anti-Wnt3a antibodies, HA-tagged version of Wnt3a ligand was employed in this assay.

Contradictory to previous conclusions, we found out that suramin was unable to affect the surface content of endogenously expressed HA-tagged Wnt3a, nor was it able to prevent adhesion of HA-tagged or untagged Wnt3a provided in a medium (Fig. 2A).

We next checked if the inhibitory effect of suramin might be due to its irreversible activity towards the Wnt protein, such as denaturation or binding, as recently shown for some natural products affecting the Wnt signaling [26]. Purified Wnt3a was incubated with 1mM suramin and the drug was subsequently removed by the buffer exchange on centrifugal filters. As shown on Fig. 2B, Wnt3a retained its full activity after this treatment. A certain decrease in both control and suramin-treated Wnt3a activity after the buffer exchange compared to the non-treated protein is attributed to the effects of accumulation of the CHAPS detergent, a necessary component of the Wnt buffer, which micelles are to some extent retained by the filter.

As suramin failed to show any direct effect on the Wnt protein, we decided to check its influence on other steps required for Wnt pathway activation. After binding to its cognate FZD surface receptors, the Wnt protein undergoes endocytosis, which is essential for the proper pathway functioning [23,27]. To analyze effects of suramin on the Wnt protein endocytosis, we analyzed its accumulation inside the cells in a pulse-chase experiment. As shown on Fig. 2C, in the absence of suramin wild-type HeLa cells readily accumulate HA-tagged Wnt3a protein provided from the conditioned medium in the course of several hours. However, presence of suramin completely abolishes this process. We next wondered if this action of suramin might be due to a general negative effect on endocytic uptake, and thus measured influence of suramin on total cell endocytic activity using fluorescently labeled dextran followed by flow cytometry quantification. In accordance with an earlier report [28], suramin did not affect this process (black line on Fig.2D). However, suramin was able to suppress internalization of fluorescently labelled transferrin in the same setting due to its known activity preventing suramin binding to the transferrin receptor [29].

Taken together, these data suggest that, rather than affecting Wnt ligand surface and receptor binding, suramin exerts its inhibitory action through the prevention of endocytic uptake of Wnt3a. Thus the 'upstream' level of action of suramin on the Wnt pathway (see Fig. 1E) is one of the components regulating Wnt ligand endocytosis.

.4.3. Suramin acts as a nucleotide analog and a reversible inhibitor of α-subunits of heterotrimeric G proteins

Heterotrimeric G proteins, being important transducers of FZD-family G protein-coupled receptors (GPCRs) in the Wnt pathway [30–32], have also been shown to regulate endocytic events, important for the amplification of the signal in the Wnt pathway [23,33]. Intriguingly, suramin has been previously shown to interfere with the ability of heterotrimeric G proteins to incorporate GTP and decouple them from their cognate GPCRs [9].

We decided to investigate this in further details and assessed the effects of suramin on α-subunits of heterotrimieric G proteins of Go/i family – key downstream effectors of Frizzled receptors [17,34,35]. We found that suramin has a dose-dependent reversible inhibition of the uptake of the fluorescent non-hydrolyzable GTP analog BODIPY-GTPγS by purified Gαo (Fig. 3AB). Gαo[Q205L] – a GTPase-deficient mutant of the G protein – is purified after recombinant production as a GTP-loaded protein, unlike the wild-type Gαo which is purified in the GDP state [21]. We find that the slow exchange of GTP from Gαo[Q205L] to BODIPY-GTPγS [21] is inhibited by suramin in a manner similar to the effect of the drug on the wild-type Gαo (Fig. S1AB).

Interestingly, the IC_{50} of these effects was comparable but lower than the IC_{50} observed for suramin inhibition of β-catenin stabilization (see Fig. 1). We argue that inhibition of heterotrimeric G proteins by suramin is the mechanism of inhibition of the 'upper' levels of the Wnt pathway, but that higher concentrations of the drug are required to show the effect in intact cells than on purified G proteins as suramin might have a reduced ability to penetrate through cell membranes.

To clarify whether suramin modulates Gα-subunits, locking them in the GDP-bound state, or whether it may rather act as a nucleotide analog, we injected suramin immediately after preloading Gαo with BODIPY-GTPγS (Fig. 3C). The resultant rapid drop in the fluorescence signal indicates loss of the GTP analog from the binding pocket of the G protein, apparently due to its substitution with the suramin molecule. Of note, this function of suramin as a nucleotide analog, competing for the nucleotide binding pocket of G proteins, is consistent with suramin functioning as purinergic antagonist [4]. Therefore, we speculate that the mechanism by which suramin acts on the 'upper' levels of the Wnt pathway is the inhibition of endocytosis by preventing the activation of the heterotrimeric G proteins of Gi/o family.

.4.4. Suramin inhibits triple-negative breast cancer cell growth *in vitro* and *in vivo*

Both proliferation and invasiveness of triple-negative breast cancer (TNBC) cells are known to depend on the overactivation of the Wnt pathway [36–38]. Moreover, it has been shown that this subtype of breast cancer is mostly dependent on overexpression of the early components of the Wnt pathway, such as the FZD_7 receptor [11,39]. We decided to check whether inhibition of Wnt signaling by suramin could also inhibit growth of TNBC cells. Indeed, we found that suramin inhibited proliferation of TNBC BT-20 cells in a concentration-dependent manner (Fig 4A). Moreover, it was able to suppress cell migration and colony formation (Fig. 4B and 4C). Interestingly, the IC_{50} of these effects is close to the IC_{50} value of suramin's inhibition of Wnt endocytosis and heterotrimeric G proteins, indicating that this effect on the 'upper floors' of the Wnt pathway might be causative for the inhibition of TNBC cell proliferation.

Encouraged by these data, we further proceeded to an *in vivo* proof of anti-TNBC activity of suramin. Being an approved drug, suramin is well-described in terms of its pharmacological properties. We analyzed NOD-SCID-gamma mice bearing intramammary xenograft of human TNBC BT-20 cells stably transfected with a luciferase reporter. Half of the mice received treatment with 300mg/kg IV dose of

suramin weekly, which was expected to produce and maintain therapeutically relevant Wnt-inhibitory plasma levels of the drug of around ~100μM. The tumor growth monitored over 5 weeks demonstrated clear inhibition of the tumor growth compared to control treatment (Fig. 5D), thus indicating efficiency of suramin as an anti-TNBC drug.

.4.5. Suramin effects on Wnt signaling are mimicked by pharmacological inhibitors of G protein signaling.

We next wanted to confirm that effects of suramin on upper levels of Wnt signaling are indeed mediated by its ability to inhibit G protein signaling. To this end we have chosen two well-characterized and broad-scope inhibitors of G protein signaling: toxin from Bordetella pertussis (pertussis toxin, PTX), which is known to decouple G proteins of Go/i family from their cognate GPCRs through attachment of ADP-ribose moiety to the Gα subunits [40], and gallein, which was shown to be medium-affinity specific and non-toxic inhibitor of Gβγ signaling [41]. Effects of these agents on Wnt signaling were analyzed on TNBC cell line BT-20 as well as HeLa cells, which were transfected by FZD_7 which has emerged as the main mediator of Wnt signaling in TNBC [39]. Indeed, such treatments resulted in significant, albeit incomplete, reduction in Wnt3a-induced β-catenin levels (Fig. 5A and 5B) as well as overall Wnt signaling as measured in TopFlash assay (Fig. 5C and 5D). Notably, the IC_{50} of gallein in Wnt signaling was found to be ~1.3μM (Fig. 5C) which correlates well with the previous data showing IC_{50} of this compound for Gβγ to be about 0.5μM [41], small discrepancy being likely due to a different cell type and assay used as well as batch-to-batch variation. Importantly, we have also observed no toxic effects of PTX, gallein or their mixture during the assay time, as measured by the parallel constitutive renilla luciferase expression (Fig. S2A, Fig. 5C), nor any effect of the agents was observed for the levels of TopFlash signal in the absence of Wnt3a stimulation (Fig. S2B), both indicating specificity of observed effect.

Incomplete effects of the drugs might be explained by necessity of simultaneous action of Gα and Gβγ subunits in Wnt signaling [42], which is reinforced by observation of additivity of the effect when both agents are used simultaneously (Fig. 5D). However, as even in this case inhibition failed to be complete, it again points towards potential involvement of G proteins from other than Go/i family or families, as suggested by other works [43–45], as well as to uniqueness of suramin as the tool for G protein-mediated signaling dissection, as its mechanism of GDP analog action presumes ability to suppress any Gα and Gβγ activity. Finally, the involvement of different G proteins depends on the cell type and Wnt/FZD landscape, as the application of PTX in some cell lines might vary from having no observable effect (unpublished observations of authors and other groups) to complete inhibition [46] of Wnt signaling.

5. DISCUSSION

Investigations of novel and approved drug compounds and small molecules in many instances resulted in the discovery of unexpected novel properties of such compounds as well as expanded our knowledge of the pathways which they regulate [47–50]. We here show that suramin, a multi-purpose drug initially developed against sleeping sickness, has the potential of being repositioned as an anti-triple-negative breast cancer (TNBC) drug. TNBC is the most aggressive type of the breast cancer, accounting for more than half of breast cancer-induced deaths, despite covering only ca. 15% of all breast cancer incidences [51]. This disproportionally high mortality is due to the lack of targeted therapies for the TNBC, which distinguishes this type of breast cancer from ER-positive and HER2-positive breast cancers [11,52]. Recently it became clear that the Wnt signaling cascade is overactivated in TNBC through overexpression of the LRP6 and FZD_7 receptors and that downregulation of this signaling pathway leads to proliferation arrest of TNBC cells *in vitro* and mouse xenograft models [37,38,53]. These observations make the Wnt signaling pathway an attractive target for the anti-TNBC drug discovery.

However, given the fact that the Wnt pathway is involved in many physiological contexts, e.g. renewal

of epithelial cells in the gastrointestinal tract, blunt suppression of the Wnt pathway, which is achieved e.g. by inhibitors acting at the downstream components of the signaling, is not desired. Instead, it is more promising to selectively target the upper levels of the pathway, where more variability among the signaling subtypes is provided [11].

Here we show that suramin has at least two targets within the Wnt signaling pathway, one in its 'lower floors', and the other in its 'upper floors' (see Fig. 1E), which are sensitive to different suramin concentrations. We further provide evidence suggesting that the suramin target in the 'upper floors' is heterotrimeric G proteins, regulating endocytosis of the Wnt protein (likely in the complex with its receptors), and that this inhibition is sufficient to block proliferation of TNBC cells *in vitro* and in the mouse xenograft model.

Endocytic regulation of cell signaling pathways has been an intensive topic of research. Initial model, suggesting that internalization of receptors from the cell surface serves to shut signaling off, dominated in the past [54]. However, many recent observations demonstrate that in several instances internalized ligand-receptor complexes continue to signal from the endocytic compartments, often in a different mode or with an increased strength [55–57]. In the latter case, one can speak of signal amplification mediated by the ligand/receptor endocytosis. Such amplification has also been observed in Wnt signaling [23,27]. Here, receptors of the FZD family were found to employ a cunning mechanism for their endocytosis, relying on their GPCR activity to recruit the heterotrimeric Go protein and the small GTPase Rab5 to promote this process [23]. We correlated this fact with the known findings concerning the ability of suramin to inhibit heterotrimeric G proteins [9,10]. We further expanded these findings, showing that suramin is a low-μM inhibitor of the α-subunit of the heterotrimeric protein Go, the main partner of FZD receptor [42,58,59] and that it acts in a competitive manner. The activities of suramin we describe here thus support earlier observations on the involvement of heterotrimeric G proteins in the Wnt pathway in fruit flies and vertebrates [17,23,60].

Our finding prompted us to re-investigate the anti-cancer properties of suramin. We find suramin to be a strong anti-cancer compound in both *in vitro* and *in vivo* settings. While previous reports already pointed out an anti-TNBC activity of suramin, alone [61,62] or in combination with other drugs [63,64], our study sheds new light on these data, indicating the anti-Wnt signaling potential of suramin as the main mode of anti-cancer action of this drug. Importantly, suramin in the Wnt-suppressing dosages does not produce any overt toxicity in mice over the period of the experiment. We find that the maximum efficiency of suramin as an anti-TNBC agent *in vivo* coincides with its ability to inhibit 'upper' events in the Wnt signaling. Therapeutic levels of suramin in plasma should be thus about 100-200μM to result in the anti-TNBC effects, explaining why suramin previously failed to show profound anti-TNBC effects in clinical trials [65,66], in which suramin plasma levels were below 50μM. Our data identify regulation of Wnt endocytosis as a promising target for anti-Wnt drugs, and suramin serves as the first example and proof of concept in creation of safe pharmacological agents with such mode of action.

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8. FIGURE LEGENDS

Figure 1. Suramin demonstrates concentration-dependent inhibition of the Wnt pathway and has at least two targets in the pathway. (A) Dose-response inhibition curves measured by TOPFlash assay (green) in stably transfected HEK293-Tf cells or β-catenin stabilization assay in L-cells (red line). (B) Suramin inhibits Wnt3a- or LiCl-induced TOPFlash response with the same efficiency. (C) Suramin inhibits Wnt3a-induced but not LiCl-induced β-catenin stabilization in both HEK293 and L-cells. (D) Dose-dependent inhibition of β-catenin stabilization by suramin in L-cells. See panel (A) for quantification. For panels (A) and (B), the data shown as mean±SEM, n=3-6; panels (C) and (D) are representatives of three experiments.

Figure 2. Suramin exerts its Wnt-inhibitory properties through inhibition of Wnt ligand endocytosis. (A) Suramin fails to suppress Wnt surface adhesion at 4°C in suspension of HEK293 cells or in adherent HeLa cells both when Wnt3a-HA ligand was supplemented as conditioned medium and when membrane-associated portion of Wnt3a-HA ligand was measured in HeLa cells stably transfected with Wnt3a-HA. (B) Purified Wnt3a retains its activity after incubation and subsequent removal of 1mM suramin. "Wnt3a" column is a reference activity induced by non-treated 0.5μg/ml Wnt3a ligand; "(Wnt3a+Sur)" is an activity of 0.5μg/ml Wnt3a ligand after treatment with and subsequent removal of 1mM suramin; "Wnt3a+Sur" is an activity mock-treated 1mM suramin solution in the Wnt3a vehicle buffer further supplemented with 0.5μg/ml of non-treated Wnt3a; "Wnt3a:1mM Sur" is an activity of 0.5μg/ml Wnt3a in continued presence of 1mM suramin. (C) 0.5mM suramin supressed intracellular accumulation of Wnt3a-HA ligand in HeLa cells as measured by amount of Wnt3a in total cell lysates. (D) Suramin is suppressing transferring uptake by inhibiting its binding to the receptor, but is not an inhibitor of general endocytosis as measured by amount of TxRed-Dextran uptake. For panels (B) and (D), the data shown as mean±SEM, n=4-6. For panel B, the significance (Student's t-test) is shown as * ($p<0.05$) for comparison of Wnt3a and (Wnt3a+Sur) columns; for the rest, p-value was <0.001. Panels (A) and (C) are representatives of four experiments.

Figure 3. Suramin is a reversible inhibitor of Gαo subunit of heterotrimeric G proteins (A) Kinetics of BODIPY-GTPyS loading in Gαo in presence or absence of different concentrations of suramin. (B) Dose-response curve of Gαo inhibition by suramin. (C) Gαo regains the ability to bind BODIPY-GTPyS after removal of suramin; injection of suramin after complete loading of Gαo with BODIPY-GTPyS results in strong drop of fluorescence consistent with displacement of BODIPY-GTPyS from Gαo. For panel (B) the data shown as mean±SEM, n=3. Panels (A) and (C) are representatives of three and two experiments, respectively.

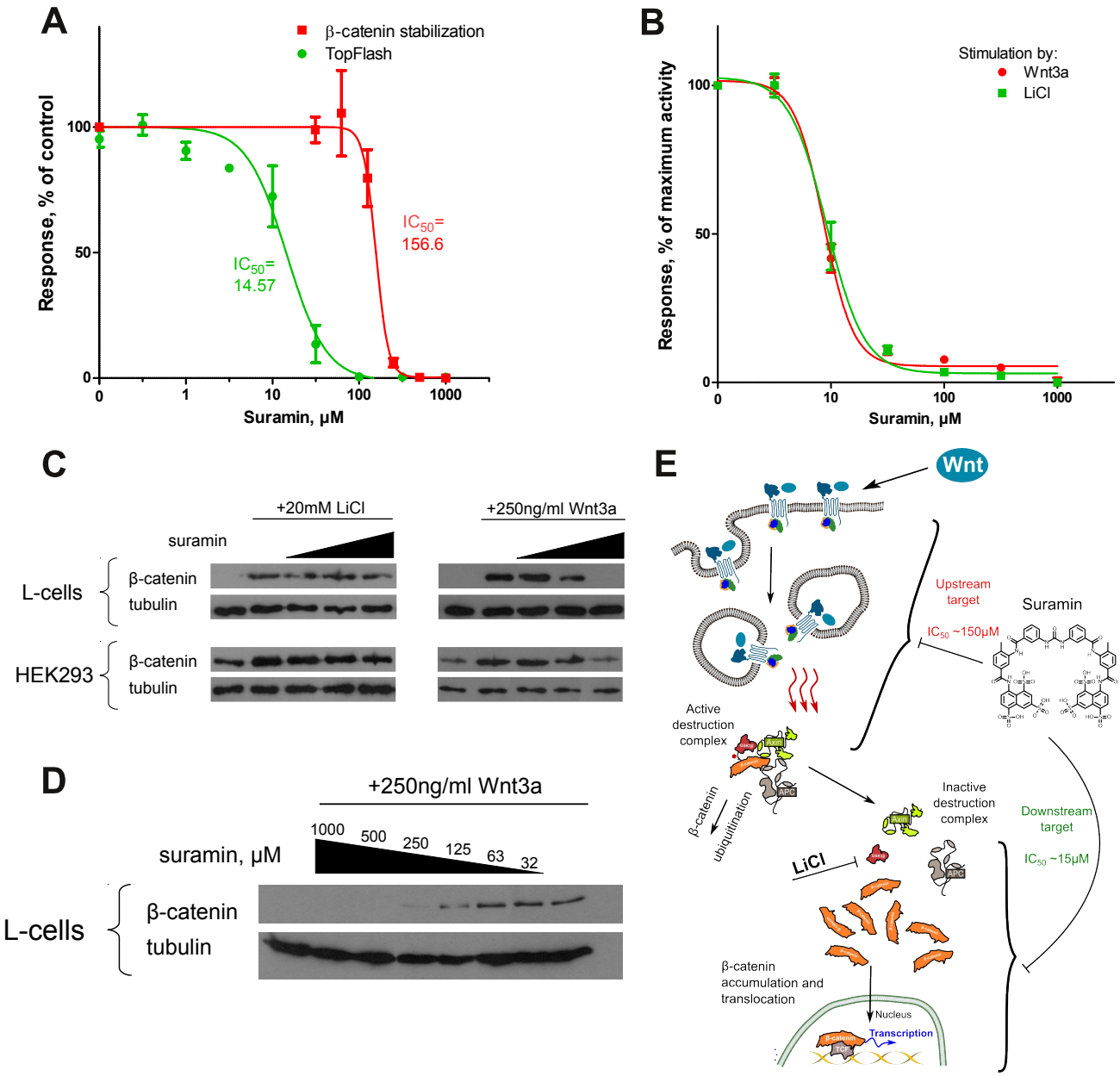
Figure 4. Anti-TNBC properties of suramin. Suramin is inhibiting growth (A), migration (B) and colony formation (C) by TNBC cell line BT-20. Weekly dosage of 300mg/kg suramin resulted in a strong decrease of xenograft BT-20 tumor size in NGS mice after 4 weeks of treatment. For panel (A), (B) and (C) the data shown as mean±SEM, n=4 to 9. For panel (D), data are shown as mean±SD from 3 (control group) or 4 (drug-treated group) animals. For panel (D), significantly different data points are marked as ** ($p<0.01$) or *** ($p<0.001$) from 2-way ANOVA with Bonferroni post-tests.

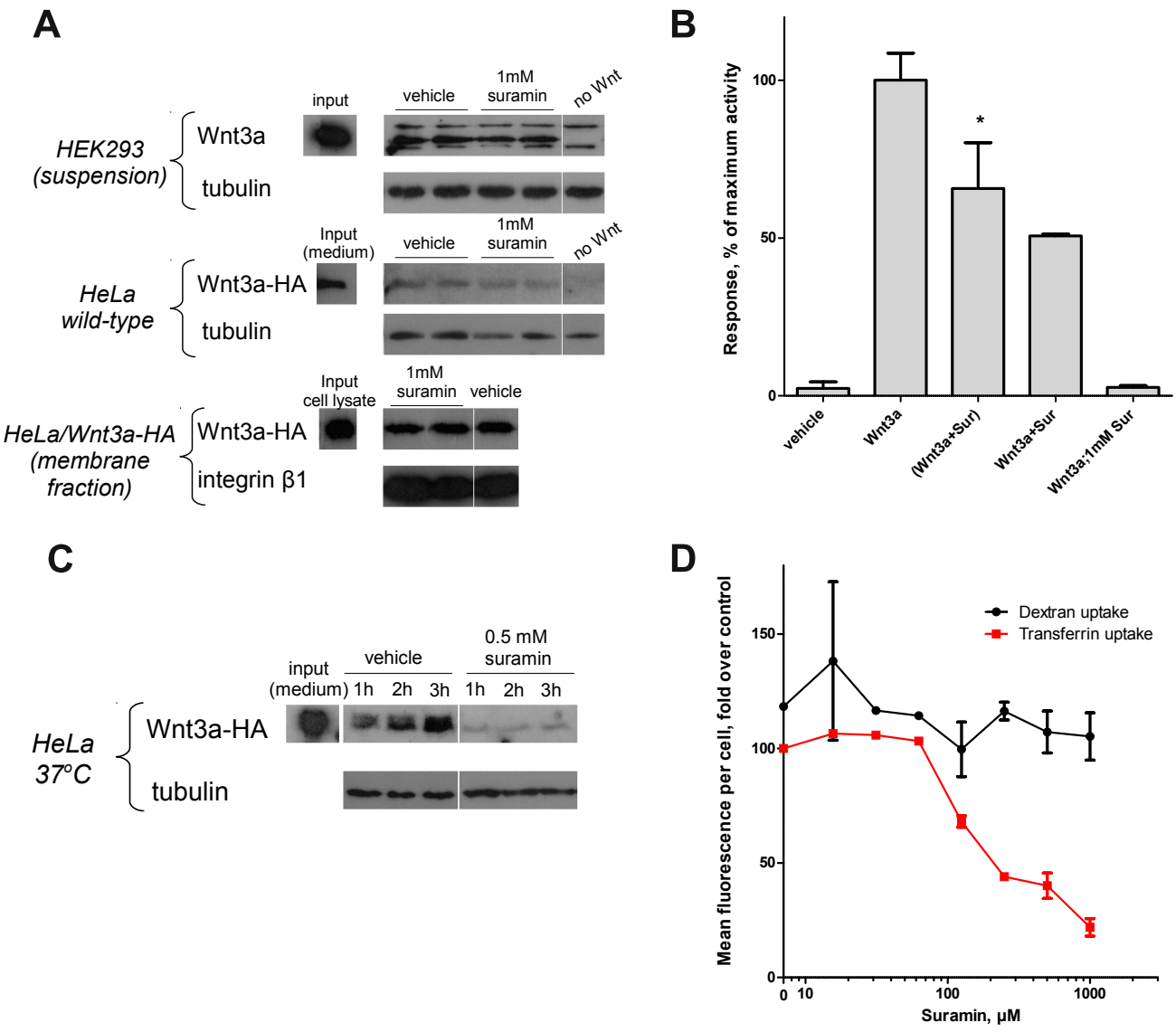
Figure 5. Effects of suramin are mimicked by pharmacological inhibitors of G protein signaling. BT-20 TNBC cells or HeLa transfected by FZD_7 pretreated by PTX (A) or gallein (general Gβγ inhibitor, B) demonstrate significantly decreased levels of β-catenin in response for Wnt3a. Representative Western blottings are shown on the left side of the panel, respective quantifications are on the right side. β-catenin/tubulin ratio was used for calculation and the data was normalized to basal levels of β-catenin in the cells (C). Gallein is able to suppress TopFlash response (red curve) induced by Wnt3a in a dose-dependent manner with IC_{50} ~1.3μM without observable toxic or unspecific effects as measured by concomitant measurement of CMV-driven Renilla luciferase levels (black curve) up to 20μM. (D) PTX

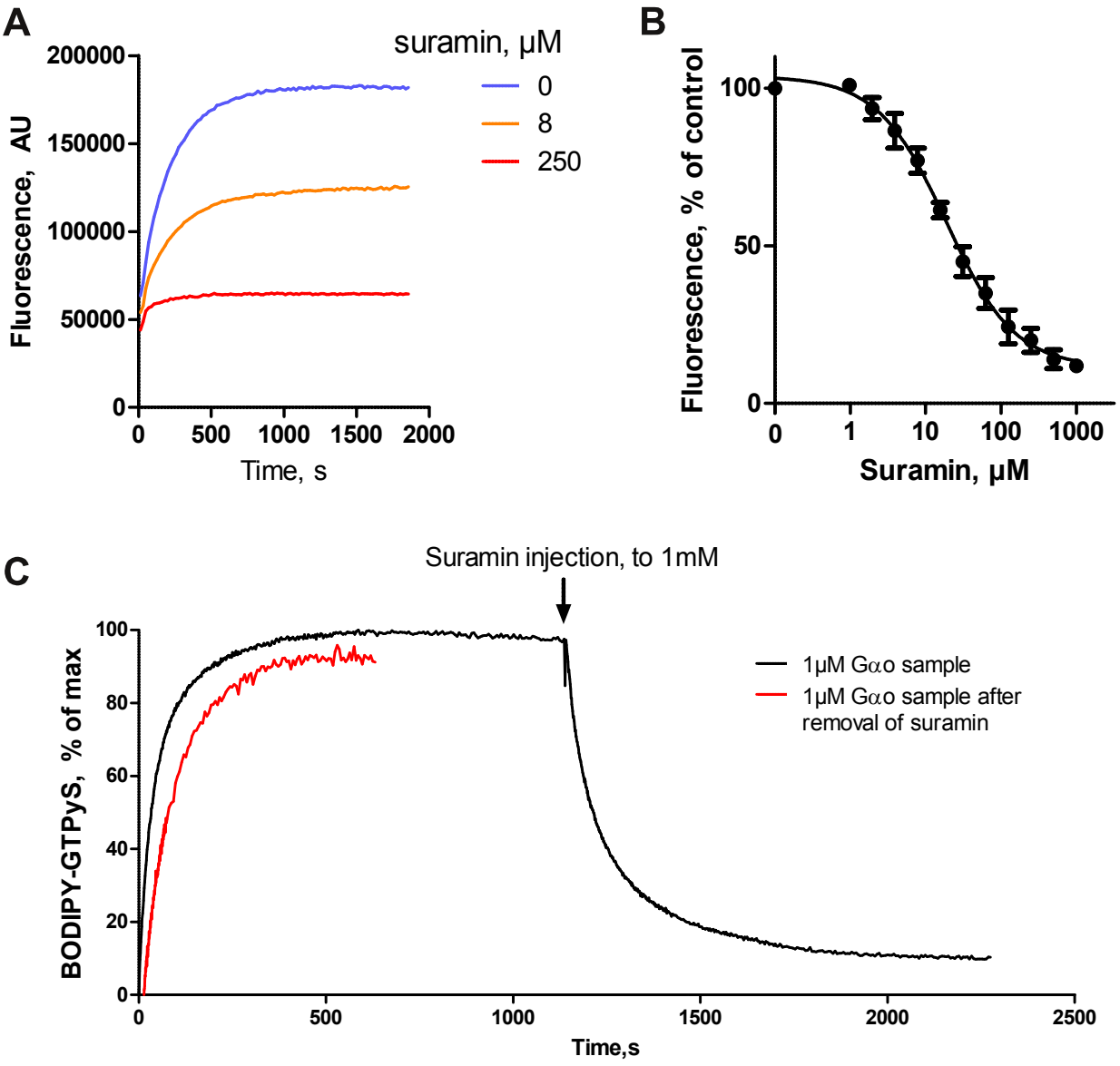
and Gallein significantly reduced Wnt-induced TopFlash levels in both FZD_7-transfected HeLa cells and BT-20 TNBC line stably transfected by TopFlash reporter; they also demonstrate significant additive effects when applied together. Data are shown as normalized mean±SEM of n=5 to 12; Western blots of panels (A) and (B) are representatives of 5-12 experiments. The statistical significance is shown as * ($p<0.05$), ** ($p<0.01$) and *** for ($p<0.001$) (Student's t-test).

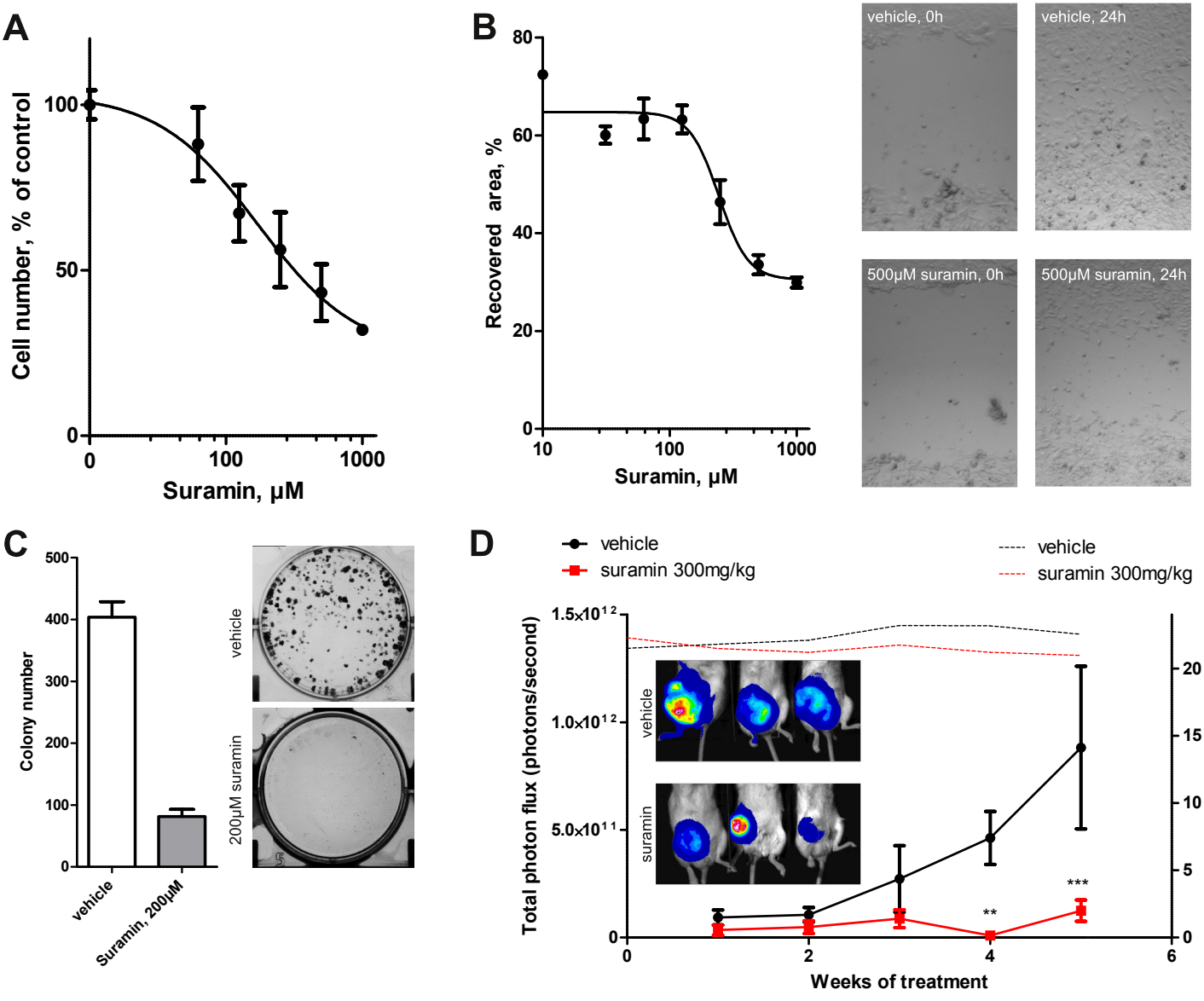
Figure S1. Suramin acts as competitive inhibitor of Gαo[Q205L] (a GTPase-deficient mutant). (A) shows representative kinetic curves of BODIPY-GTPγS binding to purified human Gαo[Q205L] in absence or presence of different suramin concentrations. (B) Plato values reached in presence of different concentrations of suramin were used to quantify inhibition of GTP-binding to Gαo[Q205L] mutant. For panel (B) the data shown as mean±SD, n=3; panel (A) is a representative of three experiments.

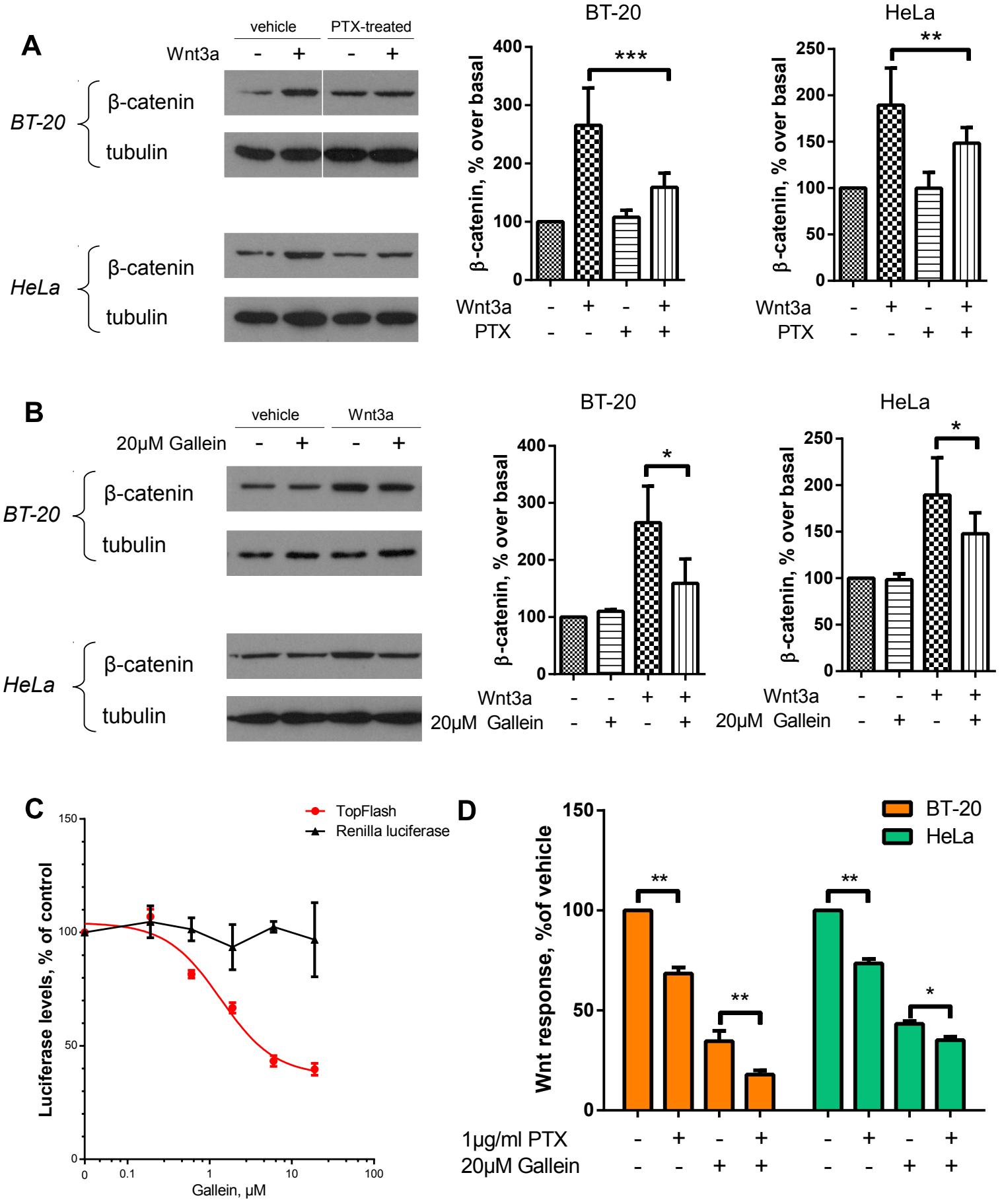
Figure S2. Pertussis toxin, gallein or their mixture exert no toxic effect or affect basal levels of TopFlash activity. (A) No observable changes were found for levels of luciferase in cells not stimulated by Wnt3a in TopFlash assay. (B) No apparent toxicity was observed after treatment with PTX, gallein or their mixture by the end of TopFlash assay as viewed by the unaffected levels of CMV-driven Renilla luciferase. Data shown as mean±SEM, n=6 to 9; no statistical differences were found by Student's t-test.

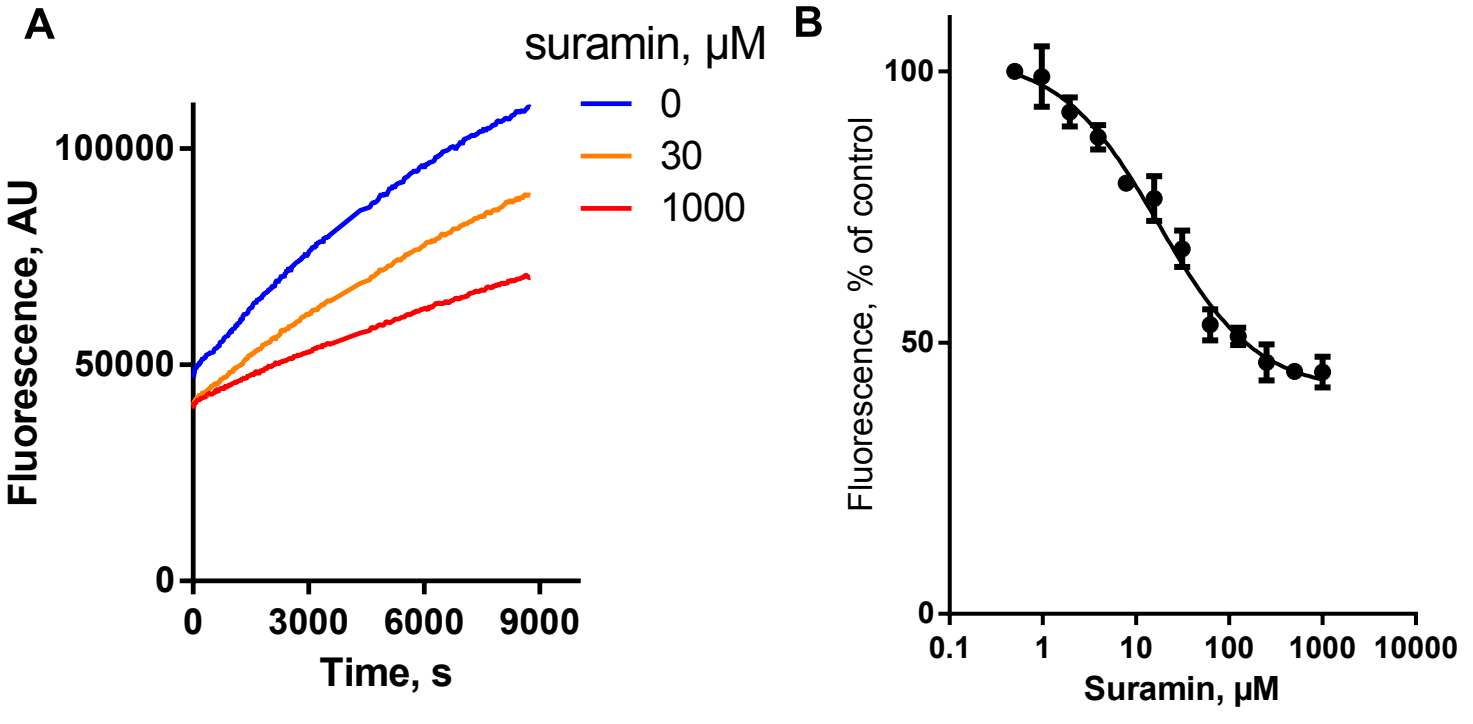

A
β-catenin stabilization
TopFlash
Response, % of control
100
50
0
0
1
10
100
1000
Suramin, μM
IC50= 156.6
IC50= 14.57
B
Stimulation by:
Wnt3a
LiCl
Response, % of maximum activity
100
50
0
0
10
100
1000
Suramin, μM
C
+20mM LiCl
+250ng/ml Wnt3a
suramin
L-cells
β-catenin
tubulin
HEK293
β-catenin
tubulin
D
+250ng/ml Wnt3a
suramin, μM
1000
500
250
125
63
32
L-cells
β-catenin
tubulin
E
Wnt
Upstream target
Suramin
IC50 ~150μM
Active destruction complex
β-catenin ubiquitination
Inactive destruction complex
Downstream target
IC50 ~15μM
LiCl
β-catenin accumulation and translocation
Nucleus
Transcription

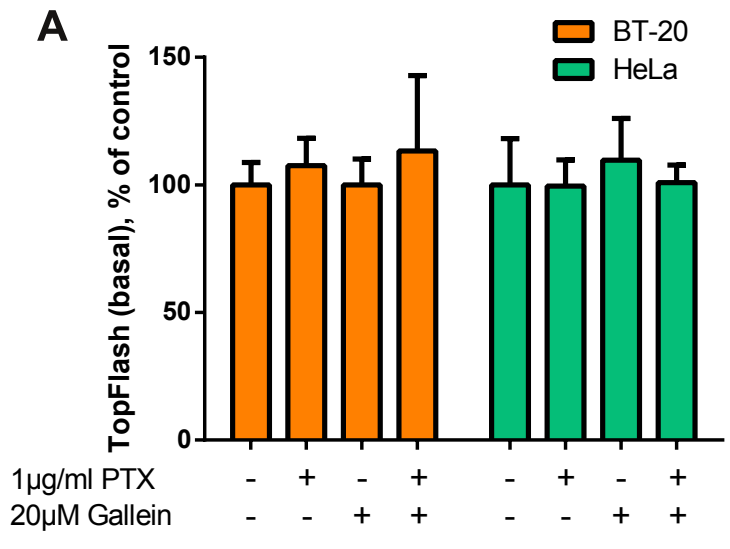

A
input
vehicle
1mM suramin
no Wnt
HEK293 (suspension)
Wnt3a
tubulin
Input (medium)
vehicle
1mM suramin
no Wnt
HeLa wild-type
Wnt3a-HA
tubulin
Input cell lysate
1mM suramin
vehicle
HeLa/Wnt3a-HA (membrane fraction)
Wnt3a-HA
integrin β1
B
Response, % of maximum activity
100
50
0
*
vehicle
Wnt3a
(Wnt3a+Sur)
Wnt3a+Sur
Wnt3a;1mM Sur
C
input (medium)
vehicle
0.5 mM suramin
1h 2h 3h 1h 2h 3h
HeLa 37°C
Wnt3a-HA
tubulin
D
Mean fluorescence per cell, fold over control
150
100
50
0
Dextran uptake
Transferrin uptake
0 10 100 1000
Suramin, μM

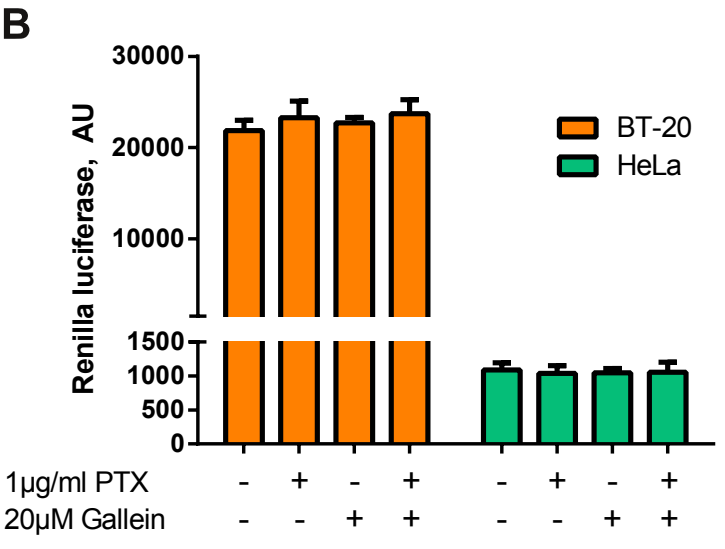

A
suramin, μM
0
8
250
Fluorescence, AU
200000
150000
100000
50000
0
0
500
1000
1500
2000
Time, s
B
Fluorescence, % of control
100
50
0
0
1
10
100
1000
Suramin, μM
C
Suramin injection, to 1mM
1μM Gαo sample
1μM Gαo sample after removal of suramin
BODIPY-GTPγS, % of max
100
80
60
40
20
0
0
500
1000
1500
2000
2500
Time,s


A
Cell number, % of control
100
50
0
0
100
1000
Suramin, μM
B
Recovered area, %
80
60
40
20
0
10
100
1000
Suramin, μM
vehicle, 0h
vehicle, 24h
500μM suramin, 0h
500μM suramin, 24h
C
Colony number
500
400
300
200
100
0
vehicle
Suramin, 200μM
vehicle
200μM suramin
D
vehicle
suramin 300mg/kg
vehicle
suramin 300mg/kg
Total photon flux (photons/second)
1.5x10^12
1.0x10^12
5.0x10^11
0
vehicle
suramin
**
***
Body weight, g
20
15
10
5
0
0
2
4
6
Weeks of treatment


A
vehicle
PTX-treated
Wnt3a - + - +
BT-20
β-catenin
tubulin
HeLa
β-catenin
tubulin
BT-20
HeLa
β-catenin, % over basal
***
**
Wnt3a - + - +
PTX - - + +
B
vehicle
Wnt3a
20μM Gallein - + - +
BT-20
β-catenin
tubulin
HeLa
β-catenin
tubulin
*
Wnt3a - - + +
20μM Gallein - + - +
C
TopFlash
Renilla luciferase
Luciferase levels, % of control
Gallein, μM
D
BT-20
HeLa
Wnt response, %of vehicle
1μg/ml PTX - + - + - + - +
20μM Gallein - - + + - - + +


A
suramin, μM
0
30
1000
100000
50000
0
Fluorescence, AU
0
3000
6000
9000
Time, s
B
100
50
0
Fluorescence, % of control
0.1
1
10
100
1000
10000
Suramin, μM


A
BT-20
HeLa
TopFlash (basal), % of control
150
100
50
0
1µg/ml PTX
20µM Gallein
- + - + - + - +
- - + + - - + +


B
BT-20
HeLa
Renilla luciferase, AU
30000
20000
10000
1500
1000
500
0
1µg/ml PTX
20µM Gallein
- + - + - + - +
- - + + - - + +