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Inhibition of Wnt signalling and breast tumour growth by the multi-purpose drug suramin through suppression of heterotrimeric G proteins and Wnt endocytosis

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Overactivation of the Wnt signalling pathway underlies oncogenic transformation and proliferation in many cancers, including the triple-negative breast cancer (TNBC), the deadliest form of tumour in the breast, taking about a quarter of a million lives annually worldwide. No clinically approved targeted therapies attacking Wnt signalling currently exist. Repositioning of approved drugs is a promising approach in drug discovery. In the present study we show that a multi-purpose drug suramin inhibits Wnt signalling 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 signalling as a promising target for anti-Wnt and anti-cancer drug discovery. Suramin, as the first example of such drug or its analogues might pave the way for the appearance of first-in-class targeted therapies against TNBC and other Wnt-dependent cancers.

Key words: drug repositioning, internalization, nucleotide analogue, triple-negative breast cancer, Wnt pathway.

INTRODUCTION

Suramin is a rather large (M_r = 1297) symmetric polysulfated 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 signalling, the developmentally and medically important pathway controlling multiple steps in embryogenesis and misactivated in many cancers [11–13]. Wnt signalling is initiated by the interaction of secreted lipoglycoproteins of the Wnt family with the cell surface receptors of the frizzled (FZD) family. Pathways initiated by Wnt ligands are diverse and different co-receptors, such as low-density lipoprotein receptor-related proteins 5/6 (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 for 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 signalling is activated, this destruction complex becomes inactivated, leading to stabilization of β-catenin and its translocation into the nucleus, leading to activation of LEF (lymphoid enhancer factor)/TCF (T-cell factor)-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 signalling 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 signalling 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 (TNBC) cells *in vitro* and in mouse models.

Abbreviations: APC, adenomatous polyposis coli; DMEM, Dulbecco's modified Eagle's medium; ER, estrogen receptor; FCS, fetal calf serum; FZD, frizzled; GPCR, G protein-coupled receptor; GSK3β, glycogen synthase kinase 3β; Gα, α-subunit (catalytic) of heterotrimeric G protein; HA, haemagglutinin; HEK, human embryonic kidney; HER2, human epidermal growth factor receptor; LRP5/6, low-density lipoprotein receptor-related proteins 5/6; NHS-SS, N-Hydroxysuccinimide-persulfide; NOD, non-obese diabetic; pCMV, cytomegalovirus promoter; PTX, pertussis toxin; RIPA, radioimmunoprecipitation assay buffer; RL, Renilla luciferase; SCID, severe combined immunodeficiency; TNBC, triple-negative breast cancer.

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MATERIALS AND METHODS

Luciferase-based assay of the Wnt-dependent transcriptional activity

Human embryonic kidney (HEK)293T and BT-20 cells stably transfected with M50 Super 8 × TOPFlash plasmid [16] were used to analyse the Wnt inhibitory activity of suramin (Sigma). The assay was performed in white tissue-culture-treated 96-well plates (Greiner). The HEK293-Tf or BT-20-Tf cells were seeded in 100 μl of Dulbecco's modified Eagle's medium (DMEM) containing 10 % fetal calf serum (FCS) at ∼10000 cells/well and subsequently stimulated by 0.5 μg/ml mouse Wnt3a purified as described [17] or 20 mM LiCl in the medium in presence or absence of the drug for 12 h. Pertussis toxin (PTX, Enzo; 1 μg/ml) and gallein (Tocris) pre-treatments were done overnight before the assay. For investigation of suramin effects on Wnt3a, purified ligand was incubated with 1 mM suramin overnight and then suramin was removed by 100-fold dilution by the Wnt3a buffer (20 mM HEPES, pH 7.4, 150 mM NaCl, 1 % CHAPS) followed by concentration using Amicon 10K centrifugal concentrators. To account for effects of increased CHAPS concentrations and residual suramin (∼1 μM in the medium during assays), an 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) according to the manufacturer's protocol. The medium was subsequently removed and 15 μl of the lysis buffer (25 mM glycylglycine, pH 7.8, 1 % Triton X-100, 15 mM $MgSO_4$, 4 mM EGTA, 1 mM DTT) were pipetted into each well. After incubation for 5 min at room temperature the 96-well plate was analysed using the Victor^3 Multilabel Counter (PerkinElmer) 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 the study by Dyer et al. [19]). The final volumes dispensed per well were 50 μl of firefly and 50 μl of *Renilla* solutions.

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. One-hour pre-incubation was used in all experiments involving suramin; PTX and gallein pre-treatment were done overnight, the HeLa cells were additionally transfected by FZD_7 subcloned in pcDNA3.1 plasmid as described in the previous section. Subsequently, purified Wnt3a or vehicle buffer were directly added into the wells to the final concentration of 1 μg/ml and incubated at 37 °C for 2 h in case of L-cells or 12 h in case of BT-20, HEK293 or HeLa cells. Subsequently the medium was removed, the cells in each well washed once with 500 μl of 1 × PBS (Biochrom AG) and lysed directly in the well by addition of 50 μl of ice-cold RIPA buffer for 10 min on ice. The cells were then resuspended, the debris was removed by 10-min centrifugation at 16000 ***g***, 4 °C, and the probes were further analysed by Western blot with antibodies against β-catenin (BD) and α-tubulin (Sigma).

Wnt internalization and surface binding

For internalization or surface adhesion of exogenous Wnt3a, wild-type HeLa cells were seeded at 5×10^5 cells/well in six-well plates. Next day, Wnt3a–HA (haemagglutinin)-conditioned medium or purified Wnt3a was added to the cells and incubated either at 37 °C (internalization) or 4 °C (surface adhesion; cells were pre-incubated on ice for 1 h prior to addition) for indicated amounts of time in presence or absence of 1 mM suramin. Subsequently the medium was removed and cells were washed 2 × with ice-cold 1 × PBS and then lysed in 1 × RIPA buffer and analysed by Western blotting.

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

BODIPY-GTPγS-binding assay

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

Transferrin and dextran uptake analysis

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

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) supplemented with 10 % FCS (PAA Laboratories GmbH). Next day after seeding, the medium was

replaced with a fresh batch containing indicated concentrations of suramin or vehicle. Every 48 h the medium was replaced with a fresh batch containing the same concentration of suramin. The cell numbers were quantified by incubation of cells for 2 h in 1 mg/ml thiazolyl blue solution in 1 × PBS followed by lysis in 50 μl of DMSO and reading of the absorbance at 570 nm.

For migration analysis, the scratch-wound assay was used. BT-20 cells were seeded at 30000 cells/well in 96-well flat-bottom plates. Next day, the monolayer in each well was wounded by a single strike of a 10 μl pipette tip. The detached cells were removed by 2 × wash with 1 × PBS. For each experimental well, a random area of the scratch was labelled and its phase-contrast picture was taken. The cells were left for 12 h in presence of indicated amounts of suramin. Afterwards, the pictures of the same area were taken and the migration of the cell front was analysed in ImageJ.

For colony formation assay, BT-20 cells were seeded at 1000 cells/well in six-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 1 × PBS, pH 7.4, and visualized by staining with Crystalline Violet solution and the number of colonies was counted.

Mouse xenograft experiments

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

RESULTS

Suramin inhibits at least two targets in the Wnt pathway

Existing reports on suramin effects in the Wnt signalling pathway [15,24,25] presume that this substance might interfere with Wnt ligand adhesion and receptor interaction. However, it was unclear to what extent this would impose on the downstream signalling 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; Figure 1A). This activity did not correspond to any significant decrease in *Renilla* luciferase levels (result 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 signalling, we also analysed 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 with that of the Wnt3a-stimulated activity (Figure 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 signalling, we used the β-catenin stabilization assay as a secondary readout monitoring pathway activation upon treatment of cells with Wnt3a and LiCl (Figure 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 (Figure 1C). This observation argues for the 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, whereas the IC_{50} for suramin inhibition of Wnt3a- and LiCl-induced transcriptional response is close to 15 μM (green curve on the Figure 1A), inhibition of the Wnt3a-induced β-catenin stabilization occurs with an IC_{50} of approximately 150 μM (Figure 1D, see red curve on the Figure 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 an IC_{50} of approximately 150 μM, whereas the other molecular target, playing at 'downstream' levels between GSK3β and the transcriptional induction, is inhibited with an IC_{50} of approximately 15 μM (Figure 1E).

Wnt3a ligand endocytosis but not surface adhesion is affected by suramin

We next decided to address in detail 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 analysed 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

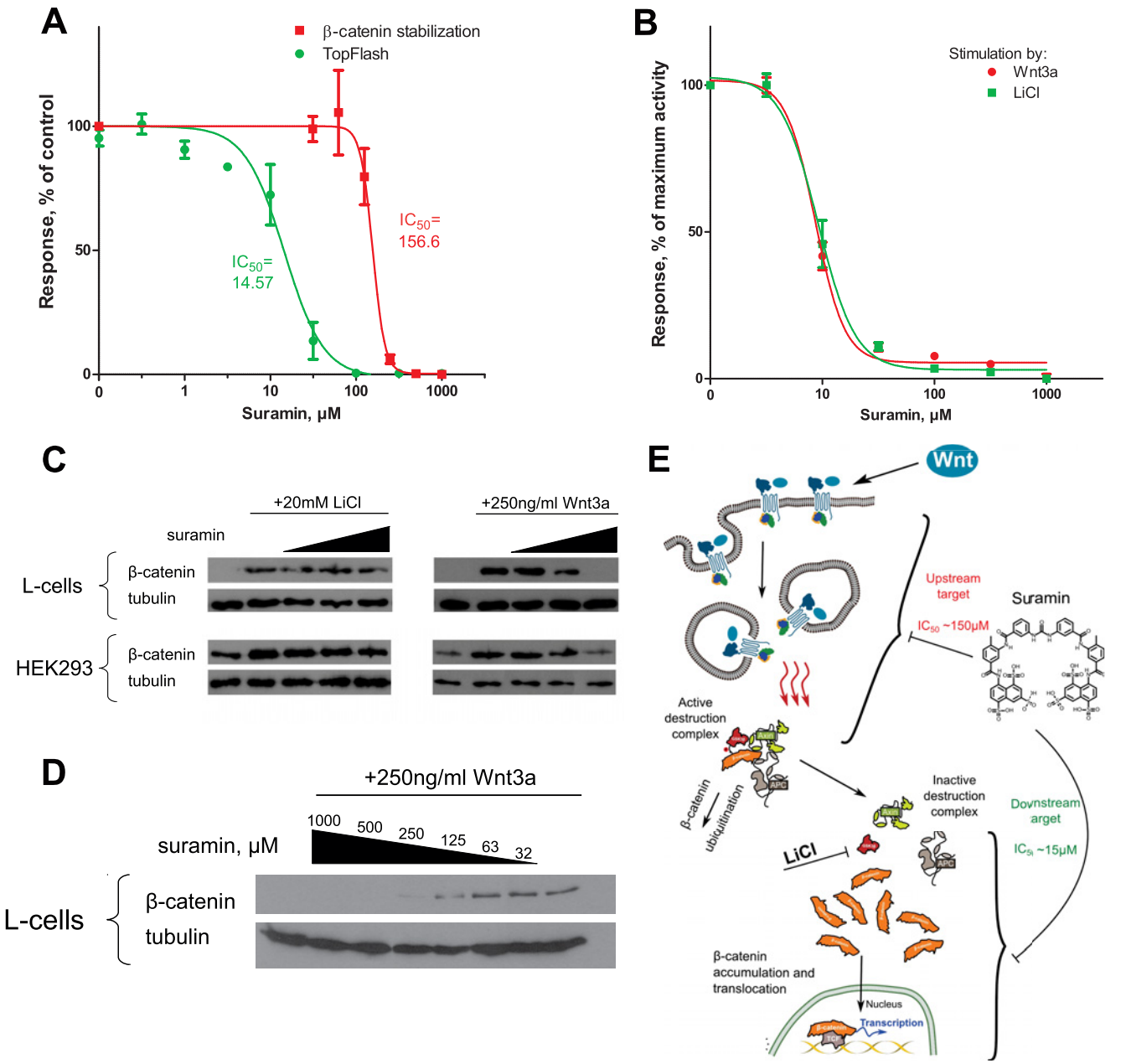

Figure 1 Suramin demonstrates concentration-dependent inhibition of the Wnt pathway and has at least two targets in the pathway

(**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 (**A**) for quantification. (**A** and **B**) Data shown as mean $\pm$ S.E.M., $n = 3$–6; (**C** and **D**) are representatives of three experiments.

anti-Wnt3a antibodies, HA-tagged version of Wnt3a ligand was employed in this assay.

Contradictory to previous conclusions, we found out that suramin was neither able 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 (Figure 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 signalling (A. Koval, C.A. Pieme, E.F. Queiroz, A. Blagodatski, J.-L. Wolfender and V.L. Katanaev, unpublished work). Purified Wnt3a was incubated with 1 mM suramin and the drug was subsequently removed by buffer exchange on centrifugal filters. As shown in Figure 2(B), Wnt3a retained its full activity after this treatment. A certain decrease in both control- and suramin-treated Wnt3a activity after the buffer exchange compared with the non-treated protein is attributed to the effects of accumulation of the CHAPS detergent, a necessary component of the Wnt buffer, whose micelles are to some extent retained by the filter.

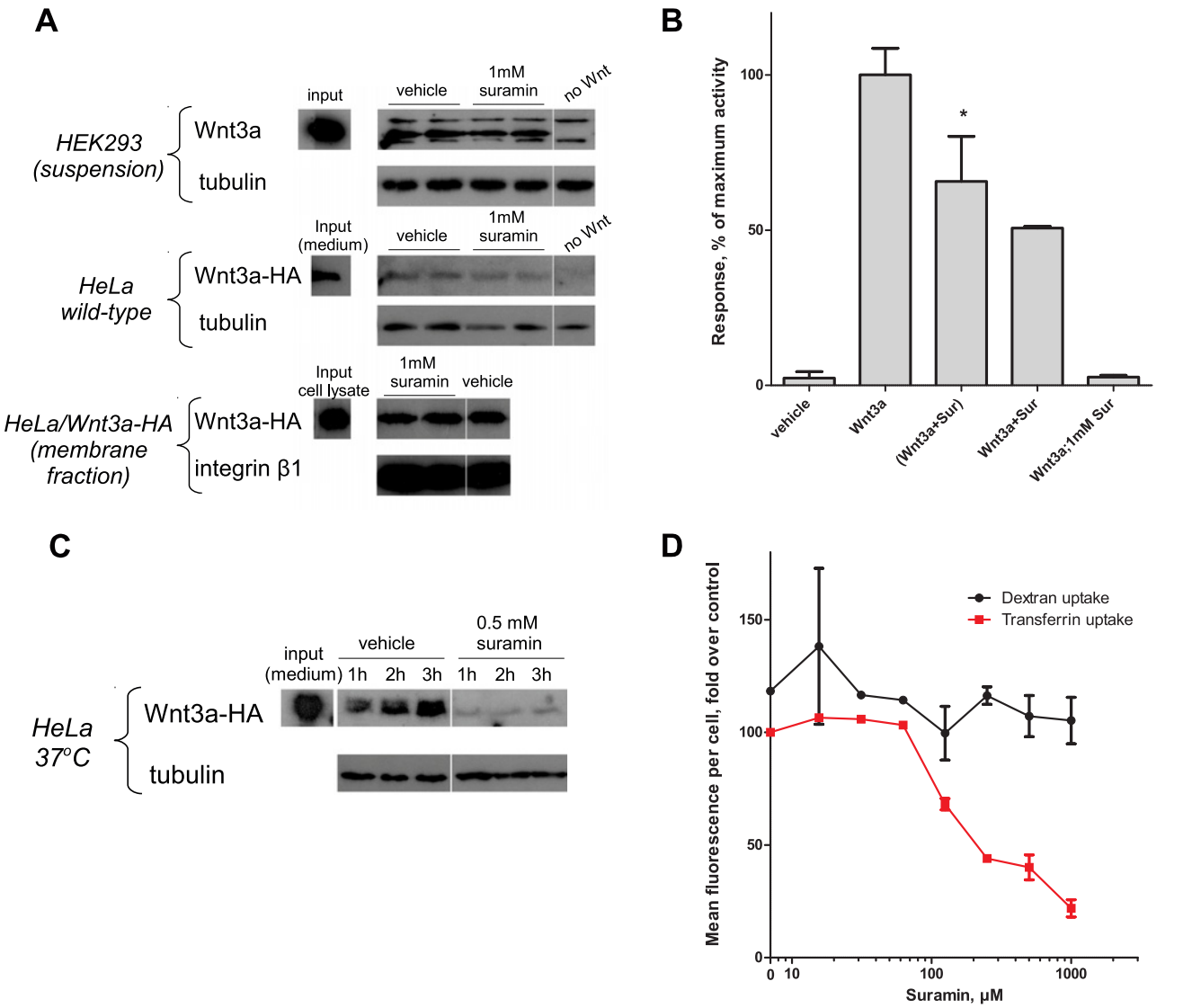

Figure 2 Suramin exerts its Wnt-inhibitory properties through inhibition of Wnt ligand endocytosis

(**A**) Suramin fails to suppress Wnt surface adhesion at 4°C in a suspension of HEK293 cells or in adherent HeLa cells both when Wnt3a–HA ligand was supplemented as conditioned medium and when the 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 1 mM suramin. 'Wnt3a' column is a reference activity induced by non-treated 0.5 μg/ml Wnt3a ligand; '(Wnt3a + Sur)' is the activity of 0.5 μg/ml Wnt3a ligand after treatment with and subsequent removal of 1 mM suramin; 'Wnt3a + Sur' is the activity of mock-treated 1 mM suramin solution in the Wnt3a vehicle buffer further supplemented with 0.5μg/ml of non-treated Wnt3a; 'Wnt3a:1 mM Sur' is the activity of 0.5 μg/ml Wnt3a in continued presence of 1 mM suramin. (**C**) Suramin (0.5 mM) supressed intracellular accumulation of Wnt3a–HA ligand in HeLa cells as measured by amount of Wnt3a in total cell lysates. (**D**) Suramin suppresses fluorescence-labeled 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. (**B** and **D**) Data shown as mean ± S.E.M., $n = 4$–6. (**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. (**A** and **C**) are representatives of four experiments.

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 proper pathway functioning [23,26]. To analyse effects of suramin on the Wnt protein endocytosis, we analysed its accumulation inside the cells in a pulse-chase experiment. As shown in Figure 2(C), 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-labelled dextran followed by flow cytometry quantification. In accordance with an earlier report [27], suramin did not affect this process (black line on Figure 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 [28]. 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 (Figure 1E) is one of the components regulating Wnt ligand endocytosis.

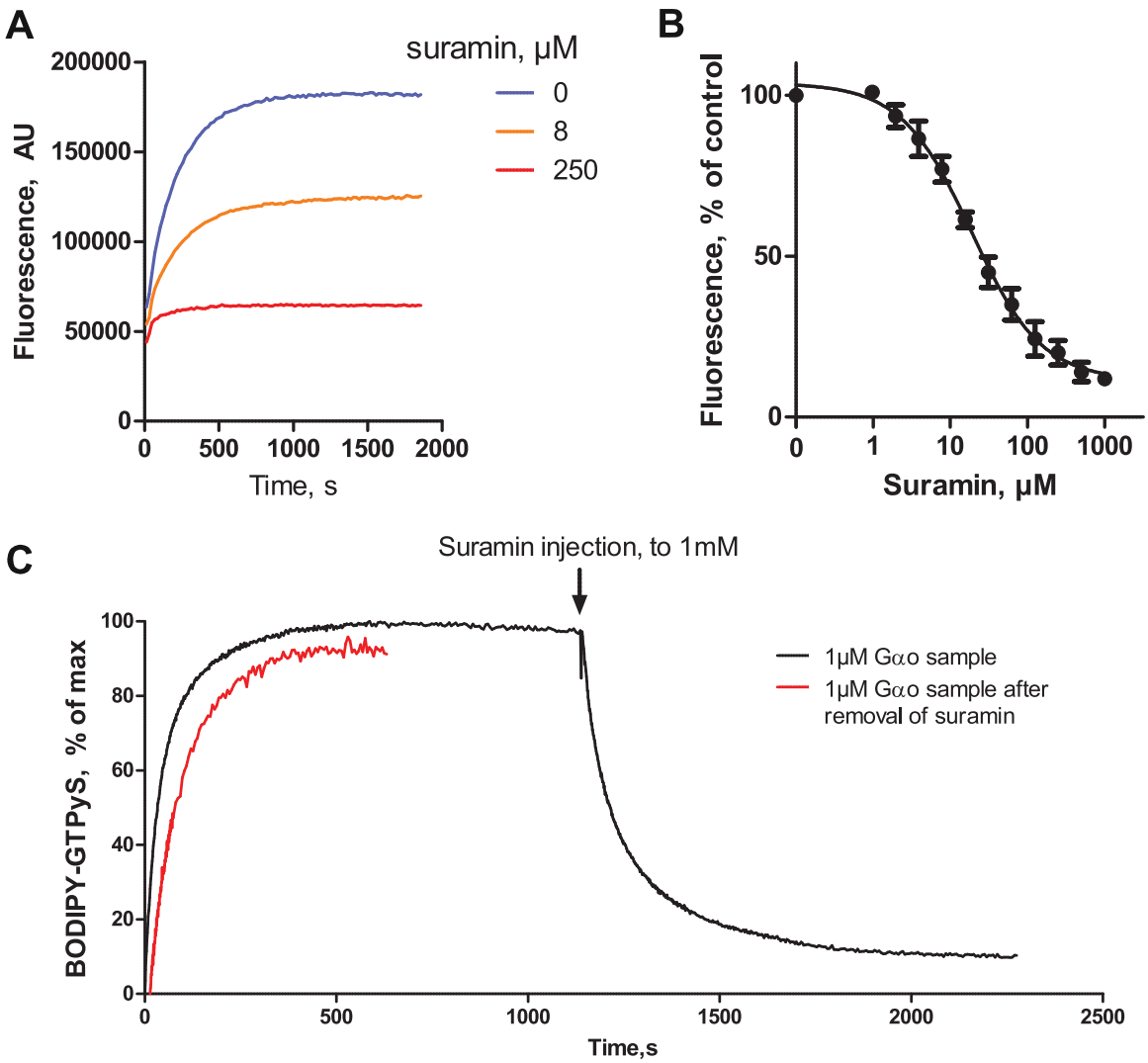

Figure 3 Suramin is a reversible inhibitor of the Gαo subunit of heterotrimeric G proteins

(**A**) Kinetics of BODIPY–GTPγS 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–GTPγS after removal of suramin; injection of suramin after complete loading of Gαo with BODIPY–GTPγS results in a strong drop of fluorescence consistent with displacement of BODIPY–GTPγS from Gαo. (**B**) Data shown as mean ± S.E.M., $n = 3$. (**A** and **C**) are representative of three and two experiments respectively.

Suramin acts as an nucleotide analogue 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 [29–31], have also been shown to regulate endocytic events, important for the amplification of the signal in the Wnt pathway [23,32]. 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 detail and assessed the effects of suramin on α-subunits of heterotrimeric G proteins of the Go/i family, key downstream effectors of FZD receptors [17,33,34]. We found that suramin has a dose-dependent reversible inhibition of the uptake of the fluorescent non-hydrolysable GTP analogue BODIPY–GTPγS by purified Gαo (Figures 3A and 3B). 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 found 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 (Supplementary Figures S1A and S1B).

Interestingly, the IC_{50} of these effects was comparable but lower than the IC_{50} observed for suramin inhibition of β-catenin stabilization (Figure 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 an nucleotide analogue, we injected suramin immediately after preloading Gαo with BODIPY–GTPγS (Figure 3C). The resultant rapid drop in the fluorescence signal indicates loss of

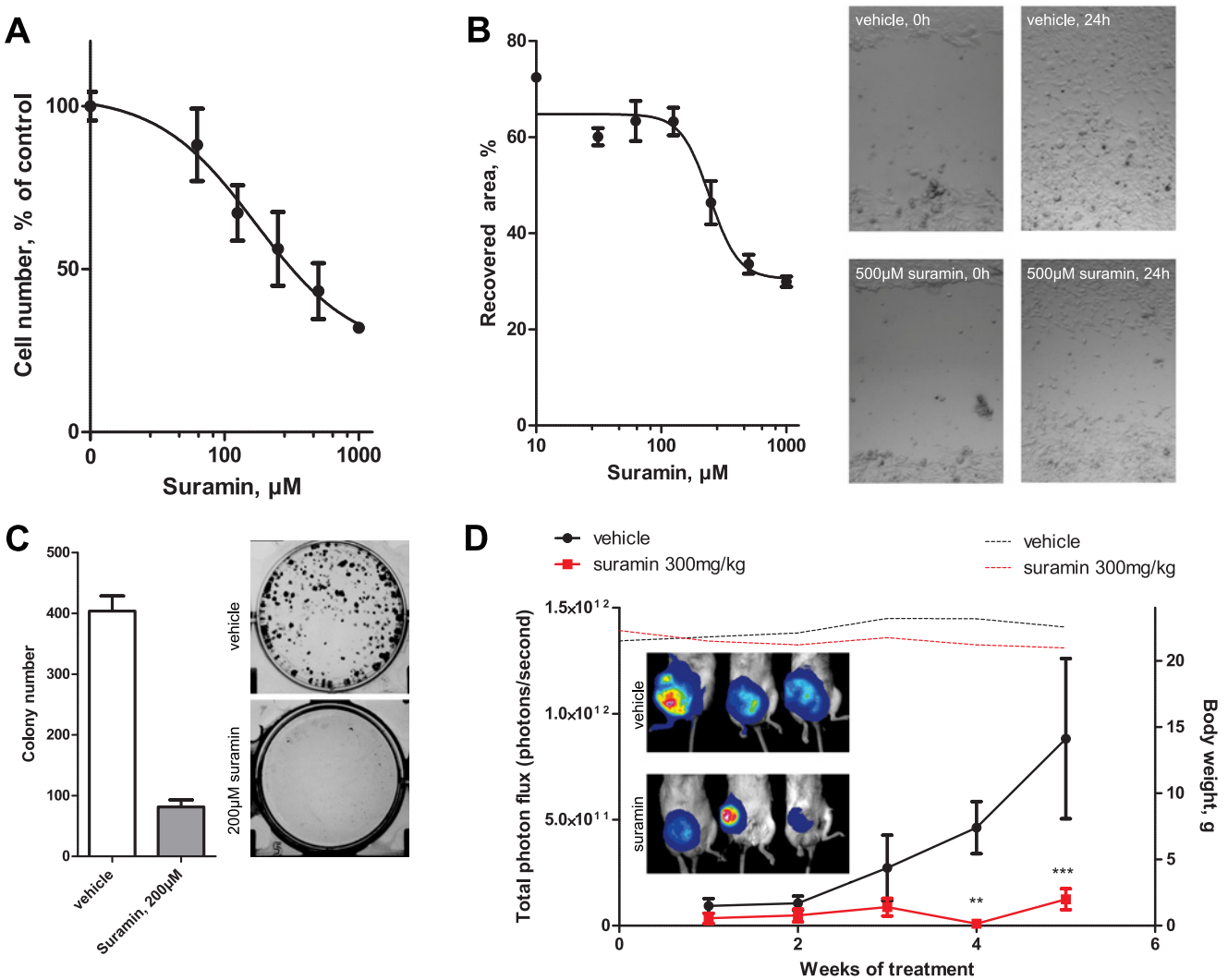

Figure 4 Anti-TNBC properties of suramin

Suramin inhibits growth (**A**), migration (**B**) and colony formation (**C**) in TNBC cell line BT-20. Weekly dosage of 300 mg/kg suramin resulted in a strong decrease in xenograft BT-20 tumour size in NGS mice after 4 weeks of treatment. (**A**–**C**) Data shown as mean ± S.E.M., $n = 4$–9. (**D**) Data are shown as mean ± S.D. from three (control group) or four (drug-treated group) animals. Significantly different data points are marked as $^{**}P < 0.01$ or $^{***}P < 0.001$ from two-way ANOVA with Bonferroni post-tests.

the GTP analogue from the binding pocket of the G protein, apparently due to its substitution with the suramin molecule. Of note, this function of suramin as an nucleotide analogue, competing for the nucleotide-binding pocket of G proteins, is consistent with suramin functioning as a 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 the Gi/o family.

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

Both proliferation and invasiveness of TNBC cells are known to depend on the overactivation of the Wnt pathway [35–38]. 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 signalling 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 (Figure 4A). Moreover, it was able to suppress cell migration and colony formation (Figures 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 analysed NOD/SCID-γ mice bearing intramammary xenograft of human TNBC BT-20 cells stably transfected with a luciferase reporter. Half of the mice received treatment with 300 mg/kg IV dose of suramin weekly, which was expected to produce and maintain therapeutically relevant Wnt-inhibitory plasma levels of the drug of approximately ~100 μM. The tumour growth monitored over 5 weeks demonstrated clear inhibition of the tumour growth

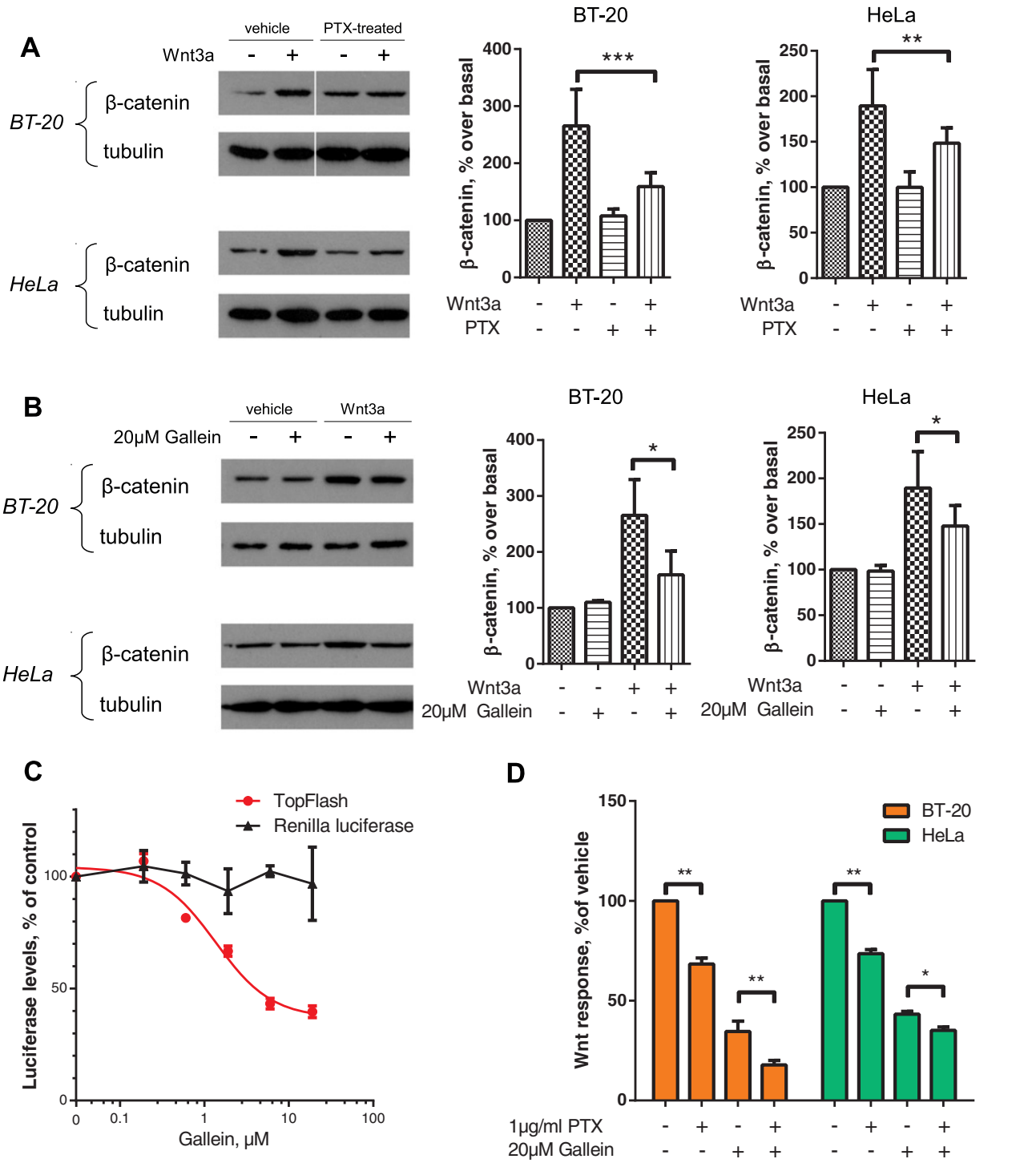

Figure 5 Effects of suramin are mimicked by pharmacological inhibitors of G protein signalling

BT-20 TNBC cells or HeLa transfected by FZD7 pre-treated by PTX (**A**) or gallein (general G$\beta\gamma$ inhibitor, **B**) demonstrate significantly decreased levels of β-catenin in response to 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 $IC_{50} \sim 1.3\ \mu M$ without observable toxic or unspecific effects as measured by concomitant measurement of CMV-driven *Renilla* luciferase levels (black curve) up to 20 μM. (**D**) PTX and gallein significantly reduced Wnt-induced TOPFlash levels in both FZD7-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 $\pm$ S.E.M. of $n = 5$–12; Western blots of (A and B) are representatives of 5–12 experiments. The statistical significance is shown as $^*P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ (Student's *t*test).

compared with control treatment (Figure 5D), thus indicating efficiency of suramin as an anti-TNBC drug.

Suramin effects on Wnt signalling are mimicked by pharmacological inhibitors of G protein signalling

We next wanted to confirm that effects of suramin on upper levels of Wnt signalling are indeed mediated by its ability to inhibit G protein signalling. To this end, we have chosen two well-characterized and broad-scope inhibitors of G protein signalling: toxin from *Bordetella pertussis* (PTX), which is known to decouple G proteins of the 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 a medium-affinity specific and non-toxic inhibitor of G$\beta\gamma$ signalling [41]. Effects of these agents on Wnt signalling were analysed 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 signalling in TNBC [39]. Indeed, such treatments resulted in significant, albeit incomplete, reduction in Wnt3a-induced β-catenin levels (Figures 5A and 5B) as well as overall Wnt signalling as measured in TOPFlash assay (Figures 5C and 5D). Notably, the IC_{50} of gallein in Wnt signalling was found to be $\sim$1.3 μM (Figure 5C) which correlates well with the previous data showing the IC_{50} of this compound for G$\beta\gamma$ to be approximately 0.5 μM [40], the small discrepancy being probably 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 (Supplementary Figure S2A; Figure 5C), nor was any effect of the agents observed for the levels of TOPFlash signal in the absence of Wnt3a stimulation (Supplementary Figure S2B), both indicating specificity of the observed effect.

Incomplete effects of the drugs might be explained by necessity of simultaneous action of Gα and G$\beta\gamma$ subunits in Wnt signalling [42], which is reinforced by observation of additivity of the effect when both agents are used simultaneously (Figure 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 the uniqueness of suramin as a tool for G protein-mediated signalling dissection, as its mechanism of GDP analogue action presumes ability to suppress any Gα and G$\beta\gamma$ 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 (A. Koval and V. Katanaev, unpublished observations) to complete inhibition [46] of Wnt signalling.

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 in the present study show that suramin, a multi-purpose drug initially developed against sleeping sickness, has the potential of being repositioned as an anti- 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 approximately 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]. Earlier it became clear that the Wnt signalling cascade is overactivated in TNBC through overexpression of the LRP6 and FZD_7 receptors and that down-regulation of this signalling pathway leads to proliferation arrest of TNBC cells *in vitro* and mouse xenograft models [36,37,52]. These observations make the Wnt signalling pathway an attractive target for 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, for example, by inhibitors acting at the downstream components of the signalling, is not desired. Instead, it is more promising to selectively target the upper levels of the pathway, where more variability among the signalling subtypes is provided [11].

In the present study, we show that suramin has at least two targets within the Wnt signalling pathway, one in its 'lower floors' and the other in its 'upper floors' (Figure 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 (probably 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 signalling pathways has been an intensive topic of research. The initial model, suggesting that internalization of receptors from the cell surface serves to shut signalling off, dominated in the past [53]. 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 [54–56]. In the latter case, one can speak of signal amplification mediated by the ligand–receptor endocytosis. Such amplification has also been observed in Wnt signalling [23,26]. 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-micromolar inhibitor of the α-subunit of the heterotrimeric protein Go, the main partner of FZD receptor [41,57,58] and that it acts in a competitive manner. The activities of suramin we describe in the present study thus support earlier observations on the involvement of heterotrimeric G proteins in the Wnt pathway in fruit flies and vertebrates [17,23,59].

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. Although previous reports already pointed out an anti-TNBC activity of suramin, alone [60,61] or in combination with other drugs [62,63], our study sheds new light on these data, indicating the anti-Wnt signalling 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 signalling. Therapeutic levels of suramin in plasma should be thus approximately 100–200 μM to result in the anti-TNBC effects, explaining why suramin previously failed to show profound anti-TNBC effects in clinical trials [64,65], in which suramin plasma levels were below 50 μM. 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 a mode of action.

AUTHOR CONTRIBUTION

Alexey Koval performed experiments, wrote parts of the manuscript and made figures; Vladimir Katanaev supervised the project, wrote parts of the manuscript; Kamal Ahmed performed animal experimentation.

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