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ORIGINAL ARTICLE

Imiquimod directly inhibits Hedgehog signalling by stimulating adenosine receptor/protein kinase A-mediated GLI phosphorylation

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Imiquimod (IMQ), a nucleoside analogue of the imidazoquinoline family, is used in the topical treatment of basal cell carcinoma (BCC) and other skin diseases. It is reported to be a TLR7 and TLR8 agonist and, as such, initiates a Th1 immune response by activating sentinel cells in the vicinity of the tumour. BCC is a hedgehog (HH)-driven malignancy with oncogenic glioma-associated oncogene (GLI) signalling activated in a ligand-independent manner. Here we show that IMQ can also directly repress HH signalling by negatively modulating GLI activity in BCC and medulloblastoma cells. Further, we provide evidence that the repressive effect of IMQ on HH signalling is not dependent on TLR/MYD88 signalling. Our results suggest a mechanism for IMQ engaging adenosine receptors (ADORAs) to control GLI signalling. Pharmacological activation of ADORA with either an ADORA agonist or IMQ resulted in a protein kinase A (PKA)-mediated GLI phosphorylation and reduction in GLI activator levels. The activation of PKA and HH pathway target gene downregulation in response to IMQ were abrogated by ADORA inhibition. Furthermore, activated Smoothened signalling, which positively signals to GLI transcription factors, could be effectively counteracted by IMQ. These results reveal a previously unknown mode of action of IMQ in the treatment of BCC and also suggest a role for ADORAs in the regulation of oncogenic HH signalling.

INTRODUCTION

Basal cell carcinomas (BCCs) are slowly growing, locally invasive tumours of the skin, which arise from the basal layer of the epidermis and from hair follicles.[1] BCC is caused by an aberrant activation of the Hedgehog (HH)/glioma-associated oncogene (GLI) signalling pathway, most frequently in response to genetic inactivation of the HH/GLI repressor Patched, or more rarely by genetic activation of the essential pathway effector Smoothened (SMO).[2,3] It comprises 80% of non-melanoma skin cancers.[4] BCC presents as several distinct subtypes, is the most common tumour in populations of European ancestry and usually occurs on sun-exposed areas.[5] The most common therapy involves surgical excision of the tumour. This can, however, be disfiguring, and non-surgical approaches are being implemented. Furthermore, BCC can become quite large and intractable, but rarely metastasizes (far less than 1% of cases). These tumours require non-surgical treatment. For treatment of malignant BCC, GDC-0449 (Vismodegib), an inhibitor of the HH signal transducer SMO, has just recently been approved, although resistance frequently evolves and severe side effects, such as muscle cramps and weight loss, possibly via the activation of non-canonical SMO signalling,[6] can result in significant drop-out rates of up to 50%.[7–10]

Small superficial BCCs are topically treated with Imiquimod (IMQ) (Figure 1a),[11,12] a synthetic nucleoside analogue of the imidazoquinoline family. The mode of action is described as immune modifying, primarily targeting sentinel cells of the skin.

IMQ initiates TLR7 and TLR8 signalling, which leads to the secretion of Th1 cytokines. This inflammatory milieu activates cytotoxic functions of CD8$^+$ cells to attack the tumour.[13,14] IMQ has also been reported to induce apoptosis.[15] Furthermore, it can bind to adenosine receptors (ADORAs), a group of G-protein-coupled receptors.[16] ADORAs are widely expressed throughout the body. They are involved in a variety of physiological processes, such as proliferation, cell death and stress signalling,[17] and the extracellular concentration of the natural ligand, adenosine, is elevated in pathological situations, such as solid tumours.[18,19] ADORAs are expressed in a range of tumours and influence immunesurveillance processes.[20] Of note, in Drosophila, adenosine has been linked to the regulation of Hh signalling by triggering the formation of the truncated transcriptional repressor form of Cubitus interruptus, the fly homologue of vertebrate GLI proteins, in haematopoietic precursor cells.[21] Whether ADORA signalling affects mammalian HH signalling has not yet been addressed.

The HH signalling pathway is well known for its role in embryonic development from flies to vertebrates.[22] In the adult organism, it is involved in the regulation of stem cell maintenance and tissue homeostasis.[23,24]

Several components of the HH pathway, including the GLIs, have been shown to localize to the primary cilium, an antenna-like cellular compartment required for sensing the three HH ligands (Sonic/Indian/Desert HH) and transforming this extracellular signal into a coordinated cellular response.[25–27] The activation of HH

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signalling by Sonic HH or Smoothened agonist (SAG) leads to ciliary translocation and activation of SMO, a process repressed by the unliganded HH receptor Patched.[28–31]

The three zinc finger transcription factors, GLI1, GLI2 and GLI3,[32–34] mediate HH signalling by controlling the HH target gene expression in response to pathway activity. GLI1 is a direct transcriptional target of GLI2 and GLI3, enhancing the overall level of GLI activator in response to HH signal activation.[35–37] GLI1 transcription, therefore, serves as a robust and sensitive functional read-out for the HH pathway activity. In the absence of HH signalling, GLI3 and, to some extent, also GLI2[38–40] act as a transcriptional repressor.[41–44] The GLI2/3 repressor activity is regulated by proteolytic processing into a C-terminally truncated transcriptional repressor form, a process involving phosphorylation of GLI2/3 by protein kinase A (PKA).[40,45] Thus, PKA has a critical role in the negative regulation of GLI activity.

We show here that IMQ negatively regulates HH/GLI signalling as evidenced by the downregulation of Gli1 mRNA and protein levels in murine BCC cells and HH-responsive human cancer cells. IMQ treatment induces phosphorylation of GLI in a PKA-dependent manner, thereby promoting the formation of GLI repressors. Our results provide evidence that the anti-tumour effect of IMQ can arise not only from the previously reported initiation of anti-tumour immune responses but also from direct effects of IMQ on aberrant HH/GLI signalling in the tumour cells. Our data also suggest that ADORAs participate in modulating oncogenic HH signalling in mammalian carcinoma cells.

RESULTS

IMQ negatively affects HH signalling in murine BCC cell lines

To test whether IMQ can directly modulate HH signalling in the absence of any immune effector cells, we treated the Patched-deficient murine BCC cell line BSZ2[46] with IMQ and measured its effect on the Hh pathway activity by quantifying the expression of two direct Hh target genes, Gli1 and Hhip. IMQ reduced mRNA expression of both target genes several fold compared with that of control-treated cells (Figure 1b). We also observed Gli1 protein downregulation (Figure 1c). Comparable results were obtained also for ASZ001 cells,[46] another Patched-deficient murine BCC cell line (Supplementary Figure 1). These results show that IMQ exerts a cell-autonomous negative effect on HH signalling by repressing the activation of canonical HH/GLI target genes.

Repression of HH/GLI signalling by IMQ is independent of the TLR7/8–MYD88 signalling axis

As IMQ is known to be a TLR7 and TLR8 agonist,[47] we asked whether these two receptors contribute to the repressive effect of IMQ on HH/GLI signalling. We first measured the expression levels of the two Tlrs and their common signal transducer Myd88 in the two murine BCC cell lines (BSZ2 and ASZ001) by real-time PCR amplification. As shown in Figure 2a, Tlr7 and Tlr8 are expressed at very low levels, if at all, in these cell lines compared with that in murine splenocytes known to express physiological levels of Tlr7/8. This is in line with the lack of TLR7/8 in other epidermal cells and cell lines, such as human HaCaT and foreskin keratinocytes.[48,49] Signalling via TLRs involves MYD88, an essential effector protein in the TLR signalling cascade.[50] To address a possible contribution of TLR/MYD88 signalling to IMQ-mediated repression of Hh signalling, we first measured the expression of Myd88 at the RNA and protein levels. As shown in Figures 2a and b, Myd88 is expressed in the cell line BSZ2 at moderate levels. To address the functional involvement of Tlr/Myd88 signalling in IMQ-mediated Hh/Gli repression, we performed stable, lentiviral RNA interference knockdown of Myd88. Of note, we found that RNA interference directed against Myd88 did not affect the ability of IMQ to repress Hh/Gli signalling in BCC cells. As shown in Figures 2b and c, we did not observe any significant difference in IMQ-mediated Gli1 repression between Myd88-expressing and Myd88-deficient cells. As first, Myd88 is dispensable for IMQ-mediated downregulation of Gli1, and second, the major known IMQ targets Tlr7/8 are not expressed at detectable levels, we conclude that IMQ does not repress Hh/Gli signalling by targeting the Tlr7/8–Myd88 signalling axis in murine BCC cell lines.

Modulation of ADORA activity contributes to IMQ-induced suppression of HH signalling by stimulating PKA-mediated GLI phosphorylation

In addition to binding to TLRs, IMQ has been shown to bind ADORAs also, thereby affecting adenylate cyclase (AC) and PKA activity.[16] This is of particular interest, as adenosine/ADORA signalling has recently been identified as a negative regulator of HH signalling in flies by the activation of PKA.[21] We therefore asked (i) whether the modulation of ADORA signalling by agonists/antagonists affects the HH/GLI pathway activity and (ii) whether IMQ and ADORA agonists are able to induce
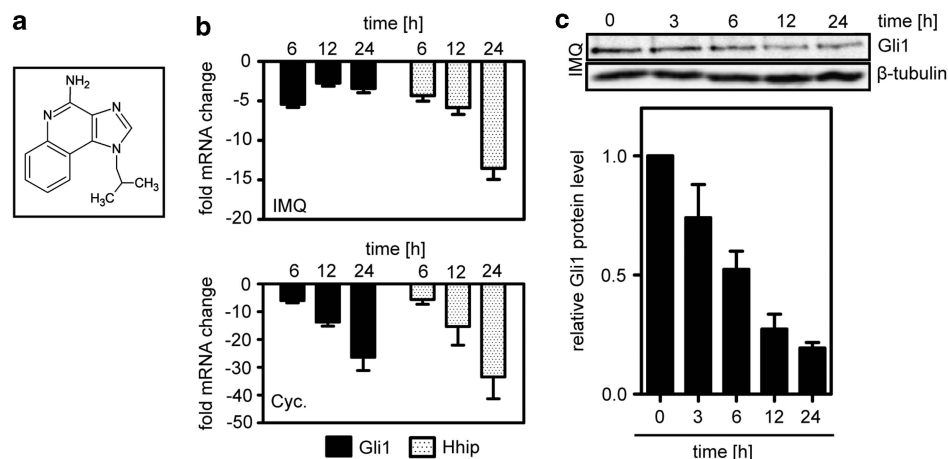

Figure 1. IMQ reduces the Hh/Gli pathway target gene expression in BSZ2, a murine Patched-deficient BCC cell line. (a) IMQ is a synthetic imidazoquinoline used in topical treatment of BCC. (b) The expression levels of two Hh/Gli target genes (Gli1 and Hhip) were measured by quantitative reverse transcriptase–PCR (qRT–PCR) upon IMQ (10 μg/ml = 42 μM) treatment. Cyclopamine (Cyc = 5 μM) served as a control inhibitor for Hh signalling. Reference gene in all qRT–PCR reactions was Rplp0. (c) Gli1 protein levels of BSZ2 cells after IMQ treatment. Bar chart shows quantification of western blots (b and c: mean values and s.e.m. of two independent experiments).
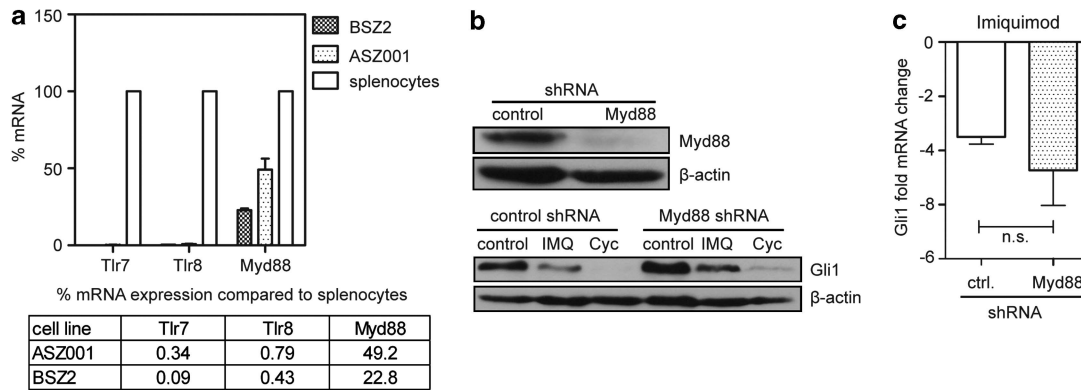
Figure 2. IMQ does not target the Tlr7/8–Myd88 signalling axis. (**a**) Relative mRNA expression levels of Tlr7, Tlr8 and Myd88 in two murine BCC cell lines. Data from murine splenocytes were set to 100%. (**b** and **c**) BSZ2 cells were treated either with control shRNA or an shRNA targeting Myd88 mRNA to investigate the possible effect of Myd88 on the IMQ-induced reduction of Gli1 expression. Cyclopamine (Cyc) is used as a control. Results shown are mean values and s.e.m. of three independent experiments.

PKA-mediated phosphorylation of GLI as mechanisms of the HH/GLI pathway repression.

To test this, we used a selective ADORA2A agonist (CGS 21680) and an antagonist (SCH 442416) to pharmacologically target this receptor. As shown in Figure 3a, the antagonist completely abrogated the IMQ-mediated downregulation of Gli1 and Hhip target gene transcription in the murine BCC cell line. We also monitored the phosphorylation status of GLI2 by using an inducible GLI2-expressing HaCaT human keratinocyte system. Here the antagonist reduced the steady-state phosphorylation of GLI2 and counteracted IMQ-induced GLI2 phosphorylation (Figures 3b and c). The ADORA2A agonist CGS 21680, on the contrary, induced steady-state phosphorylation of GLI2 and further increased IMQ-induced GLI2 phosphorylation (Figures 3b and c). These data suggest that IMQ modulates GLI transcriptional activity through ADORAs and subsequent phosphorylation of GLI2. PKA is a well-known negative regulator of GLI transcriptional activity. Phosphorylation of GLI2 and GLI3 by PKA primes for subsequent phosphorylation by GSK3β and CK1, thereby triggering proteolytic processing of GLI to its repressor forms.[40,51–53] As modulation of PKA by IMQ is mediated through ADORAs and by a direct effect on AC,[16] we hypothesized that PKA has a role in IMQ-mediated repression of HH signalling. Similar to treatment with the canonical PKA activator Forskolin, IMQ treatment of HaCaT cells clearly induced phosphorylation of GLI2. Furthermore, the AGC kinase inhibitor H89 inhibited phosphorylation of GLI2 by both IMQ and Forskolin (Figure 3d). This is consistent with a scenario where PKA is activated by IMQ promoting a change in the phosphorylation status and consequent destabilization of GLI2. In summary, these data provide evidence that IMQ negatively modulates GLI protein activity through changes in ADORA activity with subsequent activation of PKA.

IMQ promotes GLI3 repressor formation and antagonizes HH signalling in a human medulloblastoma cell line

To test the effect of IMQ on GLI processing in a more physiological system with an activated endogenous HH signal cascade, we first turned to murine BCC cells (BSZ2). As a read-out for Gli activity, we monitored the ratio of Gli3 full length to repressor forms by quantitative western blot analysis.[51] As shown in Figure 4a, IMQ reduced the ratio of full length to repressor in BSZ2 cells. Compared with cyclopamine, IMQ reduced this ratio somewhat more slowly. Still, the increase in Gli3 repressor clearly shows the repressive effect of IMQ on Hh signalling. To further address whether HH signal inhibition by IMQ is cell type- and species-

specific, or more broadly effective also in human, non-BCC cells, we analysed the effect of IMQ on inducible HH signalling, GLI expression and GLI processing, using the HH-responsive human medulloblastoma cell line (DAOY).[54,55] Notably, IMQ very potently interfered with the activation of GLI1 expression in DAOY cells treated with SAG (Figure 4b). Consistent with the model that IMQ blocks HH signalling by promoting PKA-mediated GLI phosphorylation and repressor formation, IMQ effectively re-established the GLI3 repressor form in SAG-stimulated DAOY cells (Figure 4c). As manipulations affecting the primary cilium indirectly interfere with HH signalling, we also analysed as a control the possible impact of IMQ on ciliogenesis. As shown in Figure 4d, IMQ-treated DAOY cells display normal primary cilia, indicating that IMQ does not affect HH signalling by disturbing the formation of the primary cilium. Together, these data show that IMQ is able to directly inhibit HH signalling by inducing PKA-mediated GLI phosphorylation and proteolytic repressor formation.

ADORA2A and ADORA3, but not ADORA1A and 2B, are overexpressed in human BCC

IMQ is used in the treatment of BCC. On the basis of our results indicating that ADORAs contribute to IMQ-induced suppression of HH signalling, we investigated the expression levels of ADORAs in human BCC. We tested five human BCC samples for the expression of the four ADORA mRNAs and compared the expression levels in the BCC samples with that in three normal skin samples. ADORA3 was expressed at levels similar to that of GLI1, which was overexpressed in BCCs. ADORA2A mRNA levels are higher in BCC samples compared with that in the normal skin, whereas ADORA1 and ADORA2B expression in the BCC samples is comparable to that in the normal skin. This supports the hypothesis that topical treatment with IMQ of BCCs not only activates an anti-tumour Th1 response but also engages ADORAs on BCC cells, thereby directly reducing oncogenic HH/GLI signalling.

DISCUSSION

Deregulated HH signalling drives tumourigenesis in a number of human malignancies, including medulloblastoma and BCC. Metastatic and advanced BCC can be treated using antagonists targeting the important HH signal transducer SMO.[7,56] Nevertheless, the effectiveness of SMO antagonists, such as Vismodegib, in BCC therapy is limited by tumours developing resistance or regrowth upon cessation of treatment.[57] Furthermore, up to 50% of Vismodegib-treated patients
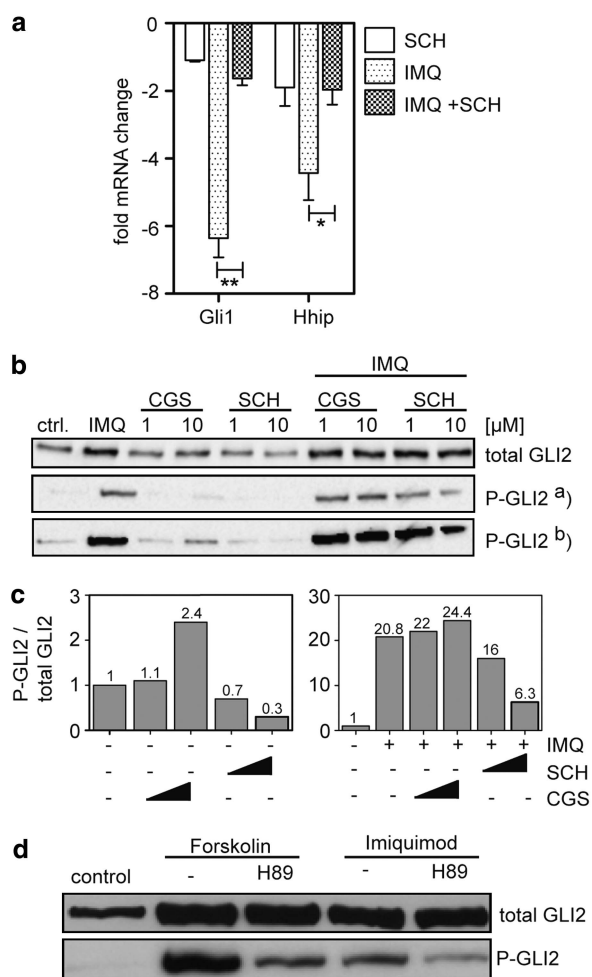
Figure 3. Modulation of ADORA activity contributes to IMQ-induced suppression of HH signalling by stimulating PKA-mediated GLI phosphorylation. (**a**) BSZ2 cells were preincubated with 10 μM ADORA2A antagonist SCH 442416 (SCH) or control before IMQ treatment (mean and s.e.m. of three independent experiments). Statistical significance (*$P < 0.05$; **$P < 0.005$) between IMQ-only and IMQ + SCH treatment is shown. (**b**) Doxycycline-inducible GLI2-expressing human keratinocytes (G2H31) were treated for 6 h with IMQ (42 μM/10 μg/ml), and ADORA2A agonist CGS 21680 hydrochloride (CGS) and ADORA2A antagonist SCH 442416 (SCH), with 1 μM or 10 μM (indicated by black triangle). A representative western blot analysis of two independent experiments is shown. GLI2 was precipitated with antibody against human GLI2. P-GLI2[a)] = short exposure; P-GLI2[b)] = long exposure. (**c**) Quantification of western blot analysis in Figure 3b is shown. Values of the ratio of P-GLI2 to total GLI2 are indicated on top of each bar. (**d**) G2H31 cells were treated for 6 h with 42 μM IMQ or 5 μM Forskolin (FSK) as a positive control, with or without the AGC kinase inhibitor H89 (5 μM). GLI2 was precipitated with antibody against human GLI2. Phosphorylated GLI2 was detected with antibody recognizing phosphorylated substrates of AGC family kinases (RXXT* or RRXS*). Representative western blot analysis of four independent experiments are shown.

discontinued treatment due to severe side effects.[8,9] Thus, combined treatment strategies become increasingly important to enhance efficacy and to circumvent the establishment of resistance. IMQ is approved for the treatment of superficial BCC. Although the immune-modulating functions of IMQ have been investigated extensively,[13,58,59] a possible direct effect on HH signalling in BCC cells has not been addressed so far. As HH

signalling is the major pathway driving the development and maintenance of BCC, we investigated the potential effect of IMQ on this pathway. Here we show that IMQ promotes PKA activity followed by HH/GLI target gene downregulation. Importantly, in contrast to the known immune modulatory effect of IMQ on sentinel cells, MYD88 is not involved in this mechanism in BCC cells. In addition, TLR7 and TLR8 are barely expressed in the tumour cell lines used. Together, this suggests that IMQ represses HH signalling independently of its immune modulatory functions, thus unraveling a new therapeutic mode of action of IMQ in BCC. Furthermore, we also observe the HH-repressive effect of IMQ in a human medulloblastoma cell line. This shows that IMQ can repress HH signalling in different tumour types and points to a general mechanism of IMQ-induced HH repression, which is not restricted to BCC cells only.

The downregulation of HH signalling is often mediated by PKA, which is recognized as an important negative regulator of GLI transcriptional activity. GLI2 and GLI3 proteins harbour several PKA target sites, which are important for proteasomal processing. Mice, homozygous for these mutant PKA sites, die in utero and show increased stability of Gli2 protein, indicating that PKA activity is essential for proper Gli2 function.[40] As GLI2 and GLI3 control the transcription of GLI1,[60,61] we propose that IMQ downregulates HH signalling and GLI1 expression, respectively, by reducing GLI2/3 activity through PKA activation. IMQ leads to phosphorylation of GLI2 (Figures 3b–d). Of note, the epitopes detected are phosphorylated substrates of AGC kinases to which PKA belongs.[62] As the PKA kinase inhibitor H89 blocks GLI2 phosphorylation induced by either IMQ or Forskolin, we conclude that IMQ, similar to Forskolin, modulates AC and subsequently PKA activity. These results are supported by data showing that IMQ can modulate the activity of AC and PKA.[16] To further strengthen the evidence for a role of PKA in IMQ-mediated repression of HH signalling, we determined the ratio of GLI3 full length to the repressor form. In two cellular cancer models (murine BCC and HH-responsive human medulloblastoma cell lines), IMQ treatment leads to increased formation of GLI3 repressor (Figures 4a and c), in line with enhanced PKA activity.

We suggest that IMQ modulates the activity of the GLI transcription factors by engaging signalling components of other pathways. PKA can be controlled by a multitude of signalling events.[63,64] ADORAs are known to engage PKA to mediate downstream signalling. Recently, it has been shown that the single Adora of Drosophila facilitates Cubitus interruptus repressor formation in haematopoietic precursor cells through PKA activation.[21] In CHO cells, Schön et al.[16] found that IMQ can bind ADORAs and modulate AC activity. We observed that all four ADORA subtypes are expressed in human BCC samples (Figure 5). Together, these data support the idea that IMQ contributes to topical therapy by repressing HH/GLI signalling in BCC through the modulation of ADORA activity. Specifically, our results suggest that binding of IMQ to ADORA2A leads to GLI2/3 phosphorylation, similar to binding of the ADORA2A agonist (CGS 21680). The quantification of GLI2 phosphorylation signals (Figure 3c) reveals that IMQ induces a phosphorylation signal significantly stronger than the signal induced by the agonist CGS 21680. This may be due to an additional receptor-independent activation of PKA by IMQ. A direct modulation of the AC activity and, thus, PKA by IMQ has been observed by Schön et al.[16] in CHO cells lacking any type of ADORA. However, the ADORA2A antagonist SCH 442416 counteracts the effect of IMQ on GLI2 phosphorylation (Figures 3b and c) and also abrogates the effect of IMQ on target gene expression in the murine BCC cells (Figure 3a). These data support the role of IMQ as an ADORA2A agonist as shown in Figures 3b and c. We can, however, not exclude effects of IMQ on HH signalling through other ADORAs, as K_i-values of IMQ to ADORA1/A2A and the A3 receptor are in a similar range[16] and we found other ADORAs to be expressed in the cell lines used in this study
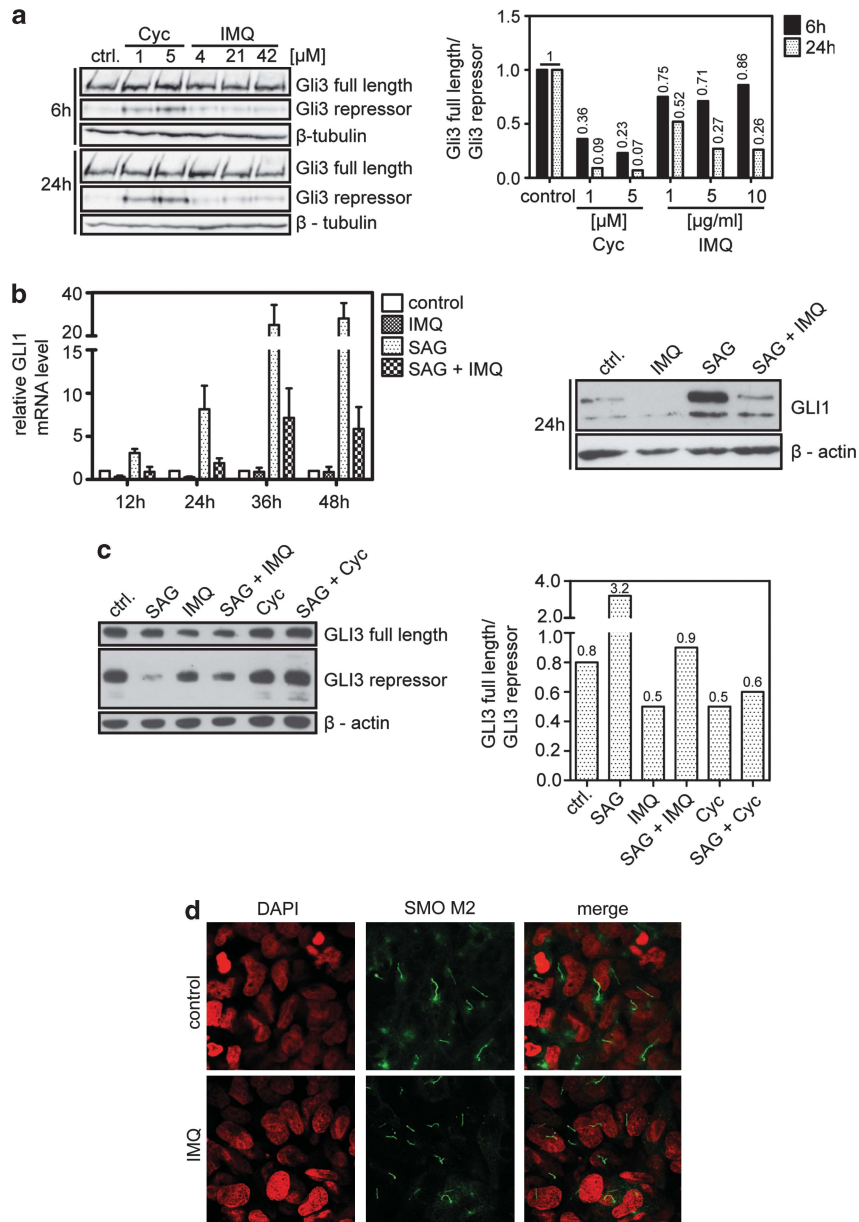
Figure 4. IMQ induces GLI3 repressor formation and antagonizes HH signalling in a human medulloblastoma cell line. (**a**) Western blot analysis (left) and corresponding quantification (right) of GLI3 full length and repressor in IMQ-treated BSZ2 and control cells. (**b**) HH signalling was activated in DAOY cells by treatment with 100 nM SAG either alone or in combination with IMQ. GLI1 mRNA levels (left panel) were measured at the indicated time points. GLI1 levels of control-treated cells (dimethyl sulfoxide only) were set to 1. Right panel shows GLI1 protein after 24 h of treatment as indicated. (**c**) Western blot analysis (left) and corresponding quantification (right) of GLI3 full length and repressor forms in DAOY cells after 48 h of IMQ treatment. Cyclopamine (Cyc) was used as an HH inhibitor control for GLI3 repressor formation. (**d**) IMQ does not disrupt the primary cilium. DAOY cells were transfected with SMOM2–green fluorescent protein (GFP) fusion construct and were treated with IMQ or control. Pictures have been taken after 24 h of treatment. SMOM2–GFP was used to identify primary cilia, as the SMOM2 mutant form constantly locates to the primary cilium.[70] Values correspond to mean values and s.e.m. of at least two independent experiments.

(Supplementary Figure 2a). As human BCCs overexpress ADORA2A and ADORA3 (Figure 5a), our experiments together strongly suggest that IMQ acts through ADORAs to repress HH signalling during BCC therapy.

As our data suggest that IMQ can interfere with HH signalling downstream of SMO on the level of PKA (Figure 5b), IMQ is of high interest and might be useful for the treatment of SMO-inhibitor-resistant tumours. As a large number of selective ADORA agonists and antagonists have already been described,[65] testing for their

potential to interfere with HH signalling may be a promising strategy to find new molecules, which can aid the development of combined therapies for HH-driven tumours.

MATERIALS AND METHODS
Cell culture and reagents
ASZ001 and BSZ2[46] cells were grown in 154-CF medium (Invitrogen, Carlsbad, CA, USA) with 2% chelexed (Bio Rad, Munich, Germany) fetal
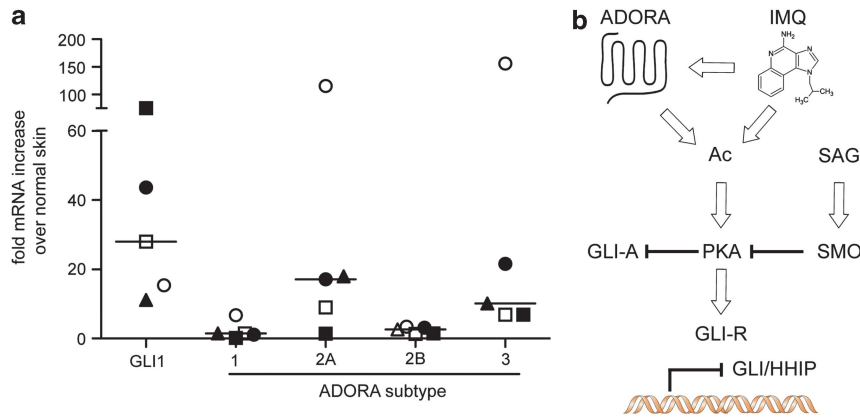
Figure 5. (**a**) Human BCCs overexpress ADORA2A and ADORA3. Expression levels of the four human ADORA subtypes in five human BCC samples is compared with that in three normal skin samples. The figure depicts the fold mRNA increase of each BCC sample to the mean of three normal skin samples. Each symbol corresponds to one tumour sample. The bars represent the mean of all five data points. GLI1 serves as a positive control for overexpression. Reference gene in all quantitative reverse transcriptase–PCR measurements was RPLP0. (**b**) Model for the effect of IMQ on GLI target gene expression. IMQ binds to ADORAs and can act receptor-independently on AC, thereby modulating PKA activity. PKA phosphorylates GLI. These events favour GLI repressor formation and subsequently lead to dowregulation of the HH target genes *GLI1* and *HHIP*. Active SMO signalling from the cilium cannot antagonize the effect of IMQ.

calf serum (PAA, Pasching, Austria), 0.05 mM $CaCl_2$, and 100 µg/ml streptomycin and 62.5 µg/ml penicillin (Invitrogen). GLI2act-HaCaT[66,67] cells were cultured in Dulbecco's modified Eagle medium (high glucose; PAA) with 10% fetal calf serum (PAA), 100 µg/ml streptomycin and 62.5 µg/ml penicillin (Invitrogen). Transgene expression in GLI2act-HaCaT cell lines was induced adding 50 ng/ml doxycycline (Sigma-Aldrich, St Louis, MO, USA). DAOY cells[54] were cultured in minimum essential medium (PAA, Pasching, Austria) with 10% fetal calf serum (PAA), 110 mg/l Na-Pyruvate, 100 µg/ml streptomycin and 62.5 µg/ml penicillin (Invitrogen). Sixteen hours before treatment with SAG (100 nM) (Calbiochem, Merck, Frankfurt, Germany), serum concentration was reduced to 0.5% and maintained throughout SAG treatment. All cell lines were cultured in a humidified incubator containing 5% CO_2 at 37 °C.

IMQ was purchased from Merck (EMD Chemicals, San Diego, CA, USA). All ADORA agonists and antagonists (CGS 21680 hydrochloride, SCH 442416 and MRS 1754) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). cAMP-dependent protein kinase inhibitor H89 was purchased from InvivoGen (San Diego, CA, USA) In all experiments, control-treated cells were incubated with dimethyl sulfoxide (Sigma-Aldrich). Dimethyl sulfoxide concentration was adjusted in every treatment to equal concentration.

Short hairpin RNA-mediated knockdown

For shRNA (short hairpin RNA)-mediated knockdown of Myd88, a validated lentiviral MISSION shRNA (NM_010851.2-1648s1c1) from Sigma-Aldrich and a non-target control shRNA were selected. Virus production and infection of cells was performed essentially.[68] Forty-eight hours post virus infection, medium was supplemented with 4 µg/ml puromycin (Sigma-Aldrich) to select for infected cells.

RNA isolation and quantitative reverse transcriptase–PCR analysis

Total RNA from BCC and normal skin was isolated with TRI-Reagent (Molecular Research Center, Inc., Cincinnati, OH, USA) followed by LiCl precipitation. Total RNA of ASZ001, BSZ2, GLI2 HaCaT and DAOY cells was isolated and purified with the High Pure RNA Isolation Kit (Roche, Mannheim, Germany). cDNA synthesis and quantitative reverse transcriptase–PCR analysis was done as described.[66] Human large ribosomal protein P0 (RPLP0) was used for normalization of sample material in quantitative reverse transcriptase–PCR analysis.[69] For primer sequences, see Supplementary Table S1.

Western blot

Cells were washed twice with phosphate-buffered saline and were processed as described in Supplementary Information.

Immunoprecipitation

For detection of phosphorylated GLI2, GLI2act-HaCaT cells were processed and subjected to western blotting for the molecules indicated as described in Supplementary Information.

Immunofluorescence

DAOY cells were grown on glass coverslips, and were fixed and stained as described in Supplementary Information.

Statistical analysis

Data are shown as mean ± s.e.m. The significance of mean comparison was assessed by two tailed Student's *t*-test. $P < 0.05$ was considered to be significant (*$P < 0.05$; **$P < 0.005$).

ABBREVIATIONS

AC, adenylate cyclase; ADORA, adenosine receptor; PKA, protein kinase A; SAG, Smoothened agonist; SMO, Smoothened.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Supplementary Information accompanies this paper on the Oncogene website (http://www.nature.com/onc)