Published in final edited form as:

Lipids. 2015 December ; 50(12): 1195–1207. doi:10.1007/s11745-015-4083-z.

3-deoxyschweinfurthin B lowers cholesterol levels by decreasing synthesis and increasing export in cultured cancer cell lines

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Abstract

The schweinfurthins have potent antiproliferative activity in multiple glioblastoma multiforme (GBM) cell lines; however, the mechanism by which growth is impeded is not fully understood. Previously, we demonstrated that the schweinfurthins reduce the level of key isoprenoid intermediates in the cholesterol biosynthetic pathway. Here in, we describe the effects of the schweinfurthins on cholesterol homeostasis. Intracellular cholesterol levels are greatly reduced in cells incubated with 3-deoxyschweinfurthin B (3dSB), an analog of the natural product schweinfurthin B. Decreased cholesterol levels are due to decreased cholesterol synthesis and increased cholesterol efflux, both of these cellular actions can be influenced by liver X-receptor (LXR) activation. The effects of 3dSB on ATP-binding cassette transporter 1 (ABCA1) levels and other LXR targets are similar to that of 25-hydroxycholesterol, an LXR agonist. Unlike 25-hydroxycholesterol, 3dSB does not act as a direct agonist for LXR α or β . These data suggest that cholesterol homeostasis plays a significant role in the growth inhibitory activity of the schweinfurthins and may elucidate a mechanism that can be targeted in human cancers such as GBM.

Introduction

The schweinfurthins, a family of natural products, potentially inhibit proliferation of several cancer cell lines of the NCI 60 cell cancer screen (NCI60),(1) yet the mechanism of this activity remains largely unknown. Interestingly, the schweinfurthins display a unique pattern

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of activity in the NCI60 when compared to anticancer agents in current clinical use, which suggests that the schweinfurthins and associated compounds represent a new class of anticancer agent.(2) Of particular interest is the potent growth inhibitory activity the schweinfurthins display against cell lines of the NCI60 Central Nervous System (CNS) panel (3). The majority of the cell lines that comprise the CNS panel were derived from human glioblastoma multiforme (GBM) tumors. Currently, the standard of care is surgical resection of the tumor mass, if operable, followed by concomitant administration of radiotherapy and the alkylating agent temozolomide. The median overall survival for this regimen, as reported in a phase III trial, is 14.6 months (4). While several studies have identified genetic abnormalities that contribute to GBM development and progression; to date, targeted therapies have been largely unsuccessful (5). For these reasons, new targets and/or therapies are needed to improve the mortality and morbidity associated with GBM.

Initial members of the schweinfurthin family, including schweinfurthin B, were isolated from the leaves of *Macaranga Schweinfurthii*. Unfortunately, this plant has proven to be an unreliable source of these compounds. To address these issues of scarcity, our group initiated a program to synthesize the natural product schweinfurthins as well as analogues thereof. These efforts have led to synthesis of 7 natural product schweinfurthins and over 80 analogues (6–16). 3-Deoxyschweinfurthin B (3dSB) was amongst the first of these analogues to be synthesized. While 3dSB lacks an A-ring hydroxyl found in schweinfurthin B, it retains the potent and selective activity (Figure 1). The increased availability of 3dSB has allowed us and others to further investigate the biological activity of the schweinfurthins.

Initial studies of schweinfurthin activity have focused largely on their antiproliferative and morphologic effects. In response to schweinfurthin incubation several cell lines adopt a spindle-like to rounded appearance, which could suggest direct or indirect involvement of Rho family proteins (6, 17). Turbyville *et. al* demonstrated that schweinfurthin A can inhibit epidermal growth factor-induced activation of ras homolog family member A (RhoA) (17). Proper localization of Rho-family proteins is necessary for efficient activation and signaling. The localization of Rho family members is mediated by post-translational isoprenylation which incorporates either a farnesyl or geranylgeranyl moiety from farnesyl diphosphate (FPP) or geranylgeranyl diphosphate (GGPP), respectively. Recently, we demonstrated that 3dSB decreases intracellular levels of FPP and GGPP in several cancer cell lines which may affect Rho signaling (18). In addition to its role in protein isoprenylation, FPP is a precursor for cholesterol synthesis suggesting that the schweinfurthins alter intracellular cholesterol levels.

Cholesterol is a vital component of cellular membranes as such cholesterol homeostasis is tightly regulated. Regulation of cholesterol levels is controlled by the opposing actions of sterol regulatory element binding protein (SREBP) and liver X-receptor (LXR) mediated transcription (19). SREBP (specifically SREBP2) transcriptional activity leads to increased cholesterol synthesis via increased expression of enzymes necessary for cholesterol synthesis and also increasing cholesterol uptake by upregulating low density lipoprotein receptor (LDLR) expression. When an excess of intracellular cholesterol is sensed by the cell, cholesterol is oxidized to form an oxysterol which is a ligand for LXR α and/or LXR β . LXR mediated transcription leads to an overall decrease in intracellular cholesterol by

increasing cholesterol efflux while decreasing cholesterol synthesis and uptake. Increased cholesterol efflux is the result of increased expression of efflux proteins ATP-binding cassette subfamily A member 1 (ABCA1) and/or ATP-binding cassette subfamily G member 1 (ABCG1). Cholesterol synthesis is depressed due to downregulation of synthetic enzymes such as squalene synthase (20). Additionally, cholesterol uptake is suppressed as a result of decreased LDLR expression, which is either due to downregulation or increased degradation by the LXR target, increased degradation of LDL receptor protein (IDOL), which ubiquitinates LDLR.(21)

Because of the effects of the schweinfurthin compounds on FPP and GGPP and our earlier observation that they induce a form of ER stress,(22) our hypothesis was that the schweinfurthins could more globally alter isoprenoid pathway metabolites and cholesterol homeostasis in a manner reminiscent of the oxysterol 25-hydroxycholesterol.(23) Herein, we describe the effects of 3dSB on cholesterol content and cholesterol synthesis in cell lines with varying sensitivity to the anti-proliferative effects of the schweinfurthins. The effects of the schweinfurthin-induced changes in cholesterol homeostasis were investigated in both sensitive and relatively insensitive cell lines. In order to identify the mechanism(s) that lead to changes in cholesterol homeostasis, we investigated the effects of 3dSB on cholesterol synthesis and cholesterol efflux mechanisms that are controlled by LXR signaling.

Materials and Methods

Cell culture and reagents- Cell culture

SF-295, U251, and OVCAR-5 cells were cultured in RPMI1640 supplemented with 10% Fetal Bovine Serum (FBS), amphotericin B, penicillin-streptomycin, and L-glutamine (media A). The A549 cells were grown in F-12 media with the same supplements mentioned above. All cell lines were maintained at 37°C in the presence of 5% CO₂. For MTT and cholesterol synthesis assays cells were cultured in either RPMI1640 (SF-295) or F12 (A549) containing 10% lipid reduced FBS, amphotericin B, penicillin-streptomycin, and L-glutamine (media B). After resuscitation, cells were not passaged beyond 4 months to maintain authenticity. The schweinfurthin analog, 3dSB, was provided by David F. Wiemer. Lovastatin, used in its open acid form, was kindly supplied by Merck & Co. Mevalactone, Paclitaxel and 25-hydroxycholesterol were purchased from Sigma. Cell lines were acquired from the Center for Cancer Research at the National Cancer Institute of NIH. The cell lines chosen for this study are based on the pattern of activity seen in the NCI60 cell line panel.(1, 24, 25) SF-295 and U251 cells are human derived glioblastoma lines which are very sensitive to schweinfurthin activity and conversely the ovarian and lung derived cell lines OVCAR-5 and A549 are resistant to the action of the schweinfurthins.

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) Assay

Cells were plated in 96-well plates at cell concentrations to yield 40–60% confluency after overnight incubation. The following day the media was replaced and cells were incubated with indicated concentrations of 3dSB. After 20 or 44 hours of incubation the media was removed and RPMI1640 without phenol red or F-12 containing 0.6 mg/mL MTT salt (15) was added. Plates were returned to culturing conditions for 4 hours. Reduction of the MTT

salt was halted by the addition of stop solution. Plates were wrapped and incubated at 37 °C with gentle shaking overnight. The assay was quantified by measuring the optical density of wells at 540 nm and 650 nm (reference wavelength) using a SpectraMax m5e (Molecular Devices, Sunnyvale, CA)

Total Cholesterol

Cells were allowed to reach approximately 60% confluency on 10 cm plates prior to compound incubation. Media was replaced and cells were incubated in the presence or absence of lovastatin (10 μ M) or varying concentrations of 3dSB. After 24 hours of incubation, cells were washed twice in PBS and incubated in trypsin for 4–6 minutes. Trypsin was inactivated by addition of an equal volume of complete media. Cells were collected and media-trypsin was removed after centrifugation at 1500 $\times$ g for 7 minutes. Cell pellet was washed twice with PBS, prior to the collection of an aliquot for cell counting. After removal of second PBS wash cells were homogenized in chloroform:Isopropyl alcohol:NP-40 (7:11:0.1). The organic phase was collected following centrifugation at 14,000 $\times$ g for 15 minutes. This phase was dried at 50°C and resuspended in cholesterol assay buffer (BioVision, USA). Cholesterol quantification was carried out according to the manufacturer's protocol (BioVision). Lovastatin has been chosen as a positive control in several of these experiments due to the known effects of this HMGCoA reductase inhibitor on cholesterol homeostasis.(26)

Cholesterol Synthesis

After reaching approximately 60% cell confluency, media A was replaced with media B. Cells were incubated with indicated concentrations of lovastatin and 3dSB. After 18 hours of incubation, 1 μ Ci/mL [C^{14}]-Acetic Acid was added to plates and returned to culturing conditions for 6 additional hours. Cells were collected and an aliquot was taken for protein quantification by the BCA method. After a wash in PBS, cells were homogenized in 50 μ L water and 200 μ L 2N KOH in methanol. To normalize extraction efficiency, samples were spiked with [H^3]-cholesterol. Cholesterol was saponified by heating samples at 70° C for 2 hours. Subsequently, 400 μ L chloroform and 100 μ L water were added and incubated at room temperature for 15 minutes. Samples were subjected to centrifugation at 10,000 $\times$ g for 4 minutes. The upper phase was discarded and the lower phase was washed 5 times with 50% methanol. After each wash the samples were centrifuged at 10,000 $\times$ g for 4 minutes and the upper phase was discarded. The lower phase was dried under a stream of nitrogen and resuspended in chloroform. Samples were resolved by thin layer chromatography Petroleum ether:diethyl ether:acetic acid (60:40:1). Cholesterol spots were identified by iodine staining and scraped into scintillation vials, into which scintillation fluid was added. Radiolabels were quantified by the Packard 2100 Tricarb liquid scintillation counter. Cholesterol values were normalized to protein content and extraction efficiency.

Western blot analysis

After indicated incubation conditions, cells were collected and lysed in buffer containing (0.15 M NaCl, 0.05 M Tris HCl, 1% w/v sodium deoxycholate, 1% w/v SDS, 1% w/v TX-100, and 1 mM EDTA, and protease inhibitor cocktail, Sigma-Aldrich). Lysate was incubated on ice for 30 minutes prior to passage through 27 G needle (27). Lysate was

centrifuged at $14,000 \times g$ for 15 minutes. Supernatant was collected and protein content was determined by the BCA method. Equal amounts of protein were resolved by SDS-PAGE. Protein was transferred to PVDF membrane. Membranes were probed for β -tubulin (Santa Cruz biotechnologies), ABCA1 (abcam), ABCG1 (abcam), and LDLR (abcam) overnight in 5% milk/TBS-T. Appropriate secondary antibodies conjugated to horseradish peroxidase were employed. Protein levels were determined by ECL detection carried out according to the manufacturer's protocol.

Cholesterol Efflux

SF-295 or A549 cells were plated in 24-well plates at 20,000 cells/well. The next day the media was removed and replaced media B containing [H^3]-cholesterol (0.5 μ Ci) and acyl-CoA cholesterol acyltransferase inhibitor, 1 μ M Sandoz 58-035. After 24 hours, cholesterol loading media was removed and cells were washed four times with PBS. Subsequently, cells were incubated in RPMI1640 or F12 media with or without 15 μ g/mL APO-AI or 20 μ g/mL HDL (Meridian Life Sciences, Memphis, TN) and indicated concentrations of 25-Hydroxycholesterol or 3dSB for 24 hours. Efflux media and one PBS wash were collected and subjected to centrifugation at $10,000 \times g$ for 2 min to remove non-adherent cells or debris. Supernatant was collected and [H^3]-cholesterol efflux was measured via liquid scintillation counting. Cells remaining on plates were incubated in 1N NaOH for 1 hour before collection and [H^3]-cholesterol measured as described above. Percent [H^3]-Cholesterol efflux was calculated using the following equation % [H^3]-cholesterol efflux = $(([H^3]\text{-cholesterol efflux media}) / ([H^3]\text{-cholesterol efflux media} + [H^3]\text{-cholesterol in cells}))$

Real-time quantitative polymerase chain reaction (qPCR)

SF295 and A549 cells were plated in six well plates and treated with 3dSB or 25-hydroxycholesterol after confluency reached approximately 60%. Each treatment was performed in triplicate. Following 24 hrs of treatment, cells were washed twice with 1X PBS and total RNA was isolated from each well using Qiashredders and the RNeasy Mini Kit (Qiagen, Valencia, CA). On column DNase digestion was performed (Qiagen, Valencia, CA). One μ g of RNA was reverse-transcribed to cDNA with the iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA). Primers were obtained from Integrated DNA Technologies (Iowa City, IA, USA) and eluted in TE Buffer (Ambion, Austin, TX). The primer sequences used for qPCR are as follows: (*ABCA1*: F TTACACAGCAACAGTTTGTGGCCC, R TGTTCGTTGTACATCCAGGGCTGA; *ABCG1*: F AACAACGCATTGCCATAGCTCGTG, R AGTCTGCATTCTGGATGGTGGACA; *ABCG1*: F TGGTGAACAACCCTCCAGTCATGT, R TTTCATCAGCGAGACCACCTGGAA; *GADP*: F TCGACAGTCAGCCGCATCTTCTTT, R ACCAAATCCGTTGACTCCGACCTT; *HMGCR*: F TATGTGCTGCTTTGGCTGCATGTC, R ATACCAAGGACACACAAGCTGGGA; *LDLR*: F TCAACACACAACAGCAGATGGCAC, R AAGGCTAACCTGGCTGTCTAGCAA; *NR1H3 (LXA- α)*: F TCTGGGATGTGCACGAATGACTGT, R TGCCCTTCTCAGTCTGTTCCACTT; *NR1H2 (LXA- β)*: F TTCGCTAAGCAAGTGCCTGGTTTC, R AGTCGTCCTTGCTGTAGGTGAAGT; *SREBF2 (SREBP2)*: F AGGCAGGCTTTGAAGACGAAGCTA, R AGTTGTACATCGGAACAGGCGGAT; *FDFT1 (SQS)*: F

AGAAGGTCCCGCTGTTACACAAC, R ATTCTCCGGCAAATGTCGGCAATC) Real-time PCR was performed with Sybr Green Master Mix (Applied Biosystems, Foster City, CA) on ABI SDS 7900 HT (Applied Biosystems, Foster City, CA). The real-time protocol consisted of 2 minutes at 50°C, 10 minutes at 95°C, followed by 40 cycles of 95°C (15 seconds) and 60°C (one minute). Dissociation curves were obtained to ensure the proper amplification of target cDNAs.

LXR α or β agonist Assay

Direct in vitro LXR α and LXR β activation were measured for 25-HC and 3dSB at concentration ranging from (0.00057–33.3 μ M) using a time-resolved fluorescence resonance energy transfer (TR-FRET) based method. LXR α and β agonist activation assays were carried out according to the LanthaScreen TR-FRET Liver X Receptor (α or β) Coactivator Assay protocol from Invitrogen. Briefly, GST-LXR (α or β) ligand-binding domain were incubated in the presence or absence of 25-HC or 3dSB dilutions in a proprietary coregulator buffer containing dithiothreitol (DTT) as a reducing agent. To detect LXR α agonism, aforementioned components were incubated with Fluorescein-TRAp220/DRIP-2 and Tb anti-GST antibody for 6 hours. LXR β agonism was determined with component co-incubation with Fluorescein-D22 and Tb anti-GST antibody for 6 hours. The TR-FRET emission ratio (518 nm/488 nm) was analyzed using a SpectraMax m5e (Molecular Devices, Sunnyvale, CA)

Statistical Methods

One-way analysis of variance (ANOVA) model was used for the analyses of cholesterol-related outcomes in different incubation environments, or different incubation times. Pair-wise comparisons with control groups were conducted using Dunnett's test. One-way analysis of covariance (ANCOVA) model was applied to investigate the relationship between MTT activity and media containing complete and lipid depleted serum for 24 or 48 hours, and the relationship between LXR activation and incubation environment. Both analyses were controlling the concentrations of 3dSB or 25-HC (log-10 transformed). Multiple test issues were adjusted by Bonferroni correction. Statistical analyses were conducted using R programming language version 3.1.2 (R Foundation). All statistical tests were two-sided at a 0.05 significance level.

Results

Cell lines grown in Lipid depleted media display increased sensitivity to 3dSB

Previous studies conducted by our group revealed that multiple myeloma cell lines are more sensitive to schweinfurthin-induced cytotoxicity when incubated in media containing lipid depleted FBS as opposed to standard media containing FBS (18). In the prior study some cell lines showed similar effects of 3dSB treatment on expression of the genes of isoprenoid metabolism under both serum replete and serum starved conditions indicating this is not likely to be simply a drug uptake dependent phenomenon. Here, we examined the effects of 3dSB incubation on the cell viability of the SF-295 (human glioblastoma multiforme derived), A549 (human lung adenocarcinoma derived), U251 (human glioblastoma multiforme derived), and OVCAR-5 (human ovarian carcinoma derived) cell lines when

grown in complete media or lipid depleted media. The SF-295 cells were the most sensitive to 3dSB-induced cytotoxicity in standard and lipid depleted media. At 24 hours, the effective concentration 50 percent (EC50) value for 3dSB in the SF-295 cells growth in complete media was between 5–10 μ M and an EC50 of approximately 1 μ M for cells grown in lipid depleted media (Figure 1B). A similar trend is observed after 48 hours of 3dSB incubation, the EC50 for cell growth in lipid depleted media is between 0.01 and 0.05 μ M and between 0.1 and 0.5 μ M in complete media (Figure 1B). Based on previous studies investigating the growth inhibitory activity of the schweinfurthin compounds in the NCI60,(28) it was expected that sensitivity to 3dSB activity would vary between the four selected cell lines in the MTT assay as well as show greater effect at the 48 hour time point. The A549 cell line was the least sensitive to 3dSB-induced cytotoxicity followed by the OVCAR-5 and U251 cell lines (Figure 1C–E). In all cell lines tested, cells were more sensitive to 3dSB-induced cytotoxicity when grown in lipid depleted serum compared to complete media (Figure 1 B–E)

Total Cholesterol and Cholesterol are altered in cells incubated with 3dSB

The increased sensitivity of cells to 3dSB-induced cytotoxicity in lipid depleted media could suggest that lipid metabolism is altered by 3dSB. In order to examine this possibility, total cholesterol levels were examined in the most sensitive (SF-295) and least sensitive (A549) cell lines after 24 hours of incubation with varying concentrations of 3dSB. We have chosen 24 hours and higher concentrations of the schweinfurthin compound to make it possible to see results in both cells lines at the same concentrations without having large growth inhibitory effects. Both cell lines display a dose-dependent decrease in total cellular cholesterol levels; however, cholesterol levels are altered to a greater extent in the SF-295 cells (Figure 2A). When incubated with 500 nM 3dSB for 24 hours, total cholesterol is decreased by approximately 65% and 25% in the SF-295 and A549 cells, respectively. In the SF-295 cells, 3dSB is more potent than lovastatin, the positive control, in lowering total cholesterol levels. 3dSB-induced effects on cholesterol level is also time dependent in the SF-295 cell line(Figure 2B). Incubation with 3dSB (500 nM) decreases total cholesterol by approximately 30% after 12 hours of incubation. To determine if the cholesterol lowering effects of 3dSB were due to decreased cholesterol synthesis, the incorporation of [C^{14}]-acetic acid into cholesterol was examined in the SF-295 and A549 cells after incubation with increasing concentrations of 3dSB for 24 hours. Interestingly, 3dSB incubation altered cholesterol synthesis to a similar extent in both the SF-295 and A549 cells (Figure 2C). 3dSB potently inhibited cholesterol synthesis in both cell lines, incubation with 25 nM 3dSB reduced cholesterol synthesis by 50–60%. Minimal cholesterol synthesis was observed in the presence of lovastatin (10 μ M) at 24 hours in the SF-295 or A549 cell lines.

3dSB and 25-Hydroxycholesterol display similar effects on expression of LXR targets in SF-295 cells

Cellular cholesterol homeostasis is maintained by the opposing actions of SREBP2 and LXR transcription factors. In order to determine the contribution of these processes on 3dSB-induced changes in cellular cholesterol level, mRNA levels of several key cholesterol homeostatic targets including ABCA1, ABCG1, HMG-CoA reductase, LDL receptor, LXR (α and β), and squalene synthase were examined using RT-PCR. SF-295 cells were

incubated with increasing concentrations of 3dSB (0.05–0.5 μ M), 25-hydroxycholesterol (1 μ g/mL), or Lovastatin (10 μ M) for 24 hours (Figure 3A). Incubation with 3dSB led to dose-dependent increase in the level of ABCA1 and ABCG1, which are targets of LXR transcription. At a 3dSB concentration of 500 nM, ABCA1 and ABCG1 levels were elevated greater than 4- and 2-fold, respectively. Levels of cholesterol homeostatic targets were also examined in the less sensitive A549 cells (Figure 3B), although at higher concentrations of 3dSB (1–10 μ M). Unlike the SF-295 cells, the effects of 3dSB incubation on ABCA1 and ABCG1 levels were minimal in the A549 cells. Incubation with 25-hydroxycholesterol induced higher levels of ABCA1 and ABCG1, but levels were not as high as those observed in the SF-295 cells.

ABCA1 protein levels increase in response to 3dSB and 25-HC incubation at 24 hours

ABCA1 and ABCG1 protein levels were examined after 24 hours of incubation with varying concentrations of 3dSB in sensitive (SF-295 and U251) and relatively insensitive (A549 and OVCAR-5) cell lines. In SF-295 cells, 3dSB induces a concentration dependent increase in ABCA1 protein levels (Figure 4A). ABCA1 is elevated at a 3dSB concentration between 50 and 250 nM. 3dSB incubation increased protein expression of ABCA1 in the A549 cells; albeit, at higher concentrations (1–10 μ M). Increased ABCA1 protein levels were demonstrated in all tested cancer cell lines. The concentration sufficient to induce increased expression of ABCA1 was lower in the sensitive cell lines than the insensitive. Interestingly, the same concentrations of 3dSB did not appear to alter ABCG1 protein levels in any of the tested cell lines (Figure 4B). At a concentration sufficient to increase ABCA1 levels, 25-HC had minimal effects on ABCG1 levels at 24 hours. The effects of 3dSB on ABCA1 protein levels is both dose- and time-dependent based on the observed effects of 3dSB (500 nM) on ABCA1 expression after varying incubation times ranging from 6 to 18-hours (Figure 4C). Incubation of SF-295 cells with 3dSB for between 6 and 12 hours is sufficient to increase ABCA1 levels. The kinetics for increased ABCA1 levels are similar between 25-hydroxycholesterol and 3dSB. Along with the increased expression of total ABCA1, incubation with 3dSB leads to the appearance of a faster migrating band. This band is not apparent in lysate examined from 25-hydroxycholesterol incubated cells.

3dSB increases cholesterol efflux through non-specific and specific mechanisms

ABCA1 serves to lower cellular cholesterol level through the specific efflux of cholesterol to APO-A1. To determine if the decrease in cellular cholesterol level induced by 3dSB were in part associated ABCA1-mediated efflux, cholesterol efflux was evaluated in the presence or absence of APO-A1 in the SF-295 and A549 cells. After 24 hours of incubation with increasing doses of 3dSB (0.025–0.5 μ M), cholesterol efflux was increased in a dose-dependent manner in comparison to the untreated control in the SF-295 (Figure 5). Total cholesterol efflux was increased by the addition of APO-A1 with 3dSB; however, the APO-A1 specific cholesterol efflux (APO-A1 specific efflux = efflux with APO-A1 - efflux in media without APO-A1) was similar to that observed in untreated control cells (Figure 5A). This data suggests that 3dSB increases cholesterol efflux through a mechanism that is not specific to the presence of APO-A1. Incubation of cells with the oxysterol 25-hydroxycholesterol, did lead to increased APO-A1 specific cholesterol efflux as expected. Intriguingly, 3dSB incubation (10 μ M) did produce APO-A1 specific cholesterol efflux after

24 hours of incubation (Figure 5B) in the A549 cells but lower concentrations showed no significant effect compared to control.

While ABCA1 prefers to transfer cholesterol to APO-A1, ABCG1 tends to transfer cholesterol to HDL and LDL. To determine if ABCG1 was contributing to cholesterol efflux, cholesterol efflux was evaluated in the presence or absence of HDL. The presence of HDL in the efflux media did not significantly alter cholesterol efflux in SF-295 or A549 cells incubated with 3dSB or 25-HC (Figure 5C and D). Incubation with 25-HC (1 μ g/mL) and lower concentrations of 3-dSB did not significantly increase cholesterol efflux in A549 cells but at higher concentrations 3-dSB showed increases whereas the increases due to both compounds were greater in SF-295 cells. These results appear consistent with the lack of perceptible changes in ABCG1 protein levels when incubated with 25-HC and 3dSB (Figure 4B).

3dSB does not serve as a LXR ligand in vitro

Due to the similarity in the effects of 3dSB with 25-hydroxycholesterol in cellular assays, we hypothesized that 3dSB could act as a direct agonist of LXR α and/or LXR β . In order to test this hypothesis, direct activation of LXR α and/or LXR β by 3dSB was examined utilizing an in vitro TR-FRET assay designed to identify LXR agonists. Increasing concentrations of 3dSB failed to induce a conformational change in the ligand binding domain of LXR α or LXR β , suggesting that 3dSB is not a ligand for either of these receptors (Figure 6A and B). Incubation with 25-HC induced a conformational change in both LXR α and β , which is indicative of a ligand.

Discussion

Natural and synthetic schweinfurthins display unique antiproliferative activity in the NCI60 cell screen,(24, 29) which encourages further investigation of their mechanism of action. Of particular interest, the schweinfurthins are potent inhibitors of cell growth in multiple cell lines derived from CNS malignancies (Figure 1B & D). The elucidation of cellular events associated with schweinfurthin treatment could identify novel targets in the treatment CNS malignancies such as GBM. Here we present data that suggests that 3dSB greatly decreases intracellular cholesterol levels, which likely contributes to its antiproliferative activity. Unlike lovastatin, which is a direct inhibitor of HMGCoA reductase, schweinfurthins do not inhibit this enzyme. We previously showed that treatment with 3-dSB does not inhibit protein prenylation which is a property of HMGCoA reductase inhibition with statins.(30) Decreased cholesterol availability could limit the formation of new biomembranes, of which cholesterol is a critical component. Interestingly, the insensitive A549 cell line is less susceptible to 3dSB-induced depletion of cholesterol than the sensitive SF-295 cell line at concentrations that do not affect cell viability at 24 hours (Figure 2A). This may suggest that cholesterol homeostasis, more specifically LXR signaling, is controlled differently in CNS malignancies.

Cholesterol has limited ability to traverse the blood-brain-barrier;(31) therefore, de novo cholesterol synthesis is likely crucial to growth and proliferation of CNS malignancies. Evidence of a nexus between CNS cancers and cholesterol metabolism comes from several

observations. An early study showing that GBM cells had a large number of LDLRs.(32) In concordance with this result in GBM cells, platelet derived growth factor (PDGF)/PDGF receptor autocrine signalling induces expression of genes under the control of SREBP such as *LDLR*.(33) This autocrine loop amplifies genes involved in cholesterol synthesis and down-regulates genes such as *cholesterol 25-hydroxylase* which synthesizes an endogenous agonist of LXR.(34) The net result of these changes would be to increase the amount of cholesterol in the neoplastic cells. Tumor profiling of brain cancer patients showed that it was possible to identify a sub-class of patients with this expression pattern indicating this is a trait of the disease and not a cell culture artifact.(33) The effects of 3dSB incubation on cholesterol synthesis are similar in the A549 and SF-295 cells, yet intracellular cholesterol levels are reduced to a greater extent in the SF-295 cells. This result suggests that SF-295 cells are more dependent upon de novo cholesterol synthesis.

Cells control cholesterol levels through the interplay of SREBP2 and LXR transcription.(35, 36) LXR activation induces increased expression of cholesterol transporters, ABCA1 and ABCG1, and decreases cholesterol synthesis by decreasing the expression of critical enzymes of the cholesterol biosynthetic pathway.(37) Similar to 25-HC, 3dSB induces the expression of ABCA1 in both sensitive and insensitive cell lines; however, increased expression of ABCA1 is observed at lower concentrations in the sensitive cell lines. Interestingly, the increased expression of ABCA1 in the SF-295 cells does not correlate with APO-A1 specific cholesterol efflux. One possibility is that cholesterol is being effluxed through ABCA1 non-specifically. It has been observed that ABCA1 in lysates from 3dSB treated cells migrates more rapidly in blots. This change in molecular character may be associated with decreased glycosylation or inhibition of a post-translational modification that may affect activity. In the A549 cells, 3dSB incubation leads to APO-A1 specific cholesterol efflux further emphasizing the differences between sensitive and insensitive cell lines.

We have previously noted that 3-dSB induces ER-stress in the SF-295 cell line as demonstrated by increasing GRP78 expression.(39) Increased ER-stress has been shown to reduce the cellular level of ABCA1 in Hepatocellular carcinoma HepG2 cells, and to cause a reduction in cholesterol efflux.(38, 40) These authors went on to show that ER-stress reduces ABCA1 expression in a manner that is independent of LXR activation. From this perspective it is interesting that a final outcome of 3-dSB treatment is increased ABCA1 levels.

The activity profiles of 25-HC, a natural LXR agonist, and 3dSB are strikingly similar in many of the experiments presented here, which, despite the ER-stress anomaly, could suggest that 3dSB is a ligand for LXR α or β . In an in vitro LXR activation assay, 3dSB was unable to activate LXR α or β . This data suggests that 3dSB is indirectly activating LXR, potentially through derepression of LXR signaling or increased free oxysterol. Recently, Burdgett et al. demonstrated that a biotinylated analogue of OSW-1, a natural product with a similar activity profile to the schweinfurthins, is able to bind to two members of the oxysterol binding protein family, OSBP and ORP4 (41). The function of these proteins is relatively unknown; however, one potential function of these proteins is to sequester oxysterols. The schweinfurthins may have the ability to disrupt the interaction between

oxysterols and oxysterol binding proteins and subsequently activate LXR. Another potential explanation would be that the schweinfurthins impair the function of a protein repressing LXR activation. A study conducted by Bowden and Ridgway demonstrates that OSBP expression/activity negatively influences the expression of ABCA1 (42). If the schweinfurthins target OSBP, perhaps this mechanism may contribute to increased expression of ABCA1. Much is left to understand regarding the association of the schweinfurthins and oxysterol binding proteins, yet the studies presented strengthen the tie between the schweinfurthins and oxysterol signaling. It is quite possible that determining the activity of the schweinfurthins will reveal nuisances to oxysterol regulation.

Lastly, the schweinfurthins and 25-HC perturb cholesterol homeostasis to a greater extent in the GBM-derived cell lines studied here. This may indicate that agents that ultimately induce LXR activation could have clinical benefit for patients diagnosed with GBM. While it is unknown if this hypothesis will translate clinically, one LXR agonist, GW3965, has demonstrated efficacy in an animal model of GBM (43). Due to the scarcity of effective interventions, agents that target cholesterol maintenance warrant further investigation.

Acknowledgments

Financial Support Statement: This project was supported in part by the Roy J. Carver Charitable Trust, the Roland W. Holden Family Program for Experimental Therapeutics, and the National Institutes of Health (5R42NS069272).

Abbreviations

3dSB	3-deoxyschweinfurthin B
LXR	Liver X receptor
NCI60	National Cancer Institute 60 cell cancer screen
CNS	Central Nervous System

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Figure 1. Cancer cell lines display increased sensitivity to 3dSB incubation in lipid depleted serum containing medium

(A) The structures of schweinfurthin B, a natural product, and 3-deoxyschweinfurthin B (3dSB), a synthetic analog. (B–E) MTT activity showing cell viability of cancer lines incubated with increasing concentrations of 3dSB (10 nM–10 μM) in media containing complete or lipid depleted serum for 24 or 48 hours. Cell lines tested included (B) SF-295 (human glioblastoma derived; Bonferroni-adjusted p-values for LDPS 24h vs. 48h, FBS 24h vs. 48h, LDPS 24h vs. FBS 24h and LDPS 48h vs. FBS 48h comparisons are <.0001, 0.0001, 0.0800 and 0.0152, respectively), (C) A549 (human lung adenocarcinoma derived; Bonferroni-adjusted p-values for LDPS 24h vs. 48h, FBS 24h vs. 48h, LDPS 24h vs. FBS 24h and LDPS 48h vs. FBS 48h comparisons are 0.0001, 0.0272, 1.0000 and 0.1134, respectively), (D) U251 (human glioblastoma derived; Bonferroni-adjusted p-values for LDPS 24h vs. 48h, FBS 24h vs. 48h, LDPS 24h vs. FBS 24h and LDPS 48h vs. FBS 48h comparisons are 0.0001, 0.0038, 1.0000 and 0.0828, respectively), and (E) OVCAR-5 (human ovarian carcinoma; Bonferroni-adjusted p-values for LDPS 24h vs. 48h, FBS 24h vs. 48h, LDPS 24h vs. FBS 24h and LDPS 48h vs. FBS 48h comparisons are 0.0018, 0.0644, 1.0000 and 0.6420, respectively).

Figure 2. The effects of 3dSB on intracellular cholesterol levels and cholesterol synthesis
(A) SF-295 and A549 cells were incubated with 3dSB (25–500 nM) and Lovastatin (10 μ M) for 24 hours. Total cholesterol (free + esterified) levels were determined using an enzymatic approach described further in “Materials and Methods”. The mean total cholesterol levels from 3 independent experiments is expressed as a percent of the of untreated controls. Standard deviation is indicated by error bars (* $p < 0.05$ vs. control, Dunnett’s test). **(B)** The time dependence of 3dSB-induced changes in total cholesterol were examined in the SF-295 cells. Cells were incubated in the presence or absence of 3dSB (500 nM) or Lovastatin (10 μ M) for 6, 12, or 18 hours. The mean total cholesterol levels from 3 independent experiments were expressed as percentage of untreated cells; the error bars are indicative of the standard deviation (* $p < 0.05$ vs. control, ** $p < 0.005$ vs. control, Dunnett’s test). **(C)** Lovastatin- and 3dSB-induced changes in cholesterol synthesis were examined after 24

hours of incubation. Cholesterol synthesis was determined by evaluating the level [C14]-Acetic Acid incorporation into total cholesterol. Bars represent the mean cholesterol synthesis in cells incubated with Lovastatin 10 uM or 3dSB (25–500 nM) expressed as a percent of untreated control. The standard deviation of mean cholesterol synthesis is shown, all treatments show $P < 0.005$ vs. control, Dunnett's test.

Figure 3. 3dSB alters the RNA expression of LXR targets in SF-295 cells, but has minimal effects on A549 cells

(A) SF-295 and (B) A549 cells were incubated with or without Lovastatin (10 μ M), 25-HC (1 μ g/mL), and increasing concentrations of 3dSB (0.05–0.5 μ M in A and 0.5–10 μ M in B) for 24 hours. The mean and standard deviation of triplicate treatments from a representative experiment is displayed. Three independent experiments were performed.

Figure 4. Increased ABCA1 protein levels induced by 3dSB are concentration- and time-dependent

(A and B) SF-295, A549, U251, and OVCAR-5 cells were incubated with or without 25-HC (1 μ g/mL) or increasing concentration of 3dSB (0.025–0.5 μ M in SF-295 cells, 0.5–10 μ M in A549 cells, 0.05–1 μ M in U251, and 0.1–5 μ M in OVCAR-5 cells) for 24 hours. (C) SF-295 cells were incubated in the presence or absence of 25-HC (1 μ g/mL) or 3dSB (0.5 μ M) for 6, 12, 18, or 24 hours. Blots were probed for ABCA1 (A and C), ABCG1 (B), and β -tubulin (loading control) expression. Experiments are representative of three independent experiments.

Figure 5. 3DSB induces non-specific cholesterol efflux in the SF-295 cells

SF-295 (A and C) or A549 (B and D) cells were incubated with or without 25-HC (1 $\mu\text{g/mL}$) or increasing concentrations of 3dSB (0.025–0.5 μM in A and C or 0.5–10 μM in B and D) for 24 hours. To determine the specificity of cholesterol efflux cells were incubated in presence or absence APO-AI (15 $\mu\text{g/mL}$) in A and B or HDL (20 $\mu\text{g/mL}$) in C and D. Results are representative of the mean and standard deviation of an experiment performed in quadruplicate. Three independent experiments were performed (* p < 0.05 vs. control condition, ** p < 0.005 vs. control condition Dunnett's test).

Figure 6. 3DSB does not directly activate LXR α or LXR β in vitro

Increasing concentrations of 25-HC or 3dSB (0.00057–33.3 μ M) were incubated with LXR α -(A) (25-HC vs. 3dSB: P-value 0.3991 by ANCOVA model) or LXR β -ligand binding domain (B) (25-HC vs. 3dSB: P-value < 0.0001) and components of LanthaScreen TR-FRET Liver X Receptor (α or β) Coactivator Assay (Invitrogen). The mean TR-FRET ratio (518 nm/488 nm) and standard deviation of one of three independent experiments performed in triplicate.