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Research Article

Inhibition of lung carcinoma cell growth by high density lipoprotein-associated α-tocopheryl-succinate

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Abstract. α-Tocopheryl-succinate (αTS) is a synthetic, anti-neoplastic derivative of α-tocopherol. Here we studied the effects of free and high-density lipoprotein subclass 3 (HDL_3)-associated αTS on the growth of human (A549) and mouse Lewis (LL2) lung carcinoma cells. Both free and HDL_3-associated αTS inhibited A549 growth in a time- and concentration-dependent manner. Treatment of A549 cells with αTS-enriched HDL_3 led to DNA fragmentation and a time-dependent decrease in immunoreactivity of poly(ADP-ribose)polymerase. Uptake experiments revealed a high capacity for selective αTS uptake in excess of holoparticle endocytosis. Overexpression of scavenger receptor class B, type I (SR-BI), the prime receptor mediating selective lipid uptake, in A549 cells resulted in significantly increased selective αTS uptake, a finding associated with complete cellular growth arrest. The present in vitro findings were verified in an in vivo model: tumor inoculation in C57BL6 was performed with either wild-type, β-galactosidase- or SR-BI-overexpressing LL2 cells. After tumor inoculation, the animals received six consecutive intravenous injections of αTS. This experimental setup resulted in significantly reduced tumor burden in animals that were inoculated with SR-BI-overexpressing LL2 cells but not in animals inoculated with wild-type or β-galactocidase-transfected cells. Based on our in vitro and in vivo findings, we propose that SR-BI could provide a novel route for HDL_3-mediated drug delivery of anti-neoplastic drugs.

Key words. HDL_3; vitamin E-succinate; A549 cells; LL2 cells; SR-BI.

Vitamin E and some of its derivatives, notably its succinate ester (RRR-α-tocopheryl-succinate; αTS) are under intense study for use as potential chemopreventive agents. In contrast to its parent compound α-tocopherol (αTocH), αTS is a potent inhibitor of tumor cell growth [1–3]. Whether growth inhibition is mediated only by αTS or also by enzymatically liberated αTocH has been disputed, but Farris and colleagues [4, 5] have shown that a non-hydrolyzable ether analog of αTS inhibits growth of leukemia cells in a manner comparable to αTS. αTS is able to inhibit DNA-synthesis [6, 7], inhibits tumor cell growth [6–9] and can induce apoptosis in a variety of different tumor cells [10].

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αTS belongs to a selected group of compounds (including retinoids, steroids and anti-estrogens) that inhibit tumor cell growth via a common pathway, the activation of transforming growth factor-β (TGF-β) [11]. As the effects of TGF-β signaling include several negative forms of regulation (induction of G1 arrest, promotion of terminal differentiation or induction of apoptosis), disruption of the TGF-β pathway could predispose to or cause cancer [reviewed in ref. 12]. Important to note here is that αTS treatment of tumor cells results in increased concentrations of biologically active TGF-β and an increase in TGF-β receptor expression [6, 8]. Besides the TGF-β signaling pathway, the Fas/Fas ligand pathway also contributes to αTS-induced apoptosis in human breast cancer cells [13, 14]. αTS converts Fas-resistant human breast cancer cells into a Fas-sensitive phenotype by a mechanism involving the translocation of cytosolic Fas to the cell surface [14]. In addition, αTS activates selected members of the mitogen-activated protein kinase family [1, 15, 16]. αTS-induced apoptosis was shown to involve caspase activation [17], as well as lysosomal and mitochondrial destabilization [18, 19].

Since αTS is subjected to intestinal hydrolysis [20], the development of non-hydrolyzable αTS derivatives or application routes other than oral application (to circumvent intestinal hydrolysis) is necessary. Due to the rather hydrophobic nature of αTS, one would expect the compound to readily associate with different classes of circulating lipoproteins after intravenous (i.v.) injection. This further implies that αTS uptake follows the receptor-mediated pathways like the classical low-density lipoprotein (LDL)-receptor [21] or scavenger receptor class B, type I (SR-BI) [22] delivering lipoprotein constituents to tissues and organs.

Based on the excessive need of rapidly dividing cancer cells for cholesterol, tumor cell growth could be impaired by lowering endogenous cholesterol biosynthesis or by inhibition of the exogenous supply of cholesterol-rich lipoproteins (in particular LDL). Indirect evidence for excessive uptake of cholesterol by tumor cells is underlined by overexpression of the LDL-receptor in a number of different tumors [reviewed in ref. 23]. On the other hand, the expression of the LDL-receptor raises the possibility of specifically targeting LDL-associated anti-tumor agents to cancer cells [23]. That this approach is feasible has been demonstrated [24–26]. Another pathway that contributes to cholesterol supply of rapidly dividing tumor cells is mediated by SR-BI, the prime receptor mediating selective lipid uptake from high-density lipoprotein (HDL) [27]. In our previous studies, we could demonstrate that selective uptake of HDL-associated cholesteryl ester (CE) via SR-BI closely correlates with the proliferation rates of human breast cancer cells and trophoblast-like cells [9, 28]. In other tumor cell models, e. g. choriocarcinoma cells [28], malignant human epithelial cells [29] or hepatoma cells [30] SR-BI was demonstrated to be involved in selective HDL-CE uptake. Importantly, overexpression of an SR-BI construct lacking the C-terminal domain (deletion of amino acids 465–509) significantly inhibited the growth of a human breast cancer cell line [31]. In addition, expression of SR-BI mRNA was significantly elevated in breast cancers when compared to non-cancerous mammary tissue [31]. This indicates that SR-BI expression correlates with the growth behavior of tumor cells.

Based on these findings, the present study aimed at elucidating uptake mechanisms of HDL-associated αTS and establishing the effect of free and HDL-associated αTS on growth rates of human and mouse lung cancer cells and the relationship to SR-BI-dependent uptake of this potentially anti-neoplastic compound.

Materials and methods

Materials

Human lung carcinoma cells (A549), mouse Lewis lung carcinoma cells (LL2) and Jurkat T cells (TIB-152) were from ATCC (Manassas, Va.). HAM's F12K and DMEM medium, trypsin, penicillin and streptomycin was from Gibco (Vienna, Austria), plasticware for cell culture was from Costar (Vienna, Austria). Cell Death Detection Elisa and a rabbit polyclonal anti-poly-(ADP-ribose)-polymerase (PARP) antibody was from Roche (Vienna, Austria). Rabbit polyclonal anti-peptide SR-BI was purchased from Abcam (Cambridge, UK). Etoposide was from Calbiochem (Bad Soden, Germany). Peroxidase-conjugated antibodies were from Chemicon (Temecula, Calif.) or Santa Cruz (sc 2004; Santa Cruz, Calif.). All solvents and other reagents used were of the highest available quality and obtained from Sigma (Vienna, Austria). RRR-αTocH and RRR-αTS were from Esai (Tokyo, Japan).

Isolation of HDL_3

Human HDL subclass 3 (HDL_3) was prepared by density gradient ultracentrifugation in a TL120 tabletop ultracentrifuge (at r_{av} 350,000 g; Beckman, Austria) as described elsewhere [32].

In vitro enrichment of human plasma and HDL_3 with αTS

Human plasma was incubated with the indicated concentrations of αTS (stock 2 M αTS in DMSO) for 3 h at 25°C on a rotary shaker (final concentration of DMSO in the samples was $\leq$ 0.05%; v/v). Thereafter, HDL_3 was isolated by ultracentrifugation as described above. The molar incorporation rates are calculated on the basis of a molecular mass of 175 kDa for HDL_3 [33]. To analyze the αTS content of in vitro-enriched human plasma and

lipoproteins isolated therefrom, 200 µl of the sample was mixed with 200 µl of water and 200 µl of ethanol and further processed and analyzed as described elsewhere [9].

Synthesis of [^{14}C]αTS

[^{14}C]αTS was synthesized from 25 µCi (446 nmol) of [^{14}C]-RRR-α-tocopherol (Amersham) as described elsewhere [9]. Radiochemical purity and product identity were assessed by coelution of the radiochemical product with an authentic αTS-standard (Sigma, Vienna) on a straight-phase HPLC column [9].

Labeling procedures

HDL_3 was iodinated with ^{125}INa using N-Br-succinimide as the oxidizing agent [34] resulting in specific activities between 200 and 400 cpm/ng protein. αTS labeling (1.5 mg HDL_3-protein/2 ml PBS) was performed by direct addition of an ethanolic [^{14}C]αTS solution, incubation at 37 °C (3 h under argon in a shaking water bath) and subsequent reisolation of HDL_3 by ultracentrifugation as described above (specific activities of 5–10 cpm/ ng protein).

A549 cell culture

Cells were grown in HAM's F12K medium supplemented with fetal calf serum (FCS; 10%, v/v), penicillin (100 U/ml) and streptomycin (100 µg/ml) and cultured under standard conditions. Culture medium was changed every 2–3 days. A549 cells were incubated on six-well plates at the density indicated in HAM's F12K containing FCS (5%, v/v). The cells were grown for 1–3 days in the absence or presence of αTS, which was added to the cells either as free compound (dissolved in DMSO), or associated with HDL_3 at the indicated concentrations. During these experiments, a vehicle control of equal amounts of HDL_3 was included. After incubation, the cells were trypsinized, harvested and counted manually by a hemacytometer in duplicates or triplicates.

Uptake experiments were conducted with the correspondingly labeled HDL_3 with confluent cells on six-well trays. Uptake studies were performed in the presence of the indicated amounts of ^{125}I-, or [^{14}C]αTS-labeled HDL_3 for the indicated times. After washing, degradation of iodinated lipoproteins was measured as non-trichloroacetic acid-precipitable radioactivity. Cells were then lysed in 0.3 M NaOH and the radioactivity was counted. An aliquot of the hydrolyzate was used to determine the cellular protein content [35]. During the present study, uptake of iodinated lipoproteins is termed holoparticle uptake, while [^{14}C]αTS uptake is expressed in terms of protein equivalents (i.e. the amount of lipoprotein uptake that would account for lipid tracer uptake calculated from the specific activity [36]). This is necessary for quantitative comparison of the two uptake pathways. Selective uptake is calculated as the difference between apparent particle uptake and holoparticle uptake (cell association and degradation).

Immunoassay of oligonucleosome release

Enrichment of mono- or oligonucleosomes was quantitatively determined by a sandwich immunoassay with a cell death detection kit (Cell Death Detection Elisa^{PLUS}) according to the manufacturer's recommendations. Cells 5×10^3 were seeded on 96-well microtiter plates and incubated in medium containing HDL_3 (5 µg protein/ 200 µl) or αTS-enriched HDL_3.

PARP cleavage

A549 cells were cultured until confluency (12-cm Petri dishes) and incubated in the presence of αTS, or αTS-enriched HDL_3 for the indicated times. Cells were then scraped in medium, centrifuged and the resulting pellet was washed twice with PBS. The pellet was resuspended in PBS and the cell number was measured on a hemacytometer. Cells were then pelleted again, lysed in sample buffer (sonication for 30 s, twice) and incubated for 15 min at 65 °C. Proteins were subjected to SDS-PAGE (5% gels), transferred to nitrocellulose, and immunoreactive bands were detected with a rabbit anti-PARP antibody, peroxidase conjugated goat anti-rabbit IgG (dilution 1:2000) and the ECL system (Amersham, Vienna, Austria). Untreated and etoposide (68 µM)-treated Jurkat T lymphoma cells were used as controls for PARP cleavage.

Intracellular αTS hydrolysis

A549 cells (5×10^4) were plated in six-well trays and received 2 ml of medium containing αTS-labeled HDL_3 (25 µg HDL_3-protein containing 10 µg αTS or 90,000 cpm [^{14}C]αTS as tracer). After 24, 48 and 72 h, the cells were washed in PBS containing BSA (twice) and PBS without BSA (twice). Cellular lipids were extracted with hexane/2-propanol and analyzed for αTS and [^{14}C]αTS by HPLC (284 nm or radiometric detection).

Immunochemical detection of SR-BI

SDS-PAGE of A549 membrane proteins [isolated as described in ref. 37] or cellular lysates was performed on 8% gels under denaturating conditions. Proteins were electrophoretically transferred to nitrocellulose membranes and immunochemical detection of SR-BI was performed with a rabbit anti-SR-BI peptide antiserum (1:1000), peroxidase-conjugated goat-anti rabbit IgG (dilution 1:2000) and subsequent ECL development.

Animals

C57BL6 mice were purchased from the research centre Himberg (Austria). Female mice of similar age (15 weeks ± 3 days) and body mass (21 ± 2 g) were used. The animals were housed at 22 °C under a constant light-dark cy-

cle and had free access to water and rodent chow (4.5% fat; 21% protein) (Sniff, Germany). During the treatment, the mice were anesthetized with Forane (Abbot Laboratories, Maidenhead, UK) and blood was collected by retro-orbital puncture.

Adenovirus transfection of mouse LL2 carcinoma cells

LL2 cells form multilayers in flasks without actually becoming confluent and were grown in DMEM containing 4 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin. The medium was adjusted to contain 1.5 g/l sodium bicarbonate, 4.5 g/l glucose and 1.0 mM sodium pyruvate and supplemented with 10% FCS. Cells were grown at 37 °C in a humidified atmosphere and 5% CO_2. At 80% confluence, the cells were harvested by trypsinization, 1×10^7 cells were replated in 150-mm petri dishes and incubated for an additional 14 h to enable cell adhesion prior to adenovirus transfection. The cells were incubated for another 5 h with recombinant adenoviruses encoding human SR-BI [38] using a multiplicity of infection (MOI) = 200 in the infection medium (proliferation medium containing 2% FCS). The same procedure was applied to A549 cells (but using MOI = 30). During infection, the plates were gently rocked every 30 min in an incubator (37 °C, 5% CO_2, humidified atmosphere). After removing infection media the cells were supplied with proliferation media containing 10% FCS and incubated for an additional 24 h prior to tumor inoculation.

Tumor inoculation in C57BL6 mice

LL2 cells used for tumor inoculation were trypsinized, centrifuged for 2 min at 30 g, washed once with PBS and resuspended in PBS at a density of 3.33×10^6 cells/ml. The cells were kept on ice until inoculation. Before inoculation, the animals were anesthetized with Forane and 1×10^6 LL2 cells were injected subcutaneously into the right flank of each mouse using a 21-gauge needle. Two sets of experiments were performed. In the first experiment, tumors were inoculated with wild-type, β-galactosidase (β-gal) and SR-BI-overexpressing (in line with another report [39], maximum expression was observed after days 3–5, then expression levels started to decline) cells (five animals per group) and the animals were injected (see below for details) with Intralipid (Fresenius Kabi, Graz, Austria) or Intralipid containing αTS. In the second experiment (performed in exactly the same way as the first one), tumors were inoculated with SR-BI- or β-gal-overexpressing LL2 cells and animals were injected with Intralipid or Intralipid containing αTS. Two days after tumor inoculation, animals were injected with 100 µl vehicle (Intralipid) or 100 µl of TS/Intralipid (100 mM TS in Intralipid) via the tail vein. This procedure results in rapid association of αTS with mouse HDL [9]. Afterwards, the mice received the same amount of Intralipid or TS/Intralipid on days 4, 7, 10 and 13, respectively. On day 14, all mice were sacrificed, tumors were removed and volumes were determined using calipers. The experiments were approved by the local ethics committee.

Statistics

Differences in cell growth were calculated by Student's t test. Differences in tumor volume were examined with one-way ANOVA followed by Dunnet's multiple comparison test. A p value of < 0.05 determined significance.

Results

Effect of free αTS and αTocH on A549 growth

In the first set of experiments, the effect of αTS and DMSO (vehicle) on cell growth was determined (table 1). At 0.01–0.05% DMSO, cell growth was unaffected within 72 h. At 0.5% DMSO, cell growth was inhibited by approximately 20% (72 h). A DMSO content of 2.5% (v/v) in the medium totally inhibited A549 growth. On the basis of these results, the DMSO content in the medium was always kept below 0.05%. Addition of increasing concentrations of αTS to the medium (5, 10 and 25 µg/ml) led to a significant time- and concentration-dependent reduction in cell numbers in comparison to controls (table 1). In contrast, when A549 cells were incubated in HAM's F12K medium supplemented with αTocH in the same concentration range, growth rates were almost unaffected (98, 99 and 97% growth in comparison to control cells after 72 h).

Effects of HDL_3-associated αTS on A549 growth

In vitro enrichment of human plasma with αTS [performed as described ref. 9] resulted in an αTS content of approximately 160 mol αTS/mol HDL_3. A549 cells were incubated in the presence of HDL_3, free αTS, or αTS-enriched HDL_3 (fig. 1A). Incubation of A549 cells in HDL_3-containing medium led to a significant (on average 1.3-fold) increase in cell numbers as compared to control cells. In contrast, αTS-enriched HDL_3 inhibited tumor cell growth by 20, 43 and 35% (after 24, 48 and 72 h, respectively) in a manner comparable to free αTS. To establish a dose-response profile of cell growth versus αTS concentrations, cells received constant amounts of HDL_3 with increasing αTS content. From data shown in figure 1B, A549 growth clearly decreased concomitantly with an increasing αTS content of HDL_3. The apparent IC50 value necessary to inhibit cell growth by 50% was approximately 7 µg αTS/25 µg HDL_3-protein/ml medium.

Oligonucleosome accumulation and PARP cleavage

To determine whether HDL_3-associated αTS inhibits A549 cell growth by induction of apoptosis, the accumulation of mono- and oligonucleosomes was followed (fig.

Table 1. Effect of DMSO, αTS and αTocH on A549 cell growth. At time zero, 5×10^4 cells were plated on six-well trays and cultivated in HAM'S F12K medium containing FCS (5%, v/v) and the indicated concentrations of DMSO, αTS (DMSO ≤ 0.05%) or αTocH (DMSO ≤ 0.05%). At the indicated time points, cells were trypsinized, harvested and counted on a hemacytometer in duplicates. Results shown represent the mean ± SD from triplicate dishes. Statistical analysis was performed with the GraphPad Prism package using Student's t test (* $p < 0.05$, ** $p < 0.005$, *** $p < 0.001$).

Addition	Cell number		
	24 h	48 h	72 h
None	84,667 ± 4,613	148,750 ± 4,507	286,330 ± 2,0731
DMSO (%, v/v)			
0.01	79,000 ± 2,646	146,670 ± 9,043	274,750 ± 5,250
0.025	78,000 ± 2,856	156,670 ± 8,509	280,000 ± 4,630
0.05	82,333 ± 5,868	146,250 ± 5,449	267,750 ± 1,225
0.5	92,334 ± 3,549*	145,100 ± 5,449	227,550 ± 9,986*
2.5	92,667 ± 2,843*	107,500 ± 3,307***	9 2,917 ± 9,043***
αTS (μg/ml)			
5	76,667 ± 1,155*	106,420 ± 13,501**	200,080 ± 11,912**
10	67,667 ± 3,055**	92,333 ± 5,508***	195,420 ± 8,808**
25	60,667 ± 2,082***	75,667 ± 2,082***	133,580 ± 7,891***
αTocH (μg/ml)			
5	82,960 ± 3,255	145,722 ± 6,673	280,030 ± 9,309
10	84,320 ± 7,982	145,496 ± 4,470	284,040 ± 5,364
25	91,372 ± 2,863*	132,678 ± 4,353*	276,594 ± 3,031

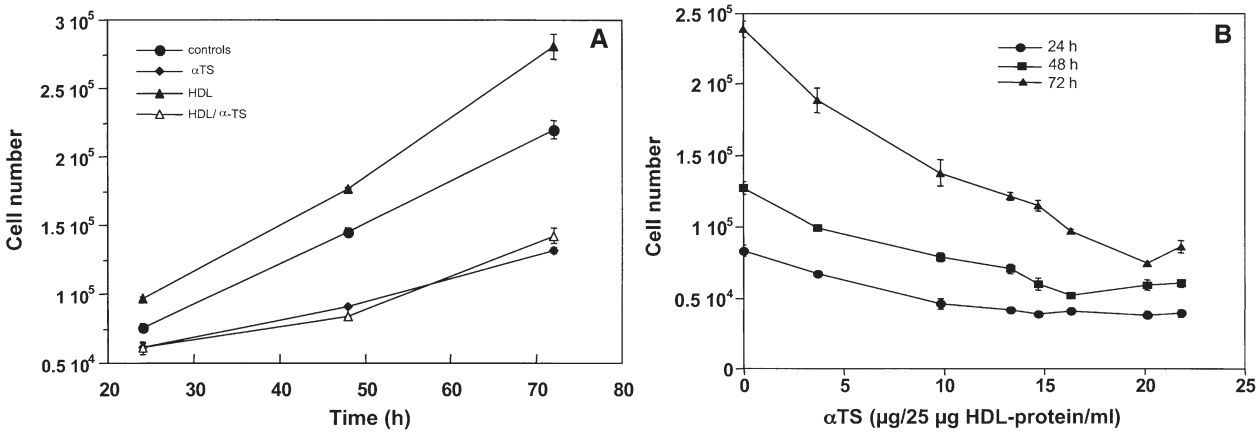

Figure 1. Effect of native and αTS-enriched HDL_3 on A549 cell growth (*A*) At time zero, 5×10^4 cells were plated on six-well trays containing 2 ml HAM's F12K medium (controls), 10 μg free αTS/ml (αTS), 25 μg/ml HDL_3-protein (HDL_3) or 25 μg/ml HDL_3-protein containing 10 μg αTS (HDL_3/αTS). At the indicated time points, the cells were washed, trypsinized and counted on a hemacytometer. Data shown represent the mean ± SD from triplicate dishes. (*B*) At time zero, 5×10^4 cells were plated on six-well trays in 2 ml HAM's F12K medium supplemented with 25 μg HDL_3-protein/ml containing the indicated αTS concentrations. HDL_3-enrichment with αTS was performed as described in Materials and methods. At the time points indicated, cells were washed, trypsinized and counted (three samples/dish). Data shown represent the mean ± SD from triplicate dishes from one representative experiment.

2A). For these experiments, freshly prepared human plasma was enriched with αTS (1–75 mM) and HDL_3 was isolated as described above. Incubation of A549 cells with αTS-enriched HDL_3 (1.7–9 μg αTS/10 μg HDL_3-protein) resulted in pronounced accumulation of mono/oligonucleosomes in cellular lysates and the cellular supernatants. Native HDL_3 was without effect on nucleosome accumulation. Within the time scale analyzed, a concentration of ≥ 4 μg αTS appeared to be necessary to induce DNA cleavage.

PARP cleavage is related to caspase-3 activation and facilitates cellular disassembly. A549 cells were treated with free and HDL_3-associated αTS, and PARP cleavage was followed by Western blot analysis (fig. 2B). The activation of caspases in Jurkat cells by etoposide (used as positive control) resulted in cleavage of intact PARP (116 kDa) and the appearance of an 86-kDa fragment within 6 h. Upon treatment of A549 cells with either free or HDL_3-associated αTS (25 μg/ml each), the immunoreactivity of the 116-kDa band decreased time-de-

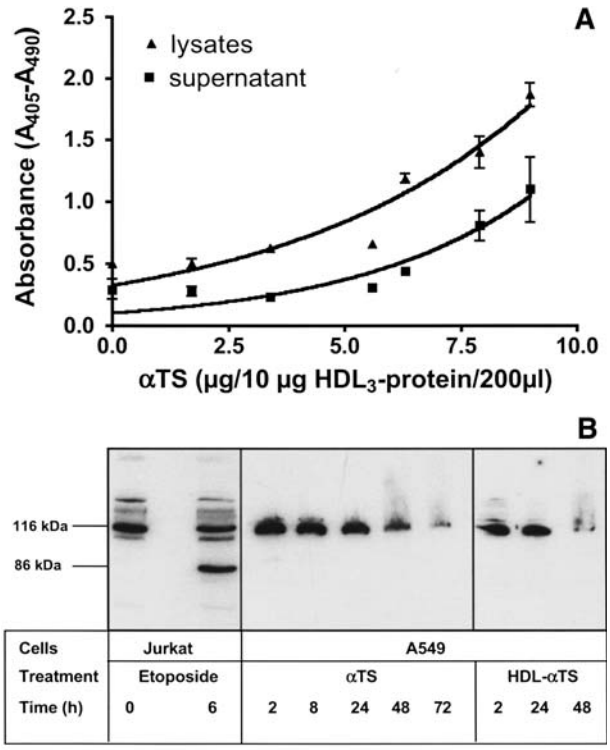

Figure 2. Oligonucleosome accumulation and PARP cleavage (*A*) To determine nucleosome fragmentation, 5×10^3 cells were seeded on 96-well microtiter plates and kept until confluency. Cells were incubated in the absence (controls) or presence of 10 µg HDL_3-protein/200 µl medium (containing the indicated concentrations of αTS for 24 h). Thereafter, the microtiter plate was centrifuged at 200 g and the supernatant was transferred to Eppendorf tubes. Cells were lysed in 200 µl lysis buffer and debris was removed by centrifugation (200 g). Twenty microliters of the supernatant and the cell lysate was analyzed by the Cell Death Detection Elisa PLUS as described in Materials and methods. Data shown represent the mean ± SD from triplicate wells. (*B*) Jurkat T cells were incubated in the absence or presence (68 µM) of etoposide. A549 cells were cultured on 12-cm petri dishes until 80% confluency in F12K medium (12 ml) containing FCS (5%) or in F12K medium containing αTS (25 µg/ml), or αTS-enriched HDL_3 (50 µg protein containing 25 µg αTS) for the indicated periods of time. Cells were then trypsinized and washed in PBS (containing 1 mM PMSF). The pellet was resuspended in PBS (500 µl), sonicated and the protein content was determined. Samples were diluted (1:40) in sample buffer, incubated at 65 °C (15 min) and separated by SDS-PAGE (5% gels; 130 µg protein/lane). After transfer to nitrocellulose, the 116-kDa full-length PARP and the 86-kDa fragment was detected by Western blot experiments as described in Materials and methods.

pendently; however, no 86-kDa cleavage fragment was detectable.

Uptake mechanisms of HDL_3-associated αTS by A549 cells

In principle, two pathways can contribute to the uptake of HDL_3-associated lipids: receptor-mediated endocytosis and SR-BI-mediated selective lipid uptake without concomitant holoparticle internalization. To evaluate the contribution of each pathway, HDL_3 was labeled with ^{125}I (to analyze the contribution of holoparticle uptake) and with [^{14}C]αTS which allows measurement of both pathways (holoparticle uptake plus selective uptake). A549 cells were incubated for 6 h in the presence of increasing HDL_3 concentrations (fig. 3). At the highest concentration used, [^{14}C]αTS uptake exceeded holoparticle uptake by 300-fold, indicating an extremely high capacity of A549 cells for selective αTS uptake.

Intracellular hydrolysis of HDL_3-associated αTS

To determine the time-dependent accumulation and intracellular hydrolysis of αTS to αTocH (which is ineffective in terms of anti-proliferative properties; table 1), A549 cells were incubated with αTS-enriched HDL_3 (to determine the efficacy of mass transfer) or [^{14}C]αTS-labeled HDL_3 (to determine hydrolysis rates). Within the time course examined, A549 cells acquired 578 ng αTS/mg cell protein from HDL_3 that was enriched with non-radioactive αTS. This is indicative of true mass transfer and not only tracer exchange. Intracellular hydrolysis of lipoprotein-associated [^{14}C]αTS appeared to be rather ineffective as hydrolysis accounted for only 0.18%/h of HDL_3-associated αTS initially present in the experiment (fig. 4).

SR-BI overexpression in A549 cells increases the sensitivity towards HDL_3-associated αTS

Next, we asked whether expression levels of SR-BI in A549 cells are a determining factor for αTS uptake and regulation of cell growth. Immunoblot analysis revealed that high-level SR-BI overexpression in A549 cells was achieved using a recombinant adenoviral system (fig. 5A). Overexpression of SR-BI resulted in significantly increased αTS uptake from HDL_3 (≈ two-fold) during the first 12 h; the β-gal construct was without effects on uptake kinetics (fig. 5B) as compared to wild-type cells. To assess the effects of SR-BI overexpression on growth rates, wild-type, β-gal- and SR-BI-transfected cells were cultivated in medium (fig. 5C), medium containing HDL_3 (fig. 5D) and medium containing αTS-enriched HDL_3 (fig. 5E). As is evident from figure 5C, D, adenoviral infection with β-gal and the SR-BI transgene resulted in decreased growth rates (on average 25% reduction) of A549 cells even in the absence of αTS. The addition of HDL_3 (fig. 5D) resulted in slightly higher cell numbers after 48 h (1.2-fold, wild type; 1.10-fold, β-gal and SR-BI overexpressors) as compared to cultures kept in medium alone. The addition of αTS-enriched HDL_3 resulted in severely decreased A549 growth (76 and 71% of controls cultured in medium; wild-type and β-gal transfected cells, respectively). Most strikingly, SR-BI overexpression resulted in total growth arrest of A549 cells when cultivated in the presence of αTS-containing HDL_3 (fig. 5E).

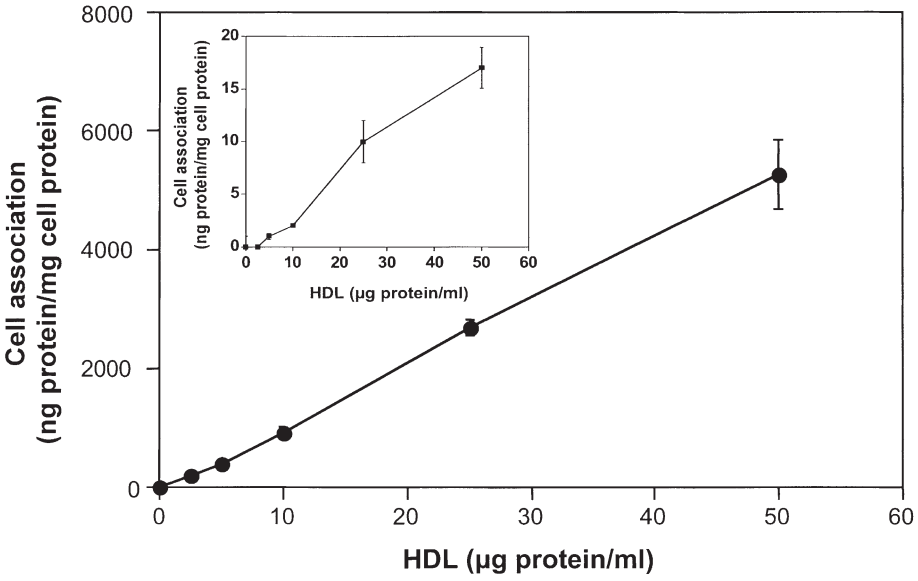

Figure 3. Uptake of HDL_3-associated αTS. A549 cells were grown on six-well trays and incubated in the presence of the indicated concentrations of ^{125}I- or [^{14}C]αTS-labeled HDL_3. After a 6-h incubation, the medium was removed and degradation of ^{125}I-HDL_3 was analyzed as described in Materials and methods. The cells were washed and lysed in NaOH to determine the cellular protein content and the cell-associated radioactivity. Holoparticle uptake (shown as inset; note the different axis scaling) represents the sum of degradation and cell association. Selective uptake was calculated as the difference between [^{14}C]αTS-HDL_3 and ^{125}I-HDL_3 holoparticle uptake. Results shown represent the mean ± SD from triplicate determinations from one representative experiment.

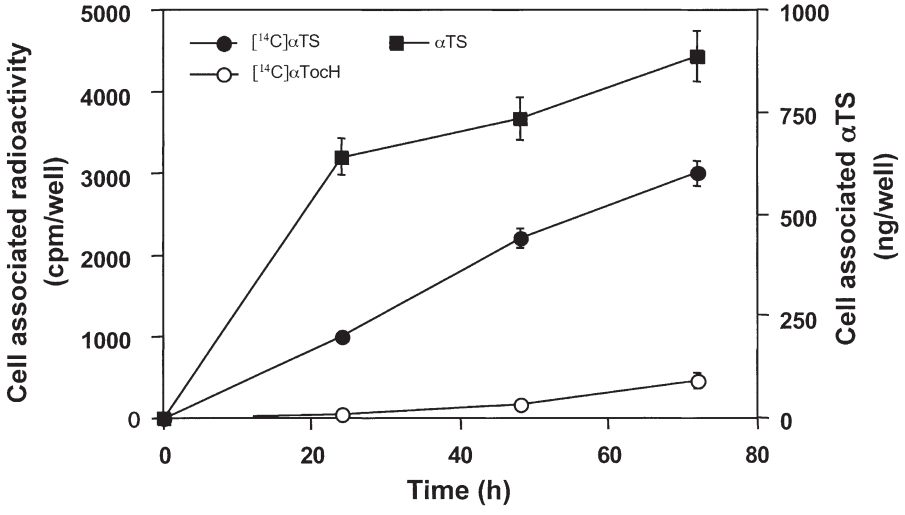

Figure 4. Intracellular hydrolysis of HDL_3-associated αTS. A549 cells were incubated in the presence of [^{14}C]αTS-HDL_3 (25 µg protein/ml) and in a parallel set of experiments, cells received HDL_3 (25 µg protein) that was enriched with unlabeled aTS (10 µg/25 µg protein) to determine mass transfer. At the indicated time points, cells were washed, cellular lipids were extracted and analyzed by HPLC (UV or radiometric detection) as described in Materials and methods. Data shown represent the mean ± SD from one representative experiment performed in triplicate.

Suppression of tumor growth by αTS in vivo

To study whether SR-BI overexpression would also lead to suppression of tumor growth in mice in response to αTS administration, solid tumors were inoculated subcutaneously in C57BL6 mice with wild-type LL2 cells and LL2 cells overexpressing either the β-gal (control vector) or the SR-BI transgene. Each of the groups was injected (days 2, 4, 7, 10 and 13) with vehicle (Intralipid) or αTS dissolved in Intralipid. On day 14, the tumors were removed and the tumor volume was determined. These experiments showed that tumor volumes were not affected by adenovirus transfection (fig. 6; 1184 ± 158, 1094 ±

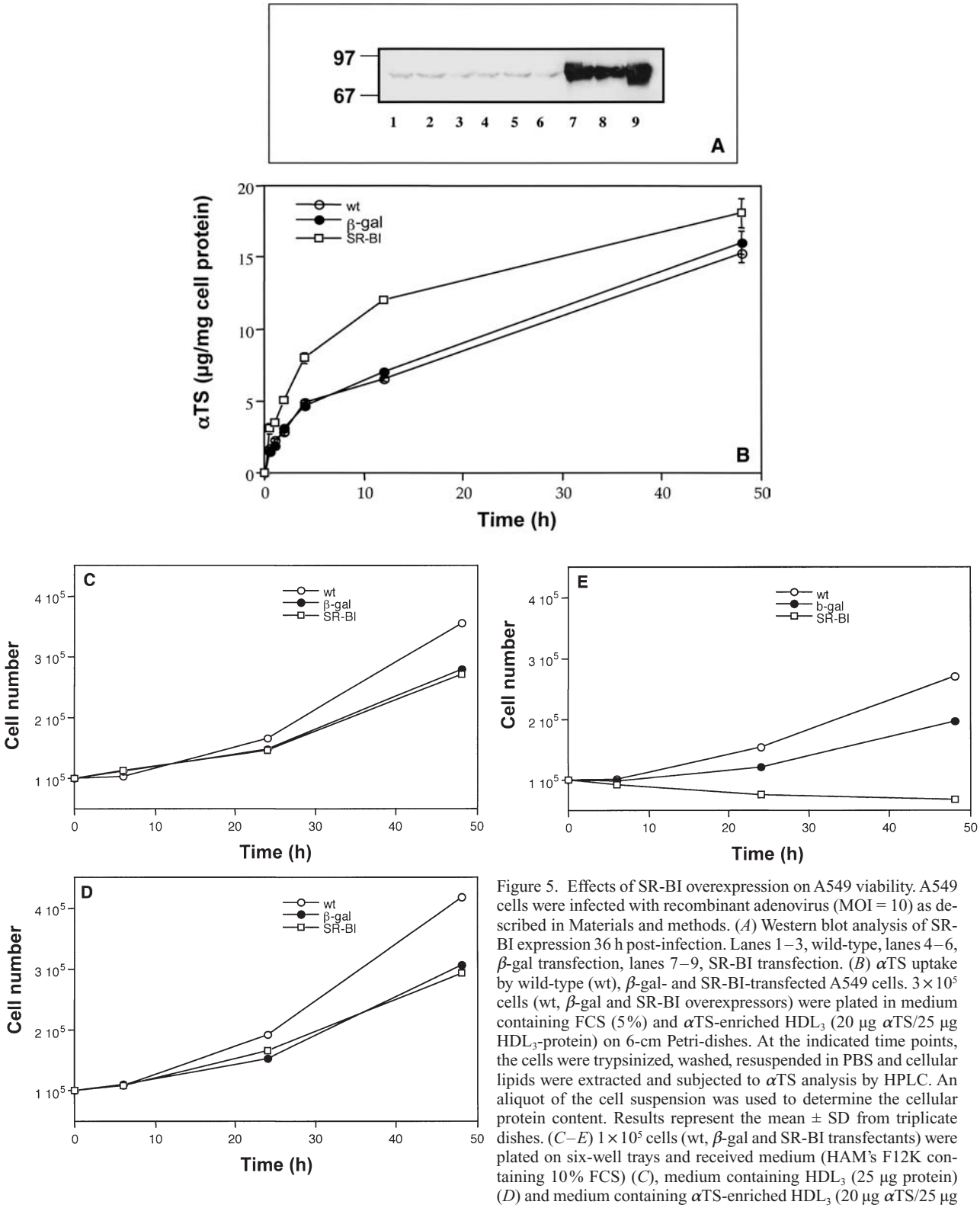

Figure 5. Effects of SR-BI overexpression on A549 viability. A549 cells were infected with recombinant adenovirus (MOI = 10) as described in Materials and methods. (*A*) Western blot analysis of SR-BI expression 36 h post-infection. Lanes 1–3, wild-type, lanes 4–6, *β*-gal transfection, lanes 7–9, SR-BI transfection. (*B*) *α*TS uptake by wild-type (wt), *β*-gal- and SR-BI-transfected A549 cells. 3×10^5 cells (wt, *β*-gal and SR-BI overexpressors) were plated in medium containing FCS (5%) and *α*TS-enriched HDL_3 (20 µg *α*TS/25 µg HDL_3-protein) on 6-cm Petri-dishes. At the indicated time points, the cells were trypsinized, washed, resuspended in PBS and cellular lipids were extracted and subjected to *α*TS analysis by HPLC. An aliquot of the cell suspension was used to determine the cellular protein content. Results represent the mean ± SD from triplicate dishes. (*C–E*) 1×10^5 cells (wt, *β*-gal and SR-BI transfectants) were plated on six-well trays and received medium (HAM's F12K containing 10% FCS) (*C*), medium containing HDL_3 (25 µg protein) (*D*) and medium containing *α*TS-enriched HDL_3 (20 µg *α*TS/25 µg HDL_3 protein) (*E*). At the indicated time points, the cells were trypsinized, washed and counted. Results shown are mean values from triplicate experiments. For more clarity, no deviation bars are shown (SD was ≤ 10%).

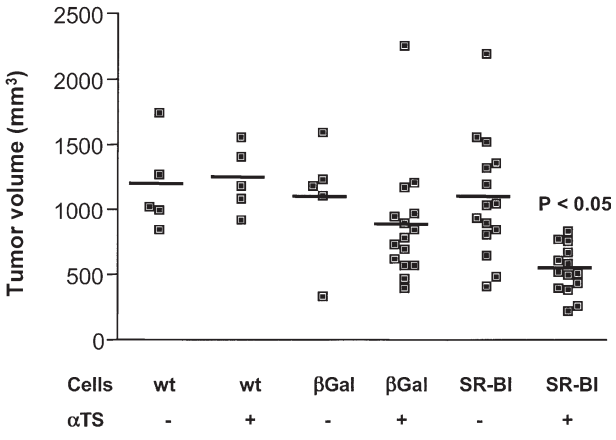

Figure 6. Tumor inoculation in C57BL6 mice with LL2 cells. LL2 cells used for tumor inoculation were cultivated as described in Materials and methods. Before inoculation, the animals were anesthetized by Forane and 1×10^6 LL2 cells were injected subcutaneously into the right flank of each mouse using a 21-gauge needle. Tumor inoculation was performed as described in Materials and methods. Two days after inoculation, mice were injected with vehicle (100 µl Intralipid) or 100 µl of TS solution (100 mM TS in Intralipid) via the tail vein. Afterwards, the mice received intravenously the same amount of vehicle or TS on days 4, 7, 10 and 13. On day 14, animals were sacrificed, the tumors were removed and their volume determined. Data shown represent the mean ± SE. Statistical difference from wild-type tumor volume observed in vehicle-injected animals was calculated with one-way ANOVA followed by Dunnett's multiple-comparison test.

207, and 1089 ± 120 mm³; wild-type, β-gal- and SR-BI-overexpressing LL2 cells, respectively). In addition, αTS injection was without statistically significant effect on tumor volumes in the wild-type and β-gal group (however, small effects might be obscured by extreme outliers in the β-gal group). In contrast, αTS administration led to a significant reduction of tumor volumes in mice inoculated with SR-BI-overexpressing LL2 cells (1089 ± 120 vs 539 ± 50 mm³; $p < 0.05$, one-way ANOVA followed by Dunnett's multiple-comparison test).

Discussion

The findings of the present study demonstrate that HDL_3 could act as a physiological αTS carrier equipped with an intrinsic targeting domain – apolipoprotein A-I – via SR-BI-dependent uptake mechanisms [40]. Our data show that human HDL_3 is capable of accommodating large amounts of αTS thus providing an extremely efficient and specific drug delivery system for a commercially available compound with pronounced anti-neoplastic potential and no toxic side-effects towards normal cells [41]. This notion is supported by several lines of evidence: (i) the capacity of HDL_3 for αTS incorporation is at least as high as described for other lipophilic compounds, e.g. 3′,5′-O-dipalmitoyl-5-iodo-2′-deoxyuridine or doxorubicin into the much larger LDL particle [42]; (ii) overexpression of SR-BI results in significantly enhanced uptake of αTS from HDL_3, findings in line with the demonstration of selective uptake of dioleyl-iodo-deoxyuridine (incorporated into synthetic HDL_3 particles) by rat liver [43]; (iii) overexpression of SR-BI in human A549 lung carcinoma cells highly improves the anti-neoplastic efficacy of HDL_3-associated αTS and (iv) SR-BI-overexpressing LL2 tumors are accessible to αTS-dependent tumor growth inhibition in vivo. From these observations, we conclude that the HDL_3/SR-BI system merits attention as a suitable potential delivery and targeting system of anti-neoplastic drugs to cancer cells.

In line with the concept that hydrophobic drugs can be incorporated into lipoproteins [23, 44], αTS readily associated with HDL_3. Enrichment of human plasma with αTS resulted in incorporation rates of about 160–200 mol αTS/mol HDL_3, the absolute incorporation rates showing a certain degree of variability between different blood donors. Our previous studies revealed that αTS associates tightly with lipoproteins since the majority of the compound can be recovered in association with LDL or HDL_3 after ultracentrifugation with a slight increase in buoyant density of αTS-enriched lipoprotein fractions [9].

Time- and concentration-dependent incubations of A549 cells with αTS-enriched HDL_3 resulted in the accumulation of mono- and oligonucleosomes, providing evidence for apoptosis probably via caspase-mediated events as repeatedly reported for other tumor cell lines incubated with αTS [17, 19, 45, 46]. However, no PARP cleavage product could be detected in A549 cells that were treated with either free or HDL_3-associated αTS. Similar observations were reported for Syrian hamster embryo cells that underwent apoptosis (characterized by pronounced DNA fragmentation) in response to topoisomerase inhibitor treatment or serum deprivation [47]. Results obtained during the present study appear to be specific for αTS, as the 86-kDa PARP cleavage product is formed in A549 cells in response to MAL-induced apoptosis [48].

A major question of the present study was whether αTS is taken up via an SR-BI-dependent pathway similar to that reported for the parent compound αTocH [38, 49]. Overexpression of SR-BI in systems as diverse as COS cells and *Xenopus laevis* oocytes (data not shown) resulted in several-fold increase in αTS uptake over wild-type cells, indicating that this is a feasible pathway for cellular αTS delivery. Most importantly, cellular delivery via selective uptake is not coupled to extensive intracellular hydrolysis of αTS, leaving most of the anti-neoplastic hemisuccinate ester intact. Overexpression of SR-BI in A549 cells resulted in much faster αTS uptake kinetics compared to non-transfected cells, while at the longest incubation times (48 h), αTS concentrations were almost similar in wild-type and SR-BI-overexpressing cells. This

indicates a limited capacity for cellular αTS uptake, in line with saturation kinetics observed during mass transfer studies (fig. 5). Adenovirus-infection of A549 cells displayed growth inhibitory properties, while the addition of HDL_3 to the medium of wild-type cells resulted in consistently enhanced growth rates. This is compatible with observations in human breast carcinoma [9] and choriocarcinoma cells [28]. Most importantly, SR-BI-overexpressing A549 cells went into complete growth arrest in response to αTS-enriched HDL_3. These findings were also confirmed by cell cycle analysis of A549 cells where αTS-enriched HDL_3 led to an impaired transition from the G1 to the S phase, an effect most pronounced for SR-BI-overexpressing cells (data not shown). This is in line with findings in αTS-treated prostate cancer cells where arrest in the G1 phase was observed [50]. These effects were ascribed to pronounced down-regulation of different modules of the cell cycle regulatory machinery (including cyclins and CDKs) in response to αTS [50].

To establish whether SR-BI expression levels correlate with the susceptibility towards αTS-dependent growth inhibition in vivo, tumors were induced in C57BL6 mice by inoculation with non-transfected and SR-BI-overexpressing LL2 cells. We here show that adenovirus transfection of LL2 cells (in contrast to A549 cells) was without effects on tumor growth, since non-transfected and transfected cells resulted in the development of nearly similar tumor burden. αTS injection displayed no inhibitory effects on tumor growth in the control and mock-transfected group. In contrast, the mean tumor volume was significantly reduced in the SR-BI-overexpressing group in response to αTS administration. This indicates that αTS associates with HDL after intravenous injection, is transported to the tumor microvasculature and taken up by tumor cell-expressed SR-BI to exhibit anti-neoplastic activity. One evident explanation for the observed beneficial effects of αTS administration in SR-BI-overexpressing tumors is increased uptake of αTS. However, analysis of tumor lipid extracts revealed almost identical αTS content in tumors inoculated with either mock-transfected or SR-BI-overexpressing LL2 cells (4.7 ± 2.5 vs 4.2 ± 2.2 ng αTS/mg tissue; concentrations comparable to other reports [51]). On the basis of our in vitro data, one might plausibly assume that increased αTS uptake by SR-BI-overexpressing LL2 tumors would be observed at the earliest time points after inoculation and this effect might be obscured at the time of tumor removal. This would further imply that the most pronounced anti-neoplastic effects of αTS should be observed at very early stages of tumor development; this is currently under investigation.

The lack of anti-neoplastic effects of αTS in the control group was surprising and is contradictory to reports for other tumor models where αTS has potent inhibitory potential towards experimentally induced mouse melanomas [52–54] and potently suppresses tumor growth in an experimental mouse model of colorectal cancer [51]. In the latter model, αTS exerted cooperative pro-apoptotic activity with tumor necrosis factor-related apoptosis-inducing ligand. Preliminary cDNA array analysis of tumor tissue performed during the present study (unpublished data) indicated that SR-BI-overexpressing tumors respond to αTS treatment with pronounced down-regulation of DAD1 (a mammalian cell death suppressor acting downstream of antiapoptotic Bcl-2 [55]).

In summary, we could show that delivery of HDL_3-associated αTS to human and mouse lung carcinoma cells via an SR-BI-dependent pathway provides a feasible and efficient system to inhibit tumor cell growth, thereby providing anti-neoplastic properties.

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