Tasisulam Sodium, an Anti-tumor Agent that Inhibits Mitotic Progression and Induces Vascular Normalization

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Abstract

LY573636-sodium ("tasisulam") is a small molecule anti-tumor agent with a novel mechanism of action currently being investigated in a variety of human cancers. In vitro, tasisulam induced apoptosis via the intrinsic pathway, resulting in cytochrome c release and caspase dependent cell death. Using high content cellular imaging and subpopulation analysis of a wide range of in vitro and in vivo cancer models, tasisulam increased the proportion of cells with 4N DNA content and phospho-histone H3 expression, leading to G2/M accumulation and subsequent apoptosis. Tasisulam also blocked vascular endothelial growth factor (VEGF), epidermal growth factor (EGF), and fibroblast growth factor (FGF)-induced endothelial cell cord formation, but did not block acute growth factor receptor signaling, (unlike sunitinib, which blocks VEGF-driven angiogenesis at the receptor kinase level) or induce apoptosis in primary endothelial cells. Importantly, in vivo phenocopying of in vitro effects were observed in multiple human tumor xenografts. Tasisulam was as effective as sunitinib at inhibiting neovascularization in a Matrigel® plug angiogenesis assay in vivo, and also caused reversible, non G2/M-dependent growth arrest in primary endothelial cells. Tasisulam also induced vascular normalization in vivo. Interestingly, the combination of tasisulam and sunitinib significantly delayed growth of the Caki-1 renal cell carcinoma model, whereas neither agent was active alone. These data demonstrate that tasisulam has a unique, dual-faceted mechanism of action involving mitotic catrostrophe and anti-angiogenesis, a phenotype distinct from conventional chemotherapies and published anti-cancer agents.
Introduction

Despite the massive resources expended over the past 20 years and a steadily declining death rate since the 1990s, cancer remains the second leading cause of death in the United States (1-2). The human genome project has provided hope for a new era of cancer therapeutics by identifying novel molecular targets for drug discovery(3-5). Despite the promise of genomics-centered drug discovery, it is likely that phenotypic-based approaches will continue to play a valuable, complementary role through interrogation of the cell as a target(6-8). Indeed many of today’s standard of care anti-cancer agents were identified using phenotypic approaches(9).

LY573636-sodium, hereafter referred to as tasisulam, is a small molecule acyl-sulfonamide that is the product of a phenotypic drug screen of >14,000 compounds. A requirement of the screen was to identify compounds that had approximately a hundred fold margin of activity between cancerous and nontransformed normal cells. In vitro, tasisulam is a potent anti-proliferative compound (free drug EC<sub>50</sub> typically in the low μM range) and displays preferential cancer cell death relative to normal, untransformed human cell lines(10-11). Furthermore, NCI COMPARE analysis indicates that tasisulam possesses a unique mechanism of action relative to other agents(10, 12). In humans, tasisulam has demonstrated activity in several different tumor subtypes, and clinical studies are ongoing or completed in soft tissue sarcoma, ovarian cancer, acute myeloblastic leukemia, metastatic breast cancer, non-small cell lung cancer, and renal cancers(13-15). In hematological malignancy cell lines, tasisulam induced apoptosis through induction of the caspase-9 dependent intrinsic apoptosis pathway, possibly resulting from a direct effect on mitochondrial processes leading to upregulation of reactive oxygen
species (ROS)(16). However, the cellular mechanism(s) underlying these effects were not well understood.

We have expanded the pre-clinical investigation of tasisulam into a wide variety of solid tumor cell lines, with a particular focus on elucidating the cellular processes underlying the anti-cancer activity of this compound. Surprisingly, tasisulam disrupted two seemingly distinct processes in cancer cell lines, neither of which appears to cause a primary induction of ROS. The first involved induction of the intrinsic apoptosis pathway in a cell cycle-dependent fashion. Using high content imaging (HCI)-based subpopulation analysis(17-18) we demonstrated that G2/M accumulation leads to caspase-3 activity and subsequent cell death. The second involved tasisulam-mediated effects on tumor vasculature. Tasisulam displayed both in vitro and in vivo anti-angiogenic effects, without inhibiting the proximal growth factor/receptor signaling or inducing endothelial cell death. In addition, tasisulam promoted vascular normalization by stabilizing the existing vasculature, reducing vessel tortuosity, and reducing hypoxia in vivo(19). Although identification of the precise cellular target(s) will require additional study, these results suggest that the broad activity of tasisulam in cancer pre-clinical models is mediated by both cytotoxic and anti-angiogenic effects.
Materials and Methods

In Vitro Cancer Cell Experimentation

Cells (non-NCI COMPARE panel) were acquired from ATCC (Manassas, VA) and cultured according to ATCC guidelines and plated in growth medium for 24 hours prior to treatment. Cell authenticity was verified by STR genotyping at RADIL no more than 6 months prior to drug testing (20). Cells were plated @ 500,000 cells/10 mL into 10 cm dishes for flow cytometry which was performed with a Beckman Coulter FC5 and cell cycle was determined using MOD fit.

For Western blotting, nitrocellulose membranes were blocked in Tris-buffered saline, 0.1% Tween-20 and 5% nonfat dry milk, probed for cleaved caspases-3 or -9, PARP/cleaved PARP (Cell Signaling Technology) and then exposed to secondary antibody for 1 hour at 25 °C. Proteins were visualized with Super Signal® West Femto enhanced chemiluminescence detection (ThermoFisher) and imaged with a Bio-Rad ChemiDoc™ XRS. For HCl, all procedures are previously described (18). For cell viability, cellular ATP production was measured by Cell Titer Glo® Assay (Promega). For assessment of in vitro biochemical proangiogenic receptor tyrosine kinase inhibitory activity, tasisulam was tested at Cerep (Poitiers, France) and Millipore (Billerica, MA) per industry accepted and validated guidelines).

In vitro Endothelial Cell Experimentation

For assessment of HUVEC migration, HUVECs (45,000/well) were plated in black 96 well collagen I plates (BD) and incubated overnight at 37°C in 5%CO2. Cells were labeled with 3.6uM CMFDA (Invitrogen) for 30 minutes in serum free medium whereupon complete growth
medium was replenished and incubated for 30 minutes. A scratch was made in each well using a pintool affixed to a MultiMek robot 96 well head. Cells were then washed using 200ul normal PBS. 100ul serum free medium was then added to each well. Cell migration was detected using the Acumen Explorer for day 0 (baseline) data collection. An additional 100ul 10% serum-containing medium with 2X compound was added into each well after imaging. The plate was cultured overnight and imaged to collect endpoint data. Migration was measured using the difference between endpoint and baseline readings for each well.

Adipose-derived stem cells (ADSCs) (Lonza) were grown in EGM® MV Microvascular Endothelial Cell Growth Medium (Lonza), and endothelial colony forming cells (ECFC) (EndGenitor Technologies) were grown in the same medium with an additional 5% FBS (Gibco). Cells were maintained in an incubator at 37 °C with 5% CO₂.

For HCl, 5,000 ECFCs/well were plated in 200 µL starvation medium (EGM+5% FBS) and cultured overnight. Fresh starvation medium was replenished +/- tasisulam for 48 hours. Cells were fixed to the plate with 50 µL concentrated Prefer (Anatech) for 30 minutes at room temperature, washed 1x with PBS, treated for 5 minutes with 100 µL 0.1% SDS and then 100 µL 0.1% Triton® X-100 in PBS. Cells were stained with Hoechst 33324 to visualize nuclei, anti-Ki67 (Neomarkers) and visualized with Alexa Fluor®-555 conjugated anti-rabbit (Invitrogen) or stained for nuclear fragmentation (“TUNEL”; In Situ Cell Death Detection Kit TMR Red, Roche) according to manufacturer’s protocol.

To assess cord formation, adipose-derived stem cells (ADSCs) were plated in basal medium (MCDB-131 media supplemented with insulin, dexamethasone, ascorbic acid,
transferrin, and tobramycin). On day 2, ECFCs were seeded on the ADSCs in basal medium. Following growth factors (VEGF, 20 ng/mL; bFGF, 50 ng/mL; or EGF, 50 ng/mL) and tasisulam addition, cultures were grown for 3 days, fixed to the plate for 30 minutes with cold 70% ethanol, immunostained with anti-human CD31 (R&D Systems)/Alex Fluor®-488 donkey anti-sheep IgG secondary (Invitrogen), anti-smooth muscle actin-Cy3 (Sigma) and Hoechst 33342.

Assessment of endothelial cell receptor signaling required a label-free technology (Cellular Dielectric Spectroscopy, CellKey), which is capable of measuring complex impedance changes in cell monolayers. Impedance (Z) is related to the ratio of voltage/current (Z=V/I) (Ohm’s law). Cells respond to receptor stimulation with rearrangements in the actin cytoskeleton causing shape, adherence and cell interaction changes, measured as changes in impedance. Recording the response of a cell to ligand stimulation allows specific evaluation of a therapeutic’s ability to block the proximal signaling pathways involved in processes such as angiogenesis. 45,000 cells/well are seeded in 150 µL of growth medium onto a microplate containing electrodes at the bottom of each well. After overnight incubation at 37 °C in 5% CO₂ incubator, the plate is loaded into the instrument where the cells are washed, and medium replaced with serum-free medium. A 30-minute equilibration to establish a baseline is followed by compound addition with impedance monitoring for 30 minutes to record effects. The baseline is reset as ligand is added at 50 ng/mL and the impedance of each well was monitored for 15 additional minutes to record ligand/receptor-induced cellular responses. Maximum change in impedance over time is reported.

In Vivo Matrigel® Plug Angiogenesis Assay
ECFC and ADSC cells were mixed at the ratio of 4:1 (2 x 10^6/0.5 x 10^6), centrifuged, resuspended in Matrigel® (BD Biosciences) on ice and sub cutaneously injected (0.2 mL/implant) into female athymic nude mice (Harlan), two implants per mouse (one/flank). 6 days post treatment mice were sacrificed and implants collected. The right implant was placed into zinc-tris fixative (Phar mingen) for IHC analysis. The left implant was flash frozen for hemoglobin assay.

For hemoglobin assay, plugs were weighed and placed into homogenization tubes. XY buffer (1% Triton® X-100, 25 mM Tris pH 7.5, 150 mM NaCl, 1mM EDTA, 1mM EGTA, HALT protease inhibitor (Pierce) added just prior to use) was added at 4x weight of the plug. Plugs were homogenized for 10 seconds at setting #4 on Fastprep FP120 machine. Samples were centrifuged 5 minutes at 12,000 rpm in 4 °C refrigerated centrifuge. Hemoglobin concentration was assessed using QuantiChrom™ Hemoglobin assay kit (BioAssay Systems) following the manufacturer’s protocol.

**In Vivo Pharmacology**

Tumor cells were mixed 1:1 with Matrigel® and implanted subcutaneously in the right rear flank of athymic nude female mice at 5.0 x10^6 cells/injection. Xenografts were grown to an average tumor volume of 200 mm^3 and the mice were randomized at baseline according to tumor volume and body weight (n=8). Physiological saline and tasiswa doses were administered daily by IV injection for 5 days followed by 2 days of rest. This cycle was repeated and tumor growth inhibition was measured.
Tumor volume was estimated using the formula: $v = l \times w^2 \times 0.536$ where $l =$ larger of measured diameter and $w =$ smaller of perpendicular diameter. Antitumor activity was calculated as a percent reduction of treated (T) tumor volume relative to untreated control (C) tumor volume $[1-(T/C)] \times 100$ and the data were log transformed to equalize variance across time and treatment groups. The data were then analyzed with a two-way repeated measures analysis of variance by time and treatment using the MIXED procedures in SAS software (version 8.2). The correlation model for the repeated measures is spatial power. The MIXED procedure is also used separately for each treatment group to calculate adjusted means and standard errors. Both analyses account for the autocorrelation within each animal and the loss of data that occurs when animals with large tumors are removed from the study early. The adjusted means and standard errors are plotted for each treatment group versus time.

**Tumor Tissue Immunofluorescence and Imaging**

Five days post treatment, xenografts were excised and placed into zinc-tris fixative. After 24 hours, tumors were trimmed, routinely processed, embedded in paraffin blocks and four micron sections were made. Slides were baked at 60 °F for 1 hour and then deparaffinized in xylene (4 X 10 minutes); rehydrated with ethanol/water immersions with final washes in TBST; blocked with Protein Block (Dako) for 30 minutes; stained with a combination of Hoechst 33324, rat anti-human CD31 (Pharmingen)/anti-rat Alexa Fluor®-488 (Invitrogen), rabbit anti-GLUT1 (Dako)/anti-rabbit Alexa Fluor®- 647 (Invitrogen), and mouse anti-Smooth Muscle Actin/Cy3 (Sigma); imaged using an iCys Laser Scanning Cytometer (CompuCyte) and a Marianas Digital Imaging Workstation configured with a Zeiss Axiovert 200M inverted fluorescence microscope (Intelligent Imaging Innovations). Mean vessel density (MVD) was
calculated as the percentage of total tissue area (Hoechst positive) that is also CD31 positive. % Pericyte Coverage of Vessels was calculated as the percentage of total CD31 area that was also colocalized with SMA staining. % Hypoxia Area was calculated as the percentage of total tissue area that is also GLUT1 positive. Mean Tortuosity Index was calculated as the % of large vessels/% of small vessels. Vascular Normalization Index was calculated as (Pericyte Coverage of Vessels)/(MVD*% Hypoxia Area*Mean Tortuosity Index) relying on both direct and surrogate markers for these parameters as explained under “Results”. Quantitative data comparisons of treatment groups were performed using the Dunnett’s analysis in JMP statistics software (SAS).

**Tumor Histopathology and Immunohistochemistry (IHC)**

Xenografts were formalin fixed, trimmed and embedded in paraffin. Three micron sections were immunohistochemically labeled using an autostainer for phosphohistone H3-serine 10 (pHH3) with a rabbit polyclonal primary antibody (Upstate) at a concentration of 1 µg/mL without antigen retrieval and with 3, 3’-diaminobenzidine as chromagen. Sections were counterstained with hematoxylin. Additional sections were stained with hematoxylin and eosin (H&E). Digital images of the immunolabeled slides were captured with the Scan Scope XT (Aperio) and evaluated using the Spectrum software (Aperio) positive pixel count algorithm.
Results

Tasisulam induced apoptosis in a broad range of in vitro cancer cell models. Tasisulam inhibited the growth of a wide range of tumor histologies, with >70% of the 120 cell lines tested displaying an anti-proliferation EC_{50} of <50 µM (Table S1). Cell lines with an EC_{50} > 50 µM were deemed resistant. Because tasisulam is highly protein bound (>99.7% bound to serum albumin), the relative free concentration of 50 µM tasisulam in cell culture media supplemented with 10% FBS is approximately 5 µM, a clinically relevant drug concentration. Overall, tasisulam induced an anti-proliferative response across a wide range of tumor histologies, suggesting that the compound likely targeted a process fundamental to cell growth. Representative in vitro anti-proliferation curves in the Calu-6 non small cell lung carcinoma (EC_{50} = 10 µM) and A-375 melanoma models (EC_{50} = 25 µM) are shown (Fig. 1A).

Tasisulam affected cell cycle. A recent report in hematological malignancy cell lines observed that induction of ROS occurred 72 hours after tasisulam treatment (16). However, in the Calu-6 cell line, glutathione levels did not change and the addition of an exogenous reducing agent, N-acetylcysteine, did not prevent cell death (Fig. S1A/B), suggesting that ROS generation may have been secondary to apoptosis-related events. FACS analysis of Calu-6 and A-375 cell lines showed that tasisulam induced a concentration-dependent increase in 4N DNA (G2/M accumulation) (Fig. 1B, top/middle ranks). Calu-6 cells demonstrated striking G2/M accumulation, likely due to asynchronous cells maintaining a relatively high basal 4N DNA population (28%). Western blotting of cleaved poly (ADP-ribose) polymerase (PARP) and activated caspase-9 confirmed an intrinsic apoptotic response (Fig. 1B, inset), while cytosolic
cytochrome C levels increased dramatically following treatment (data not shown). HeLa cells, which were largely resistant to the effects of tasisulam (EC$_{50}$ ~150 µM), displayed only a modest G2/M accumulation and only slight activation of apoptosis. Interestingly, double thymidine-blocked, synchronized Calu-6 cells treated with tasisulam were significantly delayed in progressing through G2 when drug was introduced immediately upon release from cell cycle block, or during S phase. If cells were treated after DNA replication, they were able to complete a normal mitosis (Figure S2).

**Tasisulam treatment caused apoptosis in cells with increased DNA content.** To determine if tasisulam-induced G2/M accumulation was directly linked to apoptosis, a previously described cell cycle HCI assay that focuses on cellular subpopulations was used. The HCI assay associates multiple phenotypic readouts within the same cell, as opposed to population-based readouts that cannot associate concomitant biological events at the cellular level (18). Boundaries are established for different cell cycle states based on nuclear parameters, with an emphasis on DNA content. These boundaries loosely define the classical cell cycle stages, but given the cell-level resolution of HCI in conjunction with the complexity of cell cycle biology, cell cycle stages are defined via multiple interacting parameters rather than demarcation via a single feature. Fluorescent images of tasisulam treated Calu-6 or A-375 cells probed for DNA content, tubulin, and pHH3 showed increased DNA condensation and 3-4 fold increase in pHH3 (Fig. 2A), consistent with G2/M accumulation. Similar results were observed in other sensitive, but not resistant, cancer cell lines (data not shown).
HCI subpopulation analysis revealed that only those cells with increased DNA content displayed activated caspase-3, with the extent of induction proportional to the increase in DNA content, nuclear area and pHH3 (Fig. 2B). Quantitation of G2/M accumulation corroborated FACS data whereby tasisulam’s effects were maximal after 48 hours (two cell doublings), further strengthening the hypothesis that tasisulam induces cancer cell apoptosis through a cell cycle related mechanism.

Tasisulam inhibited endothelial cell proliferation in vitro. In the original phenotypic screen, compounds were required to have a significant margin to apoptosis in malignant vs. normal cells. Tasisulam caused non-apoptotic growth arrest in human umbilical vein endothelial cells (HUVEC), prompting further study in other endothelial cell types. ECFCs treated with tasisulam and analyzed in a HCI proliferation and apoptosis assay showed dramatic decreases in the proliferation marker Ki67 at concentrations above 2 μM total tasisulam (~0.6 μM free drug), similar to the effect observed with the multi-targeted receptor tyrosine kinase inhibitor sunitinib (Fig. 3A). However, neither tasisulam nor sunitinib induced endothelial apoptosis, as measured by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL). This was in sharp contrast to the microtubule targeting agent, nocodazole, which was both anti-proliferative and pro-apoptotic (Fig. 3A). The antiproliferative effect of tasisulam was not limited to ECFCs and was also observed with endothelial cells of different origins and primary human fibroblasts (data not shown). Furthermore, in HUVECs there were no significant changes in cell cycle distribution as determined by FACS (Table S2), indicating the antiproliferative effects were non-cell cycle-dependent, suggesting that tasisulam preferentially exerts its cell cycle/pro-apoptotic effects on transformed cells.
Tasisulam inhibited in vitro endothelial cord formation and in vivo angiogenesis.

Since endothelial proliferation is a process critical for angiogenesis, tasisulam was next assessed for inhibition of in vitro endothelial cord formation, a surrogate assay that models key morphogenic features of blood vessel formation(21). Tasisulam inhibited VEGF-, FGF- and EGF-induced cords in a concentration dependent manner with EC50s of 47 nM, 103 nM and 34 nM, respectively (Fig. 3B, C). To determine if tasisulam interfered with acute proximal growth factor receptor-related signaling, a cell impedance assay was employed. Changes in impedance as a result of growth factor-induced morphological changes are a quantitative measure of receptor tyrosine kinase pathway activity (22). VEGF causes a relaxation of the junctions between cells and hence less resistance to electrical conductivity, expressed here as a decrease in impedance. EGF and FGF cause a slight increase in cell-to-cell pressure and hence a slight increase in impedance. Sunitinib, an approved angiogenesis inhibitor, completely blocked the response to VEGF, but not EGF or FGF. Tasisulam did not inhibit VEGF, FGF or EGF induced impedance changes indicating that it did not interfere with acute proximal growth factor/receptor signaling (Table 1), consistent with the fact that tasisulam does not inhibit these kinases in vitro (data not shown). In addition, tasisulam is not inhibitory to HUVEC migration in vitro, suggesting that effects on cord formation involve an as yet unexplained mechanism (data not shown).

To determine if tasisulam promotes an anti-angiogenic effect in vivo, a Matrigel® plug model of neoangiogenesis(23) was performed. After six days, the coimplanted cells in the Matrigel® formed extensive networks of human blood vessels with functional anastomoses to the mouse circulatory system (Fig. 4A). Compared to vehicle treated control animals, tasisulam
at both 25 and 50 mg/kg (corresponding to clinically relevant doses) caused a significant reduction in hemoglobin content (Fig. 4B) and mean blood vessel density by IHC labeling of Matrigel® plugs (Fig. 4C). A similar effect was seen in animals treated with a therapeutically relevant dose of sunitinib (Fig. 4C), indicating that tasisulam impaired in vivo neoangiogenesis.

**Tasisulam displayed anti-tumor efficacy in vivo.** The demonstration of in vitro cell cycle/apoptosis effects on cancer cells and in vitro and in vivo anti-angiogenic activities by tasisulam prompted us to explore its activity in tumor xenograft models. Animals received tasisulam at either 25 mg/kg or 50 mg/kg per day by intravenous injection, 5 days on/2 days off, for 2 weeks, or saline control. Tasisulam displayed dose-dependent Calu-6 xenograft anti-tumor efficacy, with a maximal reduction in tumor volume relative to control animals of 77% (Fig. 5A; p <0.001 beginning with day 27), without significant toxicity (determined using standard animal use in scientific research guidelines, including periodic assessment of animal weight and clinical status (for example, no major impact on activity, grooming, no clinical signs of distress that would warrant euthanasia, etc); formal bone marrow assessment in non-tumor bearing animals indicated minimal effects at 200 mg/kg). To confirm cell cycle effects in vivo, IHC was performed for pHH3 (Fig. 5B). Consistent with the results observed in vitro (Figures 1B and Figure 2), tasisulam treatment resulted in G2/M accumulation by pHH3 immunostain (>5 fold), and an increase in mitotic figures in vivo (Fig. 5B). As mentioned above, the strong G2/M accumulation is likely due to the increased number of 4N cells within an untreated, asynchronous Calu-6 cell population. H&E staining of tumor sections also showed increased DNA fragmentation and other apoptosis-associated cellular changes in tasisulam-treated tumors compared to controls (Fig. 5B, bottom panels, arrows). Tasisulam has demonstrated
similar anti-tumor efficacy across a range of in vivo xenografts, including colorectal (HCT-116), melanoma (A-375), gastric (NUGC-3), leukemia (MV-4-11), pancreatic (QGP-1) (Supplemental Figures S3-S4), and multiple tumorgrafts of widely varying histology (data not shown)(24).

To determine if there was a link between anti-tumor activity, indicators of G2/M accumulation/apoptosis, and anti-angiogenic activity in vivo, multiplexed immunofluorescence tissue imaging of tasisulam-and control-treated xenografts was performed, using a panel of cellular markers of tumor-associated vascularization. These, in turn, were used to assess the degree of vascular normalization, characterized by reduced vessel density, increased pericyte coverage of vessels, decreased vessel tortuosity, and decreased tumor hypoxia(19). Each feature was individually quantitated using image processing algorithms to generate a “normalization index” (see Materials and Methods). Tumors were labeled with Hoechst, anti-CD31 to visualize endothelial cells/blood vessels, anti-smooth muscle actin to visualize myofibroblasts/pericytes, and anti-GLUT1 as a surrogate marker for tissue hypoxia(25-26). While GLUT-1 does not directly measure hypoxia, in many xenograft models (including the Calu6 model) it is an accurate surrogate of hypoxia. Examination of control- and tasisulam-treated Calu-6 tumors revealed that tasisulam induced changes in several vascular morphological parameters, consistent with a vascular normalization phenotype. These changes included dose-dependent decreases in vessel density and vessel tortuosity, and trends for an increase in pericyte coverage of vessels, decrease in hypoxia and an overall increased normalization index (Fig. 5C). Similar phenotypic effects were observed in the A-375 melanoma (data not shown).
Because tasisulam and sunitinib modulated the vasculature through apparently distinct mechanisms (Table 1), the activity of these agents, alone or together, was explored in the human renal cell carcinoma mouse xenograft model Caki-1. 10 mg/kg sunitinib displayed no tumor growth delay, while both 25 mg/kg tasisulam and the tasisulam/sunitinib combination showed a modest effect out to ~ day 50. Beyond day 50, the combination displayed a durable, statistically significant tumor growth delay (minimal p<0.01 from day 26 onward) (Fig. 5D) that persisted well beyond treatment cessation, with reinitiation of tumor growth occurring nearly 70 days post dosing.
Discussion

Relatively few cancer therapeutics have anti-tumor activity mediated by both cytotoxic and anti-angiogenic effects. Tasisulam induced tumor cell apoptosis as a result of G2/M accumulation, while simultaneously normalizing tumor vasculature. The G2/M accumulation occurred in a wide range of cancer cell types but not in primary untransformed cell lines, with the effect possibly beginning in S phase prior. A more detailed look at the HCI profiles in Calu-6 and A-375 cells suggested that caspase-3 induction begins in S phase, prior to the completion of DNA replication (Figure 2B). Tasisulam is not a direct inhibitor of protein kinases \textit{in vitro} and its cell cycle effects are phenotypically distinct from those produced by cell cycle inhibitors such as microtubule poisons (e.g., paclitaxel), G2/M kinase inhibitors (e.g., Aurora or Cdk1), or topoisomerase inhibitors (doxorubicin)(6). Because tasisulam had no effect on DNA synthesis (Fig 2B and data not shown), it is possible mitotic effects are exerted through interaction with a novel target protein(s) prior to the G2 checkpoint, a hypothesis supported by the delay in cell cycle progression noted in double thymidine blocked Calu-6 cells treated with tasisulam immediately after synchronization (Figure S2).

Angiogenesis is a biological process retained by tumors that is critical for tumor growth. While the field of anti-angiogenic therapy was established largely on the basis of Folkman’s hypothesis to “starve” tumors of their blood supply(27-28), the emerging concept of “vascular normalization” has gained momentum(19). Normalization is a process by which a dense, highly tortuous and poorly delivering tumor vasculature is converted to one that is more sparse, yet very stable, mature (highly invested in pericytes), organized and functional(19). Anti-angiogenic
agents such as sunitinib induce a transient normalization window lasting ten days following the initial dose(29), with a net effect of an improvement in tumor blood flow, resulting in increased delivery of oxygen, nutrients, and theoretically, therapeutics. In the Calu-6 xenograft model tasisulam induced morphological features of vascular normalization, including increased pericyte coverage (indicative of vessel maturation) and decreased hypoxia (indicative of improved vessel functionality). Additional studies are required to confirm that tasisulam is capable of maintaining a stable vascular normalization phenotype.

It is unclear if the pro-apoptotic and anti-angiogenic effects of tasisulam are mediated by a singular target. The broad pro-apoptotic activity of tasisulam in vitro, and the finding that the dose-limiting toxicity in human clinical studies is bone marrow suppression(14) suggests a cellular target shared by hematopoietic stem cells and transformed cell lines. However, preclinical in vivo studies have also demonstrated that an endothelial cell-dependent anti-angiogenic response plays an important role in the overall anti-tumor activity of tasisulam. Further, tasisulam HCI fingerprints were clearly distinct from previously published HCI fingerprints of anti-mitotic agents that exhibit G2 phase-specific characteristics such as significantly increased nuclear area in conjunction with 4N DNA content and only slightly changed variation in Hoechst DNA staining(6-7). Altogether, these findings result in a unique phenotypic MOA for tasisulam, characterized by cell cycle-dependent apoptosis in a broad range of cancer cell lines, and anti-angiogenic effects in the absence of endothelial cell apoptosis or G2/M accumulation.

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References


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Table 1 — Maximum change in impedance following tasisulam treatment of ECFCs. Endothelial cells were treated with 50 ng/mL ligand and dZiec (extracellular impedance of the monolayer) was recorded every 2 seconds for 15 minutes. Maximum change (ohms) results are shown. VEGF causes a very marked relaxation of the junctions between cells and hence less resistance to electrical conductivity, expressed here as a decrease in impedance. EGF and FGF cause a slight increase of the cell to cell pressure and hence a slight increase in impedance. The untreated control cells here tend to relax slightly during this incubation, resulting in a slightly decreased impedance.
Figure and Table Legends

Figure 1 — Tasisulam effects in vitro. A; Calu-6 and A-375 cell sensitivity to tasisulam (structure shown at top). Cells were treated with tasisulam for two cell doublings (from 200 uM to ~200 nM; n = 3), stained with Hoechst dye and live cell number was determined using HCl as described under Materials and Methods. B; tasisulam treatment leads to G2/M accumulation and subsequent apoptosis in Calu-6 and A-375 cells, but not in HeLa cells, as determined by flow cytometry and Western blotting (inset). Replicate control and 25 µM treated samples were assayed by Western blotting for cleaved caspase-9 and cleaved PARP (C, cleaved; T, total).

Figure 2 — High content imaging subpopulation analysis of tasisulam in vitro cell cycle effects; A; tasisulam treatment leads to an accumulation of mitotic cells as visualized using HCl. B; unsupervised K-means clustering of HCl parameters shows induced caspase-3-dependent apoptosis in cells with >2N DNA content. Horizontal row correspond to individual cells, and vertical rows correspond to measured cellular parameters; total, average and variation in DNA intensity; nuclear area; pHH3, activated caspase 3. % of cells in each cell cycle stage are noted. The right hand scale corresponds to the number of standard deviations a given measurement is from the average reading for that parameter in a population of untreated asynchronous cells. See Materials and Methods for details.

Figure 3 — In vitro effects of tasisulam on endothelial and adipose-derived stem cells . A; tasisulam treatment of ECFCs caused a significant inhibition of proliferation (Ki67) over 48
hours, but did not induce apoptosis (TUNEL). B; tasisulam treatment of ECFC/ADSC co-cultures inhibits growth factor-stimulated cord formation. C; tasisulam displayed nearly equipotent inhibition of basal, VEGF, FGF, or EGF-driven cord formation assays with negligible cytotoxicity on the ADSC feeder layer.

**Figure 4 — Effects of tasisulam on blood vessel formation in vivo.** A; Matrigel plugs co-implanted (n = 7) with ECFCs and ADSCs form a functional vasculature after 6 days, which was visualized with Hoechst (all cell nuclei), anti-human CD31 (endothelial cells) and anti-SMA (myofibroblasts). Both sunitinib and tasisulam effects as assessed by hemoglobin content (B) and histological analysis of CD31-positive vessel area (C). ** indicates p < 0.01.

**Figure 5 — Tasisulam displays dose dependent anti-tumor activity, induces apoptosis, and normalizes tumor-associated vasculature in the Calu-6 non small cell lung xenograft model.** A; briefly, 8 mice/group received tasisulam at a dose of 25 mg/kg (closed squares) or 50 mg/kg (closed triangles) daily by IV injection for two weeks, on a five day on/two day off schedule (denoted by the horizontal black lines), or vehicle control (closed squares). The bars depict the standard error. B; paraffin-embedded tumor sections from control or tasisulam-treated mice were examined by immunohistochemistry for expression of the G2/M cell cycle marker pHH3 (top rank) or H&E to detect nuclear fragmentation and other apoptosis-related cellular changes (bottom rank, arrows). Bar equals 100 microns. C; tasisulam-treated tumor samples displayed morphological features of vascular normalization. At top, tumor sections from vehicle- or tasisulam-treated tumors were examined by immunofluorescence to detect endothelial cells (CD31; green), myofibroblasts/pericytes (SMA; red), regions of hypoxia (GLUT1; yellow), or all
cell nuclei (Hoechst; blue). At bottom, multiplexed quantitative tissue imaging was performed by automated microscopy as described in Materials and Methods to determine mean vessel density (MVD), and other indices used to assess tumor vascular normalization. Bars depict the standard error of 9 replicates and differences between vehicle- and tasisulam-treated sections that were significant to a p<0.05 are shown at **. D; tasisulam in combination with sunitinib induced significant tumor growth suppression in the Caki-1 renal cell carcinoma xenograft model (n = 8 animals/group). Tasisulam (25 mg/kg dosed as in Figure 5, open squares), sunitinib (10 mg/kg, BID x 14, open triangles) or both in combination (open circles) were evaluated for anti-tumor activity vs. vehicle controls (closed squares) as described for Panel A and in Materials and Methods. Dosing period is denoted by the dashed line (sunitinib) and solid line (tasisulam).
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Tasisulam Sodium, an Anti-tumor Agent that Inhibits Mitotic Progression and Induces Vascular Normalization

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