Tasquinimod is an Allosteric Modulator of HDAC4 Survival Signaling within the Compromised Cancer Microenvironment


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Abstract

Tasquinimod is an orally active anti-angiogenic drug that is currently in Phase III clinical trials for the treatment of castration resistant prostate cancer. However, the target of this drug has remained unclear. In this study we applied diverse strategies to identify the histone deacetylase HDAC4 as a target for the anti-angiogenic activity of tasquinimod. Our comprehensive analysis revealed allosteric binding (Kd 10-30 nM) to the regulatory Zn^{2+} binding domain of HDAC4 which locks the protein in a conformation preventing HDAC4/N-CoR/HDAC3 complex formation. This binding inhibited co-localization of N-CoR/HDAC3, thereby inhibiting deacetylation of histones and HDAC4 client transcription factors, such as HIF-1α, which are bound at promoter/enhancers where epigenetic reprogramming is required for cancer cell survival and angiogenic response. Through this mechanism, tasquinimod is effective as a monotherapeutic agent against human prostate, breast, bladder, and colon tumor xenografts, where its efficacy could be further enhanced in combination with a targeted thapsigargin prodrug (G202) that selectively kills tumor endothelial cells. Together, our findings define a mechanism of action of tasquinimod and offer a perspective on how its clinical activity might be leveraged in combination with other drugs that target the tumor microenvironment.
Introduction

Oral dosing with tasquinimod (TasQ), Figure 1A, results in a doubling of progression free survival in patients with metastatic castration resistant prostate cancer (CRPC) (1). Based upon these results, Phase III registration trials for metastatic CRPC are ongoing. Pre-clinical studies demonstrate that TasQ suppresses reciprocal cross-talk between cancer and tumor infiltrating host cells like endothelial cells, myeloid derived suppressor cells (MDSC), and macrophages (2-7). Such suppression inhibits tumor angiogenesis needed for cancer cell survival as documented by a variety of assays including endothelial capillary tube formation, aortic ring assay, chorioallantoic membrane assay, real-time tumor blood flow and pO2 measurements, tumor blood vessel density, and tumor hypoxic and apoptotic fractions (2-6). This suppression is significant because cancers outgrow their blood supply, which results in the tumor microenvironment becoming acidic, hypoxic, and low in nutrients (8). Continued growth in such a stressful microenvironment requires cancer and tumor infiltrating host cells to initiate epigenetic reprogramming leading to an “angiogenic switch” which involves down regulation of inhibitory genes like thrombospondin-1 (TSP-1) coupled with the up-regulation of stimulatory genes like Hypoxia Induced Factor-1α (i.e., HIF-1α) (9).

TasQ’s suppression of this epigenetic angiogenic switch (5) provides a rationale for its efficacy in pre-clinical models against primary cancer as well as its inhibition of metastasis when used as monotherapy (3,10) and explains why it enhances efficacy when combined with androgen ablation (4), taxane-based chemotherapy (4), or fractionated radiation (6), therapies known to induce a stressful tumor microenvironment. The focus of this report is the identification of TasQ’s molecular targets in order to use this information to rationally design combinational approaches to enhance therapeutic benefit in the clinic.

Materials and Methods:

Reagents: See Supplemental File 1 for source of reagents.
Recombinant wild type and C669/H675 mutated HDAC4 proteins: pcDNA vector containing human N-terminal flag-tag full length wild type HDAC4 was obtained from Addgene (Cambridge, MA, Cat # 13821).
HDAC4 protein, pcDNA vector containing human N-terminal flag-tag full length wild type HDAC4 was mutated using the Quick Change Site Directed Mutagenesis kit from Agilent Technologies (Santa Clara CA, Cat # 200523) according to manufacturer’s protocol. HEK-292T cells were transfected with these vectors and 48 hrs latter, cells washed with phosphatase inhibitor from Roche (Indianapolis IN, Cat# 0490684500); lysed in 50 mM Tris HCl pH 7.4 with 150 mM NaCl/1% Triton/ and protease inhibitor cocktail tablet from Roche (Cat# 11836170001). The resulting lysate was incubated overnight at 4°C with a 50% suspension of flag agarose from Sigma-Aldrich (Cat# A4596; lysate-agarose mixture centrifuged, washed twice, slurry transferred to chromatography column from BioRad (Hercules CA, Cat# 7311550); column washed multiple times without Triton; protein eluted with 100 µg/ml 3X flag peptide from Sigma-Aldrich (Cat# F4799); dialyzed in PBS and protein concentrated using ultra-filtration centrifugal filter concentrator [Amicon/Millipore (Cat # UFC203024PL)].

**3D-endothelial sprouting assay** was performed using a minimum of 5 replicate wells per drug dose per experiment, repeated 3 independent times as described previously (11) using HUVECs [Lonza Walkersville Inc (Walkersville, MD)] with modification that 20ul of 10X concentrated DMEM/10%FCS media conditioned by confluent human primary lung fibroblasts (Lonza) diluted to 200ul of growth factor supplemented EGM-2 media (Lonza) was used instead of fibroblast co-culture.

**Cancer models:** The source, history, and characteristics of the normal and malignant human epithelial lines used, as well as cell culture conditions for their *in vitro* maintenance and the *in vivo* protocol for their xenografting into immune-deficient nude mice are as described previously (3, 12, 13). All lines were mycoplasma negative using the MycoSensor PCR Assay kit (Agilent Technologies) and genetically authenticated within the last 6 months using STR profiling performed by the Johns Hopkins Genetic Resource Core Facility. *In vitro* growth curves were determined as described (12, 13). Animal studies were performed according to animal protocol MO09M434 approved by Johns Hopkins Animal Care and Use Committee. Daily orally TasQ dosing via the drinking water, tumor volume measurements, tumor blood vessel density determination, and tissue oxygenation expressed as mmHg were as described previously (3, 4). These experiments were repeated at 3 independent times for each xenograft.
**Biochemical assays:** Co-IP of whole cell lysates or nuclear fractions was via a kit from Active Motif (Nuclear Complex Co-IP kit cat# 54001) according to manufacturer’s protocol. For co-IP with recombinant full length flag-tagged N-CoR (i.e., amino-acid 1-2440) from Abcam (Cat # ab82239), 100ng of the protein was incubated with 20 µl anti-flag M2 affinity gel beads from Sigma-Aldrich (Cat# A2220) for 2 hr and then the mixture centrifuged, pelleted beads washed 3 times, resuspended, and 20ng of recombinant GST-tagged HDAC4 (i.e., amino acid 627-1085) obtained from BPS Bioscience (San Diego CA, Cat # 50004) added to the mixture which was incubated overnight at 4°C. The mixture was centrifuged, pellet washed 3 times, and then IB with anti-GST antibody. For co-IP with recombinant GST-tagged HDAC4, 20ng of the protein in 500 µl of pH8 buffer (i.e.,50mM Tris, 150mM NaCl, 1mM dithiothreitol , 10 µM Zn, 1% Triton-X100) was incubated with 20 µl Glutathione Sepharose 4B Beads from GE Life Sciences (Cat # 17-0756-01) for 2 hr and then the mixture centrifuged, pelleted beads washed 3 times, resuspended in pH 8 buffer, and 100ng of recombinant flag-tagged-N-CoR added. The mixture was incubated overnight at 4°C and then the mixture centrifuged, pellet washed 3 times, and then sample was IB with anti-flag antibody.

For cell based co-IP, cells transiently transfected with pcDNA vector containing either N-terminal flag-tag full length wild type HDAC4 from Addgene (Cat # 13821) or HDAC3 from Addgene (Cat # 13819) were harvested after 48hrs, anti-flag IP performed as described for isolation of recombinant proteins. IP was then IB with indicated antibody performed. The biochemical and cell based co-IP experiment were repeated independently at least 3 times with representative gels presenting the results.

For detection of acetylated HIF-1α, cells were maintained under hypoxic conditions (i.e., 2% O₂) for 24 hrs and then lysed in RIPA buffer containing added Roche protease and phosphatase inhibitors. Lysate was centrifuged, supernatant mixed with 4 µg acetylated lysine antibody and 40 µl Protein A agarose (Santa Cruz, Cat # sc-2001), incubated overnight at 4°C, mixture centrifuged and pelleted beads washed 4 times with RIPA buffer and then IB with anti-HIF-1α antibody performed. These experiments were repeated 3 independent times with a representative gel presented.

Total HDAC and isotype specific HDAC enzymatic activity was assayed on a per cell basis using the appropriate substrates as described previously (14, 15). Recombinant
human HDAC isotypes were obtained commercial as follows: HDAC1 (Millipore, Temecula, CA Cat#14-838); HDAC3 (BioMol/Enzo Life Sciences cat#SE-507); HDAC4 (Millipore cat#14-828); HDAC6 (BioMol/Enzo cat#SE-508); and HDAC8 (Millipore cat#14-609). These experiments were repeated a minimum of 3 independent times with 5 replicates per time point.

**Surface plasmon resonance:** SPR analysis was carried out with the Biacore 3000 system (GE Healthcare) as described previously (16). Sensor chips, amine coupling kit, immobilization and running buffers, and regeneration solutions were as described previously (16). Binding to Tasquinimod was determined for human full length N-terminal GST-tagged HDAC4 (Abnova cat#H00009759-P01). GST-tagged HDAC4 was immobilized onto a CM5 chip through an amine-linkage. This chip was used to determine binding of full length human N-CoR (Abcam Cat # ab82239). These experiments were repeated 3 independent times.

**Computer docking modeling:** The active vs. and inactive conformations of HDAC4 were overlaid using the Ca atoms of crystal structures previous reported (17). Protein surfaces generated and systematically examined for plausible binding pockets. The revealed binding pockets were explored by iterative docking, during which binding poses were refined by interplay of manual docking, Glide runs, and visual inspection of the modeled ligand-protein complex. More than twenty docking poses were generated. The final model selected based on the conformational energy for the ligand as well as the computed ligand-protein energy. The computational simulation was performed using Schrödinger's molecular modeling environment Maestro (www.schrodinger.com).

**HDAC4 knock-down and restoration:** Stable shRNA HDAC4 knock-down cells were generated using pLK0.1 lentiviral vector from Open Biosystems (Thermo Scientific) containing the following specific shRNA sequences: shRNA1 (CCGGCGACTCATCTTTGATGTGCTTATTCTCGAGAATAAGCTACAAGATGAGTCGTTTTT); shRNA2 (CCGGCGTGGGTTTCAACGTCAACATCTCGAGATGTTGACGTTGAAACCCACGTTTTT); shRNA3 (CCGGGCAGCTCAAGAACAAGGAGAACTCGAGTCTCCTTGTTCTTGA GCTGCTTTTT) and the non-specific shRNA sequence was (CCTAAGGTTAAGTCGCTCGCTCGAGCGAGGGCGACTTAACCTAGGTTTTT). To generate HDAC4 restoration expression vector resistant to shRNA2 knockdown, two nucleotides at
positions 9 and 12 in the sHRNA2 targeting region were changed to making it resistant to the HDAC4 shRNA without altering the amino acid sequence of wild type protein.

**HIF-1α Transcriptional Assays:** PC-3 human prostate cancer cells stably integrated with EGFP gene under a promoter containing 5 copies of the HIF-1α driven HRE of the VEGF gene as described previously (18) were exposed to different doses of TasQ under both normoxic and hypoxic conditions for 24 hrs and cell lysates analyzed by IB for GFP from 3 independent experiments. HUVECs exposed to different doses of TasQ under both normoxic and hypoxic conditions were co-transfected with 1 μg of the p2.1 expression plasmid containing firefly luciferase under a HIF-1α driven HRE of the enolase gene and 0.1 μg of pTK-Renilla control plasmid containing Renilla luciferase under a TK driven-promote using Fugene6 from Roche. The p2.1 and pTK-Renilla plasmids were a kind gift from Dr. Gregg Semenza (Johns Hopkins School of Medicine, Baltimore, MD) and have been characterized previously (19). After 6 hours, HUVEC cell lysates were analyzed with a Dual Luciferase Reporter Assay kit from Promega (Madison WI, Cat# 1910). These experiments were repeated 3 independent times with representative data reported. Human HIF-Regulated cDNA Plate Array from Signosis Inc (Sunnyvale CA, Cat# AP-0111) was performed as per instructions from the manufacturer. These experiments were repeated 3 independent times with representative data reported.

**Gene Expression Profiling:** Normal prostate samples from organ donors (n=23) were obtained as described previously (20). Castration resistant prostate cancer specimens (n=18) were autopsy specimens from 6 patients who died from PCa, as previously reported (21). The use of autopsy specimens for molecular analysis was approved by the Johns Hopkins Medicine Institutional Review Boards. Gene expression profiling was performed 3 independent times according to the guidelines provided by the Agilent Whole Genome Expression Microarray system (Agilent Technologies, Santa Clara CA), using 2-color design and the results normalized to a reference sample derived from benign prostatic hyperplasia using the standard locally weighted least squares regression (LOWESS) procedure as described previously (22).

**Statistics:** All of the values reported are presented as means ± SE of representative data generated from one of a minimum of 3 independent experiments in which there
were a minimum of 5 replicates per data point. Statistical analysis was performed by a one-way ANOVA with the Newman-Keuls test for multiple comparisons.

Results:

**Tasquinimod inhibition of the adaptive response of cancer and endothelial cells is enhanced in a stressful microenvironment**

Human cancer cells are severely stressed when xenografted as minced tissue organoids within Matrigel because these plugs initially lack a blood supply. Thus, cancer organoids must initiate an angiogenic switch, both to survive in this nutrient poor/hypoxic microenvironment (i.e., pO$_2$ in the starting Matrigel plug is 10 +/-2 mmHg vs. 40+/−9mmHg in fully oxygenated normal tissue) and to stimulate host angiogenic response. If a daily oral dose of TasQ (i.e., 10 mg/kg/day) which maintains serum drug concentration at 0.5-1µM (3) is initiated upon inoculation of Matrigel containing 15 -20 mgs of CWR22-RH human CRPC tissue suspension into castrated male nude mice, 80% of animals (n=10) are cured and tumor growth in the remaining 20% is profoundly inhibited (p<0.05), Figure 1B. In contrast, 100% of untreated animals (n=10) develop an adequate tumor blood vessel density and the cancers grow eventually killing the host. If animals are initiated on daily TasQ after tumors grow to a starting size of 0.250cc, the cancers do not regress, but subsequent growth is profoundly inhibited (p<0.05), Figure 1B. Growth inhibition is associated with 50% lower (p<0.05) tumor blood vessel density (2.6 +/- 1.4% vs. 4.8+/− 0.9% of tumor area in TasQ treated vs. untreated, respectively).

To clarify why TasQ is maximally effective when the tumor microenvironment is most compromised, in vitro experiments were utilized. Under standard culture conditions (5% CO$_2$/21%O$_2$/pH7.2 media), human prostate cancer (PC) cells (LNCaP, LAPC-4, CWR-22Rv1) and human endothelial (HUVECs) cells grow exponentially with doubling times (DT) of ~2 days. Under these standard conditions, TasQ inhibits growth (p<0.05) with IC$_{50}$ values of ~ 50µM for cancer and endothelial cells, values 50-100 fold higher than therapeutic blood levels, Figure 1C. This raises the issue of whether TasQ’s potency and/or efficacy for growth inhibition and/or cell death are increased in a stressful microenvironment. To evaluate this, LNCaPs and HUVECs were treated with 1µM TasQ (a dose that had no effect under standard in vitro conditions, but was therapeutic in vivo) in low glucose/ high CO$_2$/ low O$_2$/ acidic media to mimic the stressful
microenvironment within cancer sites. Under these compromised conditions, untreated LNCaPs and HUVECs survive, but slow their growth (DT increases from 42+/−5 to 144+/−12hrs for LNCaP and 47+/−7 to 64+/−8hrs for HUVEC; p<0.05). Addition of 1µM TasQ to such a compromised condition further decreases DT of HUVECs (88+/−9 hrs; p<0.05). In contrast, addition of 1µM TasQ to such a compromised conditions results in 52+/−9% (p<0.05) of LNCaPs dying within one week.

To evaluate whether TasQ also inhibits the functional response of endothelial cells, 3D- in vitro angiogenic sprouting/ tube formation assays were utilized. When HUVECs grown on micro-carrier beads are embedded in fibrin gels with media conditioned by normal lung fibroblasts to provide angiogenic factors (e.g., VEGF), typically secreted by cancer cells in a hypoxic microenvironment, cells “sprout” producing canalized neovessel tubes within one week, Figure 1D. If TasQ is added at culture initiation, sprouting is suppressed (p<0.05) with an IC50 value of 0.5µM, Figure 1D. In contrast, if HUVECs are allowed to pre-form 3D-neovessels and then TasQ is added, no regression occurs even at 50 µM. These results document that TasQ’s inhibition of cancer cells survival and endothelial angiogenic responses are profoundly enhanced by a stressful microenvironment.

Tasquinimod suppresses hypoxia induced decrease in histone acetylation without lowering HDAC expression or directly inhibiting HDAC activity

Survival in a stressful hypoxic microenvironment requires epigenetic reprogramming in which global transcription is decreased via decreased global histone acetylation, coupled with increased transcription of a select group of survival genes (23-26). Decreased global histone acetylation stimulated by hypoxia (2% O2) was confirmed in human PC lines and TasQ inhibits this decrease, Figure 2A. Trichostatin A (TSA) a known pan-class I/II HDAC inhibitor (27) likewise inhibits decrease in lysine 9/19 acetylated H3-histone, Figure 2A. These results suggest TasQ might be an HDAC inhibitor.

There are multiple isotypes of class I (HDAC1, 2, 3, 8), class IIa (HDAC4, 5, 7, 9) and IIb HDACs (HDAC6) (27). Normal human prostate epithelial cells (957E/hTERT) express all of the class I and IIb (HDAC6) isotypes, but HDAC4 is the only class IIa
isotype consistently detectable in these cells, Figure 2B. The lack of detectable expression of the other class Ila isotypes by normal prostate epithelial cells is not due to the quality of the antibodies used for detection, since as a positive control HDAC5 is detected in CWR22-Rv1 cell, Figure 2C, HDAC7 in MCF-7 (data not shown), and HDAC9 is detected in LNCaP cells, Figure 2B, using the appropriate antibodies. Likewise, human prostate cancer lines characteristically express all of the class I isotypes and HDAC4, with most also expressing HDAC6, but only CWR22-Rv1 cells express HDAC5 and only LNCaP cells express HDAC9, respectively, Figure 2B&C. HUVEC cells express HDAC2,3&8 of the class I and HDAC4 and 7 of the class Ila and HDAC6 of the class IIb isotypes, Figure 2D&E. Interestingly, human prostate cancer lines express 3-5 fold higher total HDAC activity than endothelial cells, Supplemental Figure 1. At 1µM, TasQ treatment does not lower the expression of any isotypes expressed by human PC lines, Figure 2C&D, or HUVECs, Figure 2D&E. TasQ even at 100µM does not inhibit total HDAC enzymatic activity in HUVECs or a series of human PC lines (LNCaP, CWR22-Rv1, PC3, or PAC-MetUT1), Supplemental Figure 1. This inability was confirmed using recombinant human proteins of type I (HDAC1, 3, and 8), type Ila (HDAC4), and type IIb (HDAC6) isotypes in biochemical assays.

**Tasquinimod binds allosterically within the regulatory Zinc domain of HDAC4**

Previous studies document an enhanced level of HDAC4 protein within PC nuclei in CRPC patients and that suppression of HDAC4 expression causes in vitro growth inhibition of a variety of solid malignancies, including PC, particularly during hypoxic stress (28-30). Gene expression array data document that HDAC4 is overexpressed by > 3 fold (p<0.05) in metastatic sites in CRPC patients (n=18) compared to normal prostate from organ donors (n=23), Supplement Figure 2A. PC lines express >5 fold higher levels of HDAC4 protein than normal prostate epithelial cells, the majority of which is in the nucleus, Supplement Figure 2B. HDAC4 is 1084 amino-acids (AA) with a nuclear localization signal (NLS) at AA244-279, a nuclear export signal (NES) at AA1051-1084, and three serines at AA246, 467, and 632 whose phosphorylation is required for binding 14-3-3, which restricts localization to the cytoplasm, Figure 3A (31). While HDAC4 lacks intrinsic DNA-binding activity, sequences within either the N-
terminal adaptor domain (AA1-648) or the C-terminal HDAC domain (AA648-1051) selectively bind a subset of DNA anchoring transcription factors as part of either repressive or stimulatory complexes at specific promoter/enhancers (31).

Within the HDAC domain, there is a zinc-bound “catalytic domain” involving AA802-950, however HDAC4 is enzymatically inactive against classic acetylated peptide substrates (14, 15). Also within the HDAC domain is a regulatory zinc-binding domain (ZRD) (AA648-751), Figure 3A. Crystallography studies document that the ZRD has two alternative conformations (17). When Zn$^{2+}$ is coordinated by C667, C669, H675, and C751, the ZRD is in an active conformation allowing binding to the transcriptional co-repressor, N-CoR/HDAC3 complex via RD3 domain of N-CoR (14, 17). In the HDAC4/N-CoR/HDAC3 complex, HDAC3 is active and deacetylates proteins tethered to the complex via binding to HDAC4 (15). Alternatively, when H665, C667, H678, and C751 are coordinated with the structural Zn$^{2+}$, the ZRD is in an inactive conformation unable to bind N-CoR/HDAC3 (17).

Using published crystal structures (17), tasquinimod docking was performed based upon energy miminization modeling. This modeling identified a specific allosteric binding site for TasQ within the inactive conformation of the ZRD, distinct from the catalytic domain, Figure 3B. Critically, specific amino acid interactions involved in this binding are only possible in inactive, not active, conformation of the ZRD, Figure 3C. TasQ was immobilized onto a Biacore chip via an amino linker and binding of human recombinant full length HDAC4 protein was determined using surface plasmon resonance (SPR). These studies demonstrated that TasQ binds HDAC4 with a Kd of 10-30 nM, Figure 3D. Additional SPR studies determined that TasQ binds to human recombinant full length HDAC4 protein immobilized onto a Biacore chip in a 1:1 manner. SPR analysis documented that recombinant human HDAC4 in which the C669 and H675 in the ZRD are mutated to alanine preventing formation of the active conformation (17) retains high affinity binding for TasQ, Supplemental Figure 3A.

**Tasquinimod phenocopies HDAC4 knock-down in endothelial and prostate cancer cells**
A series of specific and non-specific HDAC4 shRNA lentiviral constructs were tested for their ability to down regulate HDAC4 in LNCaP cells, **Supplemental Figure 3B**. The best construct (shRNA2) was then used to transduce LNCaP and HUVEC cells. In multiple transductions, cells which grew out following such shRNA2 transduction had only ~ 60% HDAC4 knock down, **Figure 4**. Even as little as a 60% reduction in HDAC4 completely inhibits HUVECs from sprouting in 3D assays, **Figure 4B** which is an identical response induced in wild type HUVECs by 1µM TasQ, **Figure 1D**. With regard to LNCaP cells, such limited HDAC4 reduction had little effect upon growth rate under these standard non-stressed conditions, **Figure 4C**. In contrast, however, under even the limited stress of exposure to 2% O₂, without increasing CO₂ and decreasing pH, 60% knock down of HDAC4 decreases survival of LNCaPs, which is a response identical to treatment with 1µM TasQ under such limited stress, **Figure 4C-lower panel**.

TasQ also phenocopies the response to HDAC4 knock-down in vivo. Similar to the situation where PCs are non-tumorigenic when TasQ treatment is initiated at inoculation, **Figure 1B**, HDAC4 shRNA2 knock-down LNCaPs are also non-tumorigenic in mice, **Supplemental Figure 3C&D**. The specificity of this response was documented by the fact that restoration of HDAC4 expression in shRNA2 knock down LNCaPs, **Figure 4D**, restores their tumorigenicity, **Figure 4E** and **Supplemental figure 3E**.

**Tasquinimod blocks the formation of HDAC4/N-CoR/HDAC3 complexes**

SPR analysis documents that full length HDAC4 protein immobilized onto a Biacore chip binds human recombinant full length N-CoR with a Kd of 1nM and that TasQ inhibits this binding in a dose-dependent manner, **Figure 5A**. In co-IP/IB assays, HDAC4/N-CoR complex formation is Zn⁺²-dependent because it is blocked by EDTA, **Figure 5B**. TasQ also blocks such complex formation with an IC₅₀ value of < 50nM, **Figure 5C**. To evaluate this blockade in a cell based system, HEK-293T human embryonic kidney cells were transfected with HDAC4. Co-IP/IB analyses documented that TasQ inhibits N-CoR/HDAC3 binding to HDAC4 with an IC₅₀ <1µM in normoxia (20% O₂), **Figure 5D &E**, and hypoxia (2% O₂), **Figure 5E**. To evaluate whether these results are unique to HEK-293Ts, LNCaPs were transfected with HDAC3. Co-IP/IB
analyses document that TasQ inhibits the binding of HDAC4 to N-CoR/HDAC3 complexes within PCs with an IC$_{50}$ of <1µM, Figure 5F.

**Tasquinimod phenocopies loss of HIF-1α transcriptional stress response induced by HDAC4 knock-down**

TasQ should inhibit HDAC4 client protein deacetylation via blocking co-localization of N-CoR associated HDAC3 deacetylase activity. One client protein is HIF-1α, which binds via its ID domain (AA603-785) to the catalytic domain (AA802-950) of HDAC4, Figure 3A (30, 32-34). Due to cessation of hydroxylation of a series of prolines and an asparagine, Hif-1α accumulates in the nucleus during hypoxia, where it is acetylated by PCAF on a series of lysines between AA10-21 and on AA674 (30, 35, 36). The N-terminal acetylated lysines are within DNA binding domain of HIF-1α (37) and are deacetylated by an HDAC4 dependent mechanism (30). Such HDAC4 binding to the ID of HIF-1α also competitively inhibits binding of factor inhibiting HIF-1α (FIH-1) and instead facilitates binding of p300HAT and dimerization with HIF-1β to form transcriptionally active HIF-1 at Hypoxia Response Elements (HREs) within promoter/enhancers of hypoxic responsive genes (19, 34). shRNA2 knock-down of HDAC4 prevents deacetylation of N-terminal lysines of HIF-1α enhancing its degradation, inhibiting its transcriptional activity, and decreasing cell survival under stressful conditions (30, 33).

To confirm these observations, HIF-1α was compared between control, HDAC4 shRNA2 knock-down, and knock down restored LNCaPs. As expected, HDAC4 knock-down decreases HIF-1α and this is reversed when HDAC4 is restored, Figure 4D. If TasQ inhibits HDAC4/N-CoR/HDAC3 dependent N-terminal lysine deacetylation of HIF-1α, then it should decrease HIF-1α which suppresses HIF-1 dependent transcription. When HEK-293T cells were transfected with HDAC4, HIF-1α is decreased by TasQ, Figure 6A. To determine if this is due to inhibition of HIF-1α /HDAC4 complex formation, LNCaPs transfected with HDAC4 were treated with TasQ under both normoxic (21% O$_2$) and hypoxic (2% O$_2$) conditions. IB of total cell extracts demonstrated that hypoxia increases HIF-1α coupled with decreasing its mobility, Figure 6B. Decreased HIF-1α mobility is due to phosphorylation which enhances its
transcriptional activity (38). Co-IP/IB of nuclear extracts documents that 1µM TasQ decreases HIF-1α/HDAC4 complex formation by >50% under both normoxic and hypoxic conditions, Figure 6B. The inhibitory effect of TasQ is not dependent upon over expression of HDAC4, because it suppresses HIF-1α by >50% under hypoxic conditions in nuclear extracts from non-transfected LNCaPs, Figure 6C. Nuclear extracts from non-transfected LNCaPs were co-IP with an anti-acetyl lysine antibody and the IP analyzed by IB with anti-HIF-1α antibody. Even though TasQ decreases nuclear HIF-1α by > two fold in the LNCaPs, Figure 6C, acetylated HIF-1α increases by 40%, Figure 6D. Thus, TasQ inhibits the nuclear fraction of N-terminal HIF-1α lysines de-acetylated under hypoxic conditions by > 3 fold.

Based upon its inhibition of HIF-1α de-acetylation, TasQ should inhibit HIF-1α dependent transcription. TasQ’s dose-response inhibition of the adaptive stress response of PC-3 human prostate cancer cells that have stably integrated a HIF-1α dependent HRE-driven green fluorescent protein (GFP) construct was evaluated to test this prediction. Like LNCaP, PC-3 predominantly has nuclear HDAC4, Supplemental Figure 2B, and HIF-1α even under normoxic conditions. In both conditions, PC-3s sense “stress” as documented by their expression of GFP. While this stress response is much greater (i.e., >100 fold increase in GFP production) under hypoxic conditions, TasQ inhibits GFP expression under both conditions, Figure 6E.

To further evaluate the generality of TasQ’s ability to inhibit HIF-1α driven survival signaling in prostate cancer cells, lactic dehydrogenase-A (LDH-A) protein expression was determined in LNCaP cancer cells exposed to 1µM TasQ under both normoxic and hypoxic conditions. These results document that expression of LDH-A, a known HIF-1α transcriptional target (19), is increased by hypoxia and that TasQ inhibits such up-regulation, Supplemental Figure 4A.

To evaluate whether TasQ’s inhibition of the HIF-1α transcriptional response also occurs in endothelial cells, HUVECs were co-transfected with a HIF-1α dependent HRE-driven firefly luciferase vector plus a CMV-driven renilla luciferase control vector and the cells exposed to normoxia vs. hypoxia. These results demonstrate that hypoxia induced HIF-1α dependent transcription in HUVECs is completely inhibited by 1µM TasQ, Supplemental Figure 4B. Likewise, a commercial HIF-1α dependent hypoxia cDNA
microarray documented that 1µM tasquinimod prevents HUVECs from adaptively upregulating transcription of stress genes under hypoxia, Figure 6F.

**Tasquinimod’s efficacy against solid malignancies and its enhancement by a combinational approach**

The previous results identify HDAC4 as a molecular target for TasQ’s suppression of epigenetic signaling required for survival in a stressful microenvironment by both cancer and endothelial cells. Such suppression is via TasQ’s allosteric binding to the ZRD of HDAC4 locking it in an inactive conformation in both cancer and endothelial cells. TasQ’s allosteric binding prevents deacetylase activity of N-CoR/HDAC3 from co-localizing with HDAC4/client transcription factors bound at the promoter/enhancers of genes, Figure 7A. HDAC4 binding with the majority of its client DNA bound-transcription factors results in decreased histone acetylation due to co-localization of HDAC3 repressing their transcription (31), Figure 7A. This explains how TasQ inhibits hypoxia stimulated global histone de-acetylation, Figure 1D. TasQ binding to HDAC4 also prevents HIF-1α/HDAC4/N-CoR/HDAC3 complex formation. This is critical because the stability and thus the transcriptional activity of HIF-1α needed for both cancer cells survival and endothelial cells angiogenesis is inhibited when its N-terminal lysines are acetylated and HDAC4 is required for their full de-acetylation, Figure 7A.

Based upon this HDAC4 allosteric binding, TasQ should be effective when used as monotherapy against multiple types of solid malignancies because ablating HDAC4 signaling not only inhibits endothelial tumor angiogenesis, but also growth/survival of a variety of human organ-specific cancers (29, 30, 39, 40). To evaluate this possibility, a series of human prostate, breast, bladder, and colon cancer xenografts were inoculated into appropriate immune-suppressed hosts and cancers allowed to reach 0.250cc before daily oral TasQ (10mg/kg/d) was initiated. As predicted, TasQ monotherapy inhibited (p< 0.05) subsequent tumor growth by a least 50% in all of these cancer types, Figure 7B.

Because TasQ’s potency and efficacy for inhibition of cancer cell survival and endothelial angiogenic response are enhanced in a stressful tumor microenvironment,
combining TasQ with agents which increase tumor microenvironmental stress should enhance therapeutic efficacy. To test this prediction, TasQ was combined with G202 which is a Thapsigargin prodrug engineered to be hydrolyzed to a cytotoxin by a protease uniquely expressed by cancer associated endothelial cells in order to selectively kill tumor endothelial cells enhancing a more stressful tumor microenvironment (12). When estrogenized female nude mice are inoculated with MCF-7 human breast cancer cells and cancers allowed to grow to a starting size of ~0.2cc before animals are given a single cycle of 2 daily IV injections of 56mg/kg of G202, cancers regress due to the death of tumor associated endothelial and cancer cells and in a subset of animals, the cancers does not regrow (12). If such breast cancers are allowed to grow to a starting size of ~0.8cc before initiating a single 2 day cycle with G202, cancers regress by >50% followed by regrowth, Figure 7C. While daily TasQ treatment (10mg/kg/d) initiated when the breast cancers are ~0.8cc inhibits subsequent tumor growth, no regression of these large cancers occurs, Figure 7C. In contrast, combining daily TasQ with G202 treatment results in regression of these large cancers, but now regrowth is inhibited (p<0.05), Figure 7C.

Discussion

Solid malignancies outgrow their blood supply producing a hypoxic tumor microenvironment. Such tumor hypoxia enhances glycolysis and amino-acid catabolism increasing lactic acid secretion producing an acidic extracellular microenvironment coupled with nutrient limitation [e.g., glucose and amino-acid deficiency] (8). To survive this stressful microenvironment, an epigenetic adaptive survival response, termed the angiogenic switch, is activated involving reciprocal interactions between cancer cells and tumor host infiltrating cells including MDSC, macrophages, and endothelial cells, (7-9). This angiogenic switch facilitates global repression of transcription coupled with selective expression of specific stress-response genes via type IIa HDAC4 dependent co-localization of type I HDAC3 deacetylase activity at specific promoter/enhancers in cancer and host tumor infiltrating cells (30, 33), Figure 7A. The importance of such deacetylase activity is documented by ability of pan- and type 1 isotype selective HDAC
inhibitors to suppress cancer growth via reduced tumor angiogenesis (30, 33).
Limitation with using pan- or type 1 isotype selective HDAC inhibitors, however, is that they up-regulate, instead of repress, transcription of a subset of unwanted survival genes [e.g., GRP78/BiP, Cox-2, FGF2, Slit2] (41-46). Thus, selective inhibition of HDAC4 signaling is a more optimal approach for blocking the angiogenic switch without activating unwanted survival signaling within cancer sites.

The present studies identify TasQ as such a high affinity HDAC4 selective negative allosteric modulator. Besides allosterically binding HDAC4, TasQ also binds with high affinity to the calcium binding protein, S100A9 (7, 16). In the presence of zinc, S100A9 undergoes a conformational change becoming a ligand for the pro-inflammatory Toll like receptor 4 (TLR4) and TasQ binding to S100A9 inhibits this binding to TLR4 (7). This is significant because myeloid-derived suppressor cells (MDSCs) from the bone marrow circulate in the blood and express S100A9 protein and TLR4 and S100A9 binding to TLR4 stimulates tumor infiltration of MDSCs (7, 47, 48). Under hypoxic conditions, tumor infiltrating MDSC differentiate via HIF-1α dependent transcription into tumor-associated macrophages (TAMs) (49). Once differentiated, TAMs secrete angiogenic factors like VEGF, FGF, TNF-α, and TGF-β (50). Thus, TasQ’s can suppress tumor angiogenesis due to inhibition of S100A9/TLR4 dependent MDSCs tumor infiltration and/or to inhibition of HDAC4/N-CoR/HDAC3 dependent deacetylation of HIF-1α by MDSCs suppressing their differentiation into TAMs.

Presently, the relative importance of both of these complementary TasQ targets for anti-cancer efficacy is being evaluated in pre-clinical models. Regardless of the results of these additional studies, the present studies document that TasQ’s therapeutic anti-cancer efficacy is enhanced when combined with an additional drug (e.g., G202) which produces a more stressful tumor micro-environment.

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References:


23. Denko N, Wernke-Dollries K, Johnson AB, Hammond E, Chiang CM, Barton MC. Hypoxia actively represses transcription by inducing negative cofactor 2 (Dr1/DrAP1) and blocking preinitiation complex assembly. J Biol Chem 2003; 278: 5744-5749.


Legends to the Figures

Figure 1. A. Chemical structure of tasquinimod (TasQ) (N-ethyl-N-phenyl-5-chloro-1,2-dihydro-4-hydroxy-1-methyl-2-oxo-3-quinoline-carboxamide B. Comparison of anti-cancer efficacy of tasquinimod (10mg/ kg/ day via drinking water) against CWR22-RH human prostate cancer xenografts (n=10/group) growing in castrated hosts initiated immediately at time of tumor inoculation vs. delayed treatment starting on day 49 post tumor inoculation when tumors were 0.250cc in size. Each symbol point is the mean tumor volume at the indicated time. SEM is <15% of the mean for each time point. p<0.05 for all treatment time points beyond day 50 compared to controls. C. Growth inhibitory response of HUVEC and indicated human prostate cell lines to one week treatment with indicated concentration of TasQ. Results are normalized to number of viable cells in control cultures not exposed to TasQ. Asterisk denotes statistical difference (p<0.05) for TasQ treated cells compared to untreated control cells. D. TasQ dose-response inhibition of 3-D endothelial cell (EC) sprouting in fibrin gels over a 7 day (D7) observation period vs. untreated (control) cells.

Figure 2. A. Tas-Q (1µM) and TSA (200nM) prevent lysine deacetylation in H3-histone at position 9 &19 induced by hypoxia in all of the human prostate cancer lines tested. β-actin was used as a loading control. B. Western blots of indicated HDAC in normal human prostate epithelial (i.e., 957E/hTERT) cells and LNCaP, PC-3, and DU-145 human PCs. Vincullin was used as a loading control. C., D., & E. TasQ(1µM) does not decrease HDAC isotypes expressed by human PC lines (i.e., LNCaP, LAPC-4, and CWR22-Rv1) or HUVECs . β-actin was used as a loading control. In D, western blot of HDAC5 was detected using SuperSignal® West Femto Maximum Sensitivity Substrate.

Figure 3. A. Schematic organization of HDAC4 protein. Numbers refer to amino acid position. Black boxes are known sites of transcription factor binding in N-terminal adapter domain. NLS refers to nuclear localization signal and NES to nuclear export
signal. S-P refers to serines which if phosphorylated allows binding to 14-3-3 protein. 

B. Computer based docking of TasQ to inactive (non N-CoR binding) conformation of regulatory zinc-binding domain (ZRD) within the catalytic domain (amino acids 648-1051) of human HDAC4. Upper magenta color ball is Zn$^{2+}$ in the ZRD and lower magenta ball is Zn$^{2+}$ in the catalytic domain. C. Interaction of TasQ with various amino-acids in the inactive conformation of ZRD. D. Surface plasmon resonance of human full length HDAC4 binding to TasQ immobilized onto a Biacore chip.

**Figure 4.** A. HDAC4 protein in HDAC4 shRNA2 knock down LNCaP and HUVEC endothelial cells. Number under lane is relative level of expression per cell normalized to untreated control cells. B. HDAC4 shRNA knock-down inhibits sprouting of HUVECs in 3-D assay. C. Upper panel- Growth of control vs. HDAC4 shRNA2 knock-down vs. 1µM TasQ treated LNCaP growth under normoxic conditions and Lower panel- under hypoxic conditions. D. HDAC4 and HIF-1α protein expression in control vs. HDAC4 shRNA2 knock-down vs. knock down restored LNCaPs. β-actin was used as a loading control. E. Tumorigenicity of HDAC4 shRNA2 knock-down vs. restored LNCaPs when xenografted into male nude mice (n=8/ group).

**Figure 5.** A. SPR determined d TasQ dose-response inhibition of N-CoR binding to HDAC4 immobilized onto a Biacore chip. B. Zn$^{2+}$ dependent binding of full length N-CoR protein binding to recombinant full length HDAC4 protein is prevented by chelation with EDTA. Flag-NCoR used as loading control. C. Binding of recombinant full length HDAC4 binding to full length N-CoR protein in the presence of Zn$^{2+}$ is inhibited by TasQ. GST-HDAC4 used as loading control. TasQ inhibits HDAC4 binding to N-CoR/HDAC3 complexes in HEK-293 cells in both normoxic (D&E) and hypoxic (E) conditions. Flag-HDAC4 used as loading control. F. TasQ inhibits HDAC4 binding to N-CoR/HDAC3 complexes in LNCaPs. Flag-HDAC3 used as loading control.

**Figure 6.** A. HDAC4 increases and 1µM TasQ decreases HIF-1α protein in HEK-293T cells. β-actin was used as a loading control. B. TasQ lowers level of HDAC4 in the nuclei of LNCaPs in both normoxia and hypoxia. Upper arrow denotes phosphorylated form. β-actin was used as a loading control for total extracts while H3-histone was used as loading control for nuclear extract. C. TasQ lowers endogenous HDAC4 in the nuclei
of LNCaPs in hypoxia. H3-histone used as loading control. D. TasQ increases the proportion of HIF-1α which is lysine-acetylated within the nuclei of LNCaPs under hypoxic. Number under lane is the relative level of expression per cell normalized to untreated control cells. β-actin was used as a loading control. E. TasQ inhibits HIF-1α dependent green fluorescent protein (GFP) expression in PC-3 human prostate cancer cells which have stably integrated a HRE/GFP construct in normoxia (signal from 25,000 cells) vs. 24 hr of hypoxia (sign from 1,250 cells). F. TasQ inhibits hypoxia induced HIF-1α dependent transcriptional stress response of HUVECs detected using a hypoxia cDNA microarray with the results normalized to β-actin. Abreviations for the genes probed are as follows: CA9, carbonic anhydrase IX; CTSD, cathepsin D; Glut-1, glucose transporter-1; Glut-3, glucose transporter-3; HIF-1, hypoxia induced factor-1α; HIF-2, hypoxia induced factor-2α; MMP2, matrix metalloproteinase 2; MXi-1, Max interactor-1; NDRG, N-myc downstream regulated-1; NDRG2, N-myc downstream regulated-2; PAI-1, plasminogen activator inhibitor-1.

**Figure 7.** A. Overview of TasQ’s mechanism of action. Under hypoxia/stressful microenvironment conditions, HDAC4/N-CoR/HDAC3 complex binds via HDAC4 to DNA bound transcription factors allowing HDAC3 to deacetylate histones locally repressing basal transcription within the nucleus of endothelial and cancer cells. Also during such stress, HIF-1α accumulates in the nuclei where PCAF acetylates its N-terminal lysines between AA11-21 and at position 674 in its inhibitory domain (ID). HDAC4/N-CoR/HDAC3 complexes bind via HDAC4 to the acetylated lysine 674 in the ID of HIF-1 co-localizing N-CoR/HDAC3 resulting in deacetylation of N-terminal lysines between AA11-21 of HIF-1 facilitating binding of p300HAT and HIF-1β needed for formation of HIF-1 complex which via p300 acetylates histones in cell survival/angiogenesis genes stimulating their transcription. TasQ binding allosterically locks ZRD of HDAC4 in an inactive conformation preventing basal gene repression and survival/angiogenesis gene transcription needed for the angiogenic switch. B. TasQ’s efficacy against a diverse series of human solid cancer xenografts. Results are expressed as percent inhibition of cancer growth based upon comparison of tumor volume (N=5-10 cancer bearing mice per cancer subtype) in TasQ treated (10mg/kg/day via drinking water) vs. untreated mice over a month observation period.
C. Therapeutic response of established (0.8cc) MCF-7 human breast cancers growing in mice given two daily IV injections at 56mg/kg of a tumor endothelial targeted cytolytic prodrug (G202) alone and in combination with 10mg/kg/d oral TasQ (N=8 per grouping). Results are presented as relative tumor size normalized to tumor volume at initiation of treatment. p<0.05 for combination (combo) group vs. either monotherapies after day 49.
Figure 1

A. Chemical structure of TasQ.

B. Graph showing mean tumor volume over days post tumor inoculation, with inhibition of EC sprouting calculated as:
- TasQ 0.5 µM: 50%
- TasQ 1 µM: 75%
- TasQ 10 µM: 90%

C. Bar graph comparing percent of control for different cell lines:
- HUVEC
- LAPC-4
- CWR22Rv1
- LnCaP

D. Images showing inhibition of EC sprouting:
- D0 Control
- D7 Control
- D7 0.5 µM
- D7 1 µM
- D7 10 µM

Inhibition of EC Sprouting:
- TasQ 0.5 µM: 50%
- TasQ 1 µM: 75%
- TasQ 10 µM: 90%
Figure 2

A. Hypoxic vs. Normoxic Conditions

<table>
<thead>
<tr>
<th></th>
<th>LNCaP</th>
<th>LAPC4</th>
<th>CWR22 - Rv1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>TasQ</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>TSA</td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

- Acetyl-H3
- β-actin

B. HDAC Expression in Various Cell Lines

<table>
<thead>
<tr>
<th></th>
<th>957E/MTERT</th>
<th>LNCaP</th>
<th>PC3</th>
<th>DU145</th>
</tr>
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<tbody>
<tr>
<td>HDAC 1</td>
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<tr>
<td>HDAC 2</td>
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<td>HDAC 3</td>
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<tr>
<td>HDAC 9</td>
<td></td>
<td></td>
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<tr>
<td>Vincullin</td>
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</tr>
</tbody>
</table>

C. HDAC Expression in LNCaP, LAPC-4, and CWR22-Rv1

- HDAC 1
- HDAC 2
- HDAC 3
- HDAC 4
- HDAC 6
- HDAC 8
- β-actin

D. HDAC Expression under Control and 1 μM TasQ Conditions

- LNCaP
- LAPC4
- CWR22R
- HUVEC

- HDAC 5
- β-actin
Figure 3

A. Protein domain diagram showing the NLS (Nuclear Localization Signal), ZBD (Zinc Finger Domain), Catalytic Domain, and NES (Nuclear Export Signal) regions. Key interactions include 14-3-3 binding, N-CoR binding, and HIF-1α binding.

B. Structural view of the protein domain, highlighting the interactions and regions mentioned in A.

C. Close-up view of a specific interaction or binding site, showing residues and their interactions.

D. Kinetic response graph showing the response (RU) over time (s) and HDAC4 concentration (nM).
**Figure 4**

**A.**

<table>
<thead>
<tr>
<th></th>
<th>LNCaP</th>
<th>LNCaP HDAC4 Knock down</th>
<th>HUVEC</th>
<th>HUVEC HDAC4 Knock down</th>
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<td><img src="image7" alt="Image" /></td>
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<tr>
<td></td>
<td>1.00</td>
<td>0.44</td>
<td>1.00</td>
<td>0.48</td>
</tr>
</tbody>
</table>

**B.**

![Image](image9)

**C.**

**Normoxic Conditions**

- Control
- shRNA
- Tasquinimod

**D.**

**LNCaP**

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>HDAC4 KD</th>
<th>HDAC4 KD * restored</th>
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<td><img src="image17" alt="Image" /></td>
<td><img src="image18" alt="Image" /></td>
</tr>
</tbody>
</table>

**E.**

**Tumor Volume cc**

- LnCaP-HDAC 4 knockdown
- LnCaP-HDAC 4 knockdown-restored
Figure 5

A. 

Response (RU) vs. HDAC4 for NCoR/No Comp, NCoR/1µM TasQ, NCoR/10µM TasQ, and NCoR/100µM TasQ.

B. 

IP: Flag-NCoR

1 mM EDTA - + IP: IgG

IB: GST-HDAC4

IB: Flag-NCoR

C. 

IP: GST-HDAC4

Control, 50 nM TasQ, 100 nM TasQ

IB: Flag-NCoR

IB: GST-HDAC4

D. 

IP: Flag-HDAC4

Control, 1 µM TasQ, 10 µM TasQ, 25 µM TasQ, -ve Control

IB: NCoR

IB: HDAC3

IB: Flag-HDAC4

E. 

IP: Flag-HDAC4

1 µM TasQ

Control, 1 µM TasQ, 10 µM TasQ, -ve control

IB: HDAC3

IB: Flag-HDAC4

normoxic hypoxic

F. 

Total Extract - + IP: Flag-HDAC3

1 µM TasQ - +

IB: HDAC4

IB: Flag-HDAC3
Figure 6

A. Flag-HDAC4

<table>
<thead>
<tr>
<th></th>
<th>Normoxic</th>
<th>Hypoxic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tasquinimod</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

IB: HIF-1α
IB: β-actin

B. Nuclear Extract
IP: Flag-HDAC4

<table>
<thead>
<tr>
<th></th>
<th>Total extract</th>
<th>Normoxic</th>
<th>Hypoxic</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1 μM TasQ</td>
<td>1 μM TasQ</td>
</tr>
</tbody>
</table>

IB: HIF-1α
IB: β-actin
IB: H3-histone

C. IB: HIF-1α
IB: β-actin
IB: H3-histone

D. IP: Acetyl-Lysine

IB: HIF-1α
IB: β-actin
IB: H3-histone

E. IB: GFP

F. Bar graph

Expression Relative to β-Actin

- Normoxic
- Hypoxic
- Hypoxic + 1 uM TasQ
Figure 7

A.

B.

<table>
<thead>
<tr>
<th>Human Cancer Xenografts</th>
<th>Percent Inhibition of Cancer Growth by Tasquinimod</th>
</tr>
</thead>
<tbody>
<tr>
<td>LAPC-4 (prostate)</td>
<td>78 +/- 11</td>
</tr>
<tr>
<td>LNCaP (prostate)</td>
<td>57 +/- 8</td>
</tr>
<tr>
<td>CWR22-RH (prostate)</td>
<td>84 +/- 12</td>
</tr>
<tr>
<td>MCF-7 (mammary)</td>
<td>52 +/- 7</td>
</tr>
<tr>
<td>TSU (bladder)</td>
<td>59 +/- 10</td>
</tr>
<tr>
<td>HCT-116 (colon)</td>
<td>50 +/- 5</td>
</tr>
</tbody>
</table>

C.

- Control
- G202
- TasQ
- Combo

Days

Relative tumor size

35 40 45 50 55 60
Tasquinimod is an allosteric modulator of HDAC4 survival signaling within the compromised cancer microenvironment


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