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

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Abstract

Tasquinimod is an orally active antiangiogenic 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 antiangiogenic activity of tasquinimod. Our comprehensive analysis revealed allosteric binding (Kd 10–30 nmol/L) to the regulatory Zn2+ binding domain of HDAC4 that locks the protein in a conformation preventing HDAC4/N-CoR/HDAC3 complex formation. This binding inhibited colocalization 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. Cancer Res; 73(4); 1386–99. ©2012 AACR.

Introduction

Oral dosing with tasquinimod (TasQ; Fig. 1A) results in a doubling of progression free survival in patients with metastatic castration-resistant prostate cancer (CRPC; ref. 1). Based on these results, phase III registration trials for metastatic CRPC are ongoing. Preclinical studies show that TasQ suppresses reciprocal cross-talk between cancer and tumor infiltrating host cells such as 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 downregulation of inhibitory genes such as thrombospondin-1 coupled with the upregulation of stimulatory genes such as hypoxia-induced factor-1α (i.e., HIF-1α; ref. 9).

TasQ's suppression of this epigenetic angiogenic switch (5) provides a rationale for its efficacy in preclinical 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 to use this information to rationally design combinational approaches to enhance therapeutic benefit in the clinic.

Materials and Methods

Reagents

Reagents: G202 (GenSpera); Tasquinimod (Active Biotech Research AB); Trichostatin-A (Sigma-Aldrich); Acetyllysine-7-amino-methylcoumarin (i.e., total HDAC substrate) and trifluoromethy-acetyllysine7-amino-methylcoumarin (i.e., HDAC4 specific substrate; Bachem Inc.). Antibodies (Ab) against the indicated antigen: acetyl-histone H3; rabbit polyclonal Ab (Millipore, Cat # 06-599); acetyl-lysine: rabbit

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Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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monoclonal Ab (Millipore, Cat # 06-933); Flag; clone M2;
mouse monoclonal Ab (Sigma-Aldrich, Cat # F3165); GFP;
mouse monoclonal Ab (Clontech, Cat # 632375); GST; rabbit
polyclonal Ab (Cell Signaling, Cat # 2622); HDAC1(H-11);
mouse monoclonal Ab (Santa Cruz, Cat # sc-8410); HDCA2
(C-8); mouse monoclonal Ab (Santa Cruz, Cat # sc-9959);
HDAC3(B-12); mouse monoclonal Ab (Santa Cruz, Cat # sc-
17795); HDAC4; rabbit polyclonal Ab (Active Motif, Cat #
40969); HDAC5: rabbit polyclonal Ab (Cell Signaling, Cat #
sc-2082); HDAC6(H-300); rabbit polyclonal Ab (Santa Cruz,
Cat # 11420); HDAC7(C-18); goat polyclonal Ab (Santa Cruz,
Cat # sc-11491); HDAC8(H-145); rabbit polyclonal Ab (Santa
Cruz, Cat # 11405); HDAC9 9 Ab was a gift from Dr. Arthur
Zelent (Institute of Cancer Research, Sutton, Surrey, United
Kingdom); HIF-1α(H-206); rabbit polyclonal Ab [Santa Cruz,
Cat # sc-10790 (IP)]; HIF-1α; mouse monoclonal Ab [BD
Transduction Laboratories, Cat # 610958 (IB)]; HIF-1α(H-
206); rabbit polyclonal [Bethyl Laboratories, Cat # A300-286A
(II)]; N-CoR; rabbit polyclonal Ab (Bethyl Laboratories, Cat #
A301-146A).

Recombinant wild-type and C669/H675-mutated HDAC4
proteins
pcDNA vector containing human N-terminal flag-tag full-
length wild-type histone deacetylase 4 (HDAC4) was obtained
from Addgene (Cat # 13821). To produce recombinant C669/
H675 mutated 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 (Cat # 200523) according to manu-
facturer’s protocol. HEK-293T cells were transfected with these
vectors and 48 hours latter, cells washed with phosphate
inhibitor from Roche (Cat# 090084500); lysed in 50 mmol/L
Tris HCl pH 7.4 with 150 mmol/L 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 trans-
ferred to chromatography column from BioRad (Cat# 7311550);
column washed multiple times without Triton; protein eluted
with 100 μg/mL 3X flag peptide from Sigma-Aldrich (Cat#
F4799); and dialyzed in PBS and protein concentrated using
ultrafiltration centrifugal filter concentrator [Amicon/Millipore
(Cat # UFC203024PL)].

Three-dimensional (3D)-endothelial sprouting assay was
conducted using a minimum of 5 replicate wells per drug dose
per experiment, repeated 3 independent times as described
previously (11) using human umbilical vein endothelial cells
(HUVECs; Lonza Walkersville, Inc.) with modification that
20 μL of 10X concentrated Dulbecco’s Modified Eagle’s
Media/10%FCS media conditioned by confluent human pri-
mary lung fibroblasts (Lonza) diluted to 200 μL of growth
factor supplemented EGM-2 media (Lonza) was used instead of
fibroblast coculture.

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 short tandem repeat pro-
filing conducted by the Johns Hopkins Genetic Resource
Core Facility. In vitro growth curves were determined as
described (12, 13). Animal studies were conducted accord-
ing to animal protocol M009M434 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 oxy-
genation expressed as mmHg were as described previously
(3, 4). These experiments were repeated at 3 independent
times for each xenograft.

Biochemical assays
Coimmunoprecipitation (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 1-2,440) from Abcam (Cat #
ab82239), 100 ng of the protein was incubated with 20 μL
antiflag M2 affinity gel beads from Sigma-Aldrich (Cat# A2220)
for 2 hours and then the mixture centrifuged, pel-
letted beads washed 3 times, resuspended, and 20 ng of
recombinant glutathione S-transferase (GST)-tagged HDAC4
(i.e., amino acid 627-1,085) obtained from BPS Bioscience
(Cat # 50004) added to the mixture, which was incubated
overnight at 4°C. The mixture was centrifuged, pellet washed
3 times, and then immunoblotted (IB) with anti-GST anti-
body. For co-IP with recombinant GST-tagged HDAC4, 20 ng
of the protein in 500 μL of pH8 buffer (i.e., 50 mmol/L Tris,
150 mmol/L NaCl, 1 mmol/L dithiothreitol, 10 μmol/L Zn,
1% Triton-X100) was incubated with 20 μL Glutathione
Sepharose 4B Beads from GE Life Sciences (Cat # 17-
0756-01) for 2 hours and then the mixture centrifuged,
pelleted beads washed 3 times, resuspended in
pH 8 buffer, and 100 ng 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 antiflag 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 48 hours,
antiflag IP conducted as described for isolation of recombinant
proteins. IP was then IB with indicated antibody conducted.
The biochemical and cell-based co-IP experiment were repeat-
ed independently at least 3 times with representative gels
presenting the results.

For detection of acetylated HIF-1α, cells were main-
tained under hypoxic conditions (i.e., 2% O2) for 24 hours
and then lysed in radioimmunoprecipitation assay buffer
(RIPA) buffer containing added Roche protease and phos-
phatase 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 conducted. 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, 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**

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 TasQ 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 versus inactive conformations of HDAC4 were overlaid using the Ca atoms of crystal structures previously 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 20 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 conducted using Schrödinger's molecular modeling environment Maestro (www.schrodinger.com).

**HDAC4 knockdown and restoration**

Stable shRNA HDAC4 knockdown cells were generated using pLKO.1 lentiviral vector from Open Biosystems (Thermo Scientific) containing the following specific shRNA sequences: shRNA1 (CCGGCGACTCATCTTTGTAGTATTTTCTCTGAGAATTACGCTACAAGATGAGTCGTATTTTT); shRNA2 (CCGGCGTGGTTTCAACGCTACATTCGAGATGTTTAAGGTTAAGTGCTCTTGTCTTATTTT); and shRNA3 (CCGGCGGACICTCAAGACACGGAGAATCGAGTCTCTTGTCTTGTGTGCTTTTTTT). The nonspecific shRNA sequence was (CCTGGAATACGCTCCGCTTAGCTGACGTTAATACGCTTGGTTT); to generate HDAC4 restoration expression vector resistant to shRNA2 knockdown, 2 nucleotides at positions 9 and 12 in the shRNA2 targeting region were changed to make it resistant to the HDAC4 shRNA without altering the amino acid sequence of wild-type protein.

**HIF-1α transcriptional assays**

PC3 human prostate cancer cells stably integrated with EGFPgene under a promoter containing 5 copies of the HIF-1α-driven hypoxia response element (HRE) of the VEGF gene as described previously (18) were exposed to different doses of TasQ under both normoxic and hypoxic conditions for 24 hours 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 cotransfected 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 p-thymidine kinase (pTK)-Renilla control plasmid containing Renilla luciferase under a TK-driven promoter using Fugene6 from Roche. The p2.1 and pTK-Renilla plasmids were a kind gift from Dr. Gregg Semenza (Johns Hopkins School of Medicine) and have been characterized previously (19). After 6 hours, HUVEC cell lysates were analyzed with a Dual Luciferase Reporter Assay kit from Promega (Cat# 1910). These experiments were repeated 3 independent times with representative data reported. Human HIF-regulated cDNA plate array from Signosis, Inc. (Cat# AP-0111) was conducted 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). CRPC 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 conducted 3 independent times according to the guidelines provided by the Agilent Whole Genome Expression Microarray system (Agilent Technologies), 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 procedure as described previously (22).

**Statistics**

All of the values reported are presented as means ± SE of representative data generated from 1 of a minimum of 3 independent experiments in which there were a minimum of 5 replicates per data point. Statistical analysis was conducted by a 1-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., pO₂ in the
starting Matrigel plug is 10 ± 2 mmHg vs. 40 ± 9 mmHg in fully oxygenated normal tissue) and to stimulate host angiogenic response. If a daily oral dose of TasQ (i.e., 10 mg/kg/d), which maintains serum drug concentration at 0.5 to 1 μmol/L (3), is initiated upon inoculation of Matrigel containing 15 to 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; Fig. 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 0.250 cc, the cancers do not regress, but subsequent growth is profoundly inhibited (P < 0.05; Fig. 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 used. Under standard culture conditions (5% CO2/21%O2/ pH7.2 media), human PC cells (LNCaP, LAPC-4, and CWR-22Rv1) and HUVECs grow exponentially with doubling times of approximately 2 days. Under these standard conditions, TasQ inhibits growth (P < 0.05) with IC50 values of approximately 50 μmol/L for cancer and endothelial cells, values 50- to 100-fold higher than therapeutic blood levels (Fig. 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 1 μmol/L TasQ (a dose that had no effect under standard in vitro conditions, but was therapeutic in vivo) in low glucose/high CO2/low O2/acidic media to mimic the stressful microenvironment within cancer sites. Under these compromised conditions, untreated LNCaPs and HUVECs survive, but
slow their growth (doubling time increases from 42 ± 5 to 144 ± 12 hours for LNCaP and 47 ± 7 to 64 ± 8 hours for HUVEC; P < 0.05). Addition of 1 μmol/L TasQ to such a compromised condition further decreases doubling time of HUVECs (88 ± 9 hours; P < 0.05). In contrast, addition of 1 μmol/L TasQ to such a compromised condition results in 52 ± 9% (P < 0.05) of LNCaPs dying within 1 week.

To evaluate whether TasQ also inhibits the functional response of endothelial cells, 3D in vitro angiogenic sprouting/tube formation assays were used. When HUVECs grown on microcarrier 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 1 week (Fig. 1D). If TasQ is added at culture initiation, sprouting is suppressed (P < 0.05) with an IC50 value of 0.5 μmol/L (Fig. 1D). In contrast, if HUVECs are allowed to preform 3D neovessels and then TasQ is added, no regression occurs even at 50 μmol/L. These results document that TasQ’s inhibition of cancer cells survival and endothelial angiogenic responses are profoundly enhanced by a stressful microenvironment.

**Tasquinomod 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 (Fig. 2A). Trichostatin A (TSA) a known pan-class I/II HDAC inhibitor (27) likewise inhibits decrease in lysine 9/19 acetylated H3-histone (Fig. 2A). These results suggest TasQ might be an HDAC inhibitor.

There are multiple isotypes of class I (HDAC1, 2, 3, and 8), class IIa (HDAC4, 5, 7, and 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 (Fig. 2B). The lack of detectable expression of the other class IIa isotypes by normal prostate epithelial cells is not due to the quality of the antibodies used for detection, as a positive control HDAC5 is detected in CWR22-Rv1 cell (Fig. 2C), HDAC7 in MCF-7 (data not shown), and HDAC9 is detected in LNCaP cells (Fig. 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 (Fig. 2B and C). HUVEC cells express HDAC2, 3, and 8 of the class I and HDAC4 and 7 of the class IIa and HDAC6 of the class IIb isotypes (Fig. 2D and E). Interestingly, human prostate cancer lines express 3- to 5-fold higher total HDAC activity than endothelial cells (Supplementary Fig. S1). At 1 μmol/L, TasQ treatment does not lower the expression of any isotypes expressed by human PC lines (Fig. 2C and D) or HUVECs (Fig. 2D and E). TasQ even at 100 μmol/L does not inhibit total HDAC enzymatic activity in HUVECs or a series of human PC lines (LNCaP, CWR22-Rv1, LNCaP).
Tasquinimod binds allosterically within the regulatory Zinc domain of HDAC4

Previous studies document an enhanced level of HDAC4 protein within PC nuclei in patients with CRPC 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 more than 3-fold (P < 0.05) in metastatic sites in patients with CRPC (n = 18) compared with normal prostate from organ donors (n = 23; Supplementary Fig. S2A). PC lines express more than 5-fold higher levels of HDAC4 protein than normal prostate epithelial cells, which is not in the nucleus (Supplementary Fig. S2B). HDAC4 is 1,084 amino acids (AA) with a nuclear localization signal (NLS) at AA244-279, a nuclear export signal (NES) at AA1051-1084, and 3 serines at AA246, 467, and 632 whose phosphorylation is required for binding 14-3-3, which restricts localization to the cytoplasm (Fig. 3A; ref. 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; Fig. 3A). Crystallography studies document that the ZRD has 2 alternative conformations (17). When Zn\(^{2+}\) is coordinated with the structural Zn\(^{2+}\) in the ZRD, the ZRD is in an active conformation allowing binding to the transcriptional corepressor, 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, H675, 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), TasQ docking was conducted based upon energy minimization modeling. This modeling identified a specific allosteric binding site for TasQ within the inactive conformation of the ZRD, distinct from the catalytic domain (Fig. 3B). Critically, specific amino acid interactions involved in this binding are only possible in inactive, not active, conformation of the ZRD (Fig. 3C). TasQ was immobilized onto a Biacore chip via an amino linker and binding of human recombinant full-length HDAC4 protein was determined using SPR. These studies showed that TasQ binds HDAC4 with a Kd of 10 to 30 nmol/L (Fig. 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 (Supplementary Fig. S3A).

Tasquinimod phenocopies HDAC4 knockdown in endothelial and prostate cancer cells

A series of specific and nonspecific HDAC4 shRNA lentiviral constructs were tested for their ability to downregulate HDAC4 in LNCaP cells (Supplementary Fig. S3B). The best construct (shRNA2) was then used to transduce LNCaP and HUVEC cells in multiple transductions, cells that grew out following such shRNA2 transduction had only approximately 60% HDAC4 knockdown (Fig. 4A). Even as little as a 60% reduction in HDAC4 completely inhibits HUVECs by 1 μmol/L TasQ (Fig. 1D). With regard to LNCaP cells, such limited HDAC4 reduction had little effect upon growth rate under these standard nonstressed conditions (Fig. 4C). In contrast, however, under even the limited stress of exposure to 2% O\(_2\), without increasing CO\(_2\) and decreasing pH, 60% knockdown of HDAC4 decreases survival of LNCaPs, which is a response identical to treatment with 1 μmol/L TasQ under such limited stress (Fig. 4C, bottom).

TasQ also phenocopies the response to HDAC4 knockdown in vivo. Similar to the situation where PCs are non-tumorigenic when TasQ treatment is initiated at inoculation (Fig. 1B), HDAC4 shRNA2 knockdown LNCaPs are also nontumorigenic in mice (Supplementary Fig. S3C and S3D). The specificity of this response was documented by the fact that restoration of HDAC4 expression in shRNA2 knockdown LNCaPs (Fig. 4D) restores their tumorigenicity (Fig. 4E and Supplementary Fig. S3E).

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 1 nmol/L and that TasQ inhibits this binding in a dose-dependent manner (Fig. 5A). In co-IP/IB assays, HDAC4/N-CoR/HDAC3 complex formation is Zn\(^{2+}\) dependent because it is blocked by EDTA (Fig. 5B). TasQ also blocks such complex formation with an IC\(_{50}\) value of less than 50 nmol/L (Fig. 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 IC\(_{50}\) < 1 μmol/L in normoxia (20% O\(_2\); Fig. 5D and E), and hypoxia (2% O\(_2\); Fig. 5E). To evaluate whether these results are unique to HEK-293Ts, LNCaPs were transfected with HDAC3. Co-IP/IB analyses documented that TasQ inhibits the binding of HDAC4 to N-CoR/HDAC3 complexes within PCs with an IC\(_{50}\) of less than 1 μmol/L (Fig. 5F).
Tasquinimod phenocopies loss of HIF-1α transcriptional stress response induced by HDAC4 knockdown

TasQ should inhibit HDAC4 client protein deacetylation via blocking colocalization 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 (Fig. 3A; refs. 30, 32–34). Because of cessation of hydroxylation of a series of prolines and an asparagine, HIF-1α accumulates in the nucleus during hypoxia, where it is acetylated by p300/CBP associated factor (PCAF) acetyltransferase 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 and instead facilitates binding of p300HAT and dimerization with HIF-1β to form transcriptionally active HIF-1α at HREs within promoter/enhancers of hypoxic responsive genes (19, 34). shRNA2 knockdown 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 knockdown, and knockdown-restored LNCaPs. As expected, HDAC4 knockdown decreases HIF-1α and this is reversed when HDAC4 is restored (Fig. 4D). If TasQ inhibits HDAC4/N-CoR/HDAC3-dependent N-terminal lysine deacetylation of HIF-1α, then it should decrease HIF-1α that suppresses HIF-1α-dependent transcription. When HEK-293T cells were transfected with HDAC4, HIF-1α is decreased by TasQ (Fig. 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% O₂) and hypoxic (2% O₂) conditions. IB of total cell extracts showed that hypoxia increases HIF-1α coupled with decreasing its mobility that enhances its transcriptional activity (38). Co-IP/IB of nuclear extracts documents that 1 μmol/L TasQ decreases HIF-1α/HDAC4 complex formation by more than...
50% under both normoxic and hypoxic conditions (Fig. 6B). The inhibitory effect of TasQ is not dependent upon overexpression of HDAC4, because it suppresses HIF-1α by more than 50% under hypoxic conditions in nuclear extracts from nontransfected LNCaPs (Fig. 6C). Nuclear extracts from nontransfected LNCaPs were co-IP with an antiacetyl lysine antibody and the IP analyzed by IB with anti-HIF-1α antibody. Even though TasQ decreases nuclear HIF-1α by more than 2-fold in the LNCaPs (Fig. 6C), acetylated HIF-1α increases by 40% (Fig. 6D). Thus, TasQ inhibits the nuclear fraction of N-terminal HIF-1α lysines deacetylated under hypoxic conditions by more than 3-fold.

On the basis of its inhibition of HIF-1α deacetylation, 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 GFP construct was evaluated to test this prediction. Like LNCaP, PC-3 predominantly has nuclear HDAC4 (Supplementary Fig. S2B) 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 (Fig. 6E).

To further evaluate the generality of TasQ’s ability to inhibit HIF-1α-driven survival signaling in prostate cancer cells, lactate dehydrogenase-A (LDH-A) protein expression was determined in LNCaP cancer cells exposed to 1 µmol/L 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 upregulation (Supplementary Fig. S4A).

To evaluate whether TasQ’s inhibition of the HIF-1α transcriptional response also occurs in endothelial cells, HUVECs were cotransfected with a HIF-1α-dependent HRE-driven firefly luciferase vector plus a cytomegalovirus-driven renilla luciferase control vector and the cells exposed to normoxia versus hypoxia. These results show that hypoxia induced HIF-1α-dependent transcription in HUVECs is completely inhibited by 1 µmol/L TasQ (Supplementary Fig. S4B). Likewise, a commercial HIF-1α-dependent hypoxia cDNA microarray documented that 1 µmol/L TasQ prevents HUVECs from adaptively upregulating transcription of stress genes under hypoxia (Fig. 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 colocalizing with HDAC4/client transcription factors.

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 (HIF-1) colocalizing N-CoR/HDAC3, resulting in deacetylation of N-terminal lysines between AA11–21 of HIF-1, facilitating binding of p300/HAT 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 (10 mg/kg/d via drinking water) versus untreated mice over a month observation period. C, therapeutic response of established (0.8 cc) MCF-7 human breast cancers growing in mice given 2 daily intravenous injections at 56 mg/kg of a tumor endothelial targeted cytolytic prodru (G202) alone and in combination with 10 mg/kg/d oral TasQ (N = 8/group). Results are presented as relative tumor size normalized to tumor volume at initiation of treatment. P < 0.05 for combination (combo) group versus either monotherapies after day 49.
bound at the promoter/enhancers of genes (Fig. 7A). HDAC4 binding with the majority of its client DNA bound-transcription factors results in decreased histone acetylation due to colocalization of HDAC3 repressing their transcription (31; Fig. 7A). This explains how TasQ inhibits hypoxia stimulated global histone deacetylation (Fig. 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 deacetylation (Fig. 7A).

On the basis of 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 0.250 cc before daily oral TasQ (10 mg/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 (Fig. 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 that increase tumor environmental stress should enhance therapeutic efficacy. To test this prediction, TasQ was combined with G202, which is a Thapsigargin prodrgue engineered to be hydrolyzed to a cytotoxin by a protease uniquely expressed by cancer associated endothelial cells 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 approximately 0.2 cc before animals are given a single cycle of 2 daily IV injections of 56 mg/kg of G202, cancers regress due to the death of tumor associated endothelial 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 approximately 0.8 cc before initiating a single 2-day cycle with G202, cancers regress by more than 50% followed by regrowth (Fig. 7C). While daily TasQ treatment (10 mg/kg/d) initiated when the breast cancers are approximately 0.8 cc inhibits subsequent tumor growth, no regression of these large cancers occurs (Fig. 7C). In contrast, combining daily TasQ with G202 treatment results in regression of these large cancers, but now regrowth is inhibited \( P < 0.05 \); Fig. 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; ref. 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 colocalization of type 1 HDAC3 deacetylase activity at specific promoter/enhancers in cancer and host tumor infiltrating cells (refs. 30, 33; Fig. 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 upregulate, instead of repress, transcription of a subset of unwanted survival genes [e.g., GRP78/BiP, Cox-2, fibroblast growth factor (FGF2), and Shh; refs. 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 proinflammatory Toll-like receptor 4 (TLR4) and TasQ binding to S100A9 inhibits this binding to TLR4 (7). This is significant because 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 DSC differentiate via HIF-1α-dependent transcription into tumor-associated macrophages (TAM; ref. 49). Once differentiated, TAMs secrete angiogenic factors such as 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 anticancer efficacy is being evaluated in preclinical 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 microenvironment.

Disclosure of Potential Conflict of Interest

No potential conflicts of interest were disclosed.

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