Inhibition of Galectin-1 Sensitizes HRAS-driven Tumor Growth to Rapamycin Treatment

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Abstract. *The goal of this study was to develop combinatorial application of two drugs currently either in active use as anticancer agents (rapamycin) or in clinical trials (OTX008) as a novel strategy to inhibit Harvey RAS (HRAS)-driven tumor progression. HRAS anchored to the plasma membrane shuttles from the lipid ordered (L_o) domain to the lipid ordered/lipid disordered border upon activation, and retention of HRAS at these sites requires galectin-1. We recently showed that genetically enforced L_o sequestration of HRAS inhibited mitogen-activated protein kinase (MAPK) signaling, but not phosphatidylinositol 3-kinase (PI3K) activation. Here we show that inhibition of galectin-1 with OTX008 sequestered HRAS in the L_o domain, blocked HRAS-mediated MAPK signaling, and attenuated HRAS-driven tumor progression in mice. HRAS-driven tumor growth was also attenuated by treatment with mammalian target of rapamycin (mTOR) inhibitor rapamycin, and this effect was further enhanced in tumors driven by L_o -sequestered HRAS. These drugs also revealed bidirectional cross-talk in HRAS pathways. Moreover, dual pathway inhibition with OTX008 and rapamycin resulted in nearly complete ablation of HRAS-driven tumor growth. These findings indicate that membrane microdomain sequestration of HRAS with galectin-1 inhibition, coupled with mTOR inhibition, may support a novel therapeutic approach to treat HRAS-mutant cancer.*

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The rat sarcoma (RAS) genes most prominently associated with cancer, Harvey RAS (HRAS), neuroblastoma RAS (NRAS) and Kirsten RAS (KRAS), are ubiquitously expressed and have overlapping, yet non-redundant functions (1). RAS proteins cycle between an active GTP-bound and an inactive GDP-bound state. Active RAS stimulates mitogenic and survival signal transduction by coupling to effectors including rapidly accelerated fibrosarcoma (RAF) kinase for propagation of the mitogen-activated protein kinase (MAPK) pathway [RAS/RAF/MAPK kinase (MEK)/extracellular regulated kinase (ERK)], and phosphatidylinositol 3-kinase (PI3K) pathways (2). Mutations locking RAS in the constitutively active (*i.e.* GTP-locked) state are highly transforming and induce tumor formation (3), and the combined set of somatic or inherited activating RAS mutations altogether are associated with as many as ~30% of human malignancies (1, 4).

The mammalian target of rapamycin complex 1 (mTORC1) complex functions as a point of convergence from multiple signaling networks, and aberrant signaling has been implicated in pathologies including cancer (5). The prototypic pathway of mTOR activation is through the PI3K/protein kinase B (PKB/AKT) pathway. PI3K is a phospholipid kinase that is activated by multiple mechanisms, such as interaction with activated RAS oncogenes, or by directly coupling to receptor tyrosine kinase complexes. However, the mTORC1 complex is also subject to AKT-independent activation. For example, mTOR is activated by mitogenic signaling through activation of the RAS/MEK/ERK pathway. The PI3K/AKT or RAS/MEK/ERK pathways induce phosphorylation of distinct residues in the mTORC1-negative regulator, TSC2, each resulting in activation of the mTORC1 complex (5).

During the life-cycle of RAS proteins, a series of lipid modifications within the C-terminal targeting domains support anchorage of the RAS proteins to lipid bilayers such as the plasma membrane (6). Localization within membrane microdomains at the plasma membrane is a critical factor in RAS signaling, and is isotype-specific.

HRAS anchors to lipid rafts, also known as the lipid-ordered (L_o) domain, while inactive (GDP-bound), and is shuttled to the Lo/lipid-disordered (L_d) domain border and forms nanoclusters upon activation by GTP coupling, such that effector signaling by interaction with GTP-HRAS emanates from these sites (7).

The process which regulates this shuttle is largely unknown, although at least one scaffold protein, galectin-1 (GAL1), has been identified as a critical part of this process. GAL1 belongs to a family of carbohydrate-binding proteins, with high affinity for β -galactosides. GAL1 overexpression has been observed in several tumor types, and has been associated with tumor progression (8). GAL1 contains a prenyl-binding pocket, which interacts with the farnesyl group in GTP-HRAS, independently of lectin function. This interaction is thought to alter the orientation of the HRAS globular domain with respect to the plasma membrane, and thereby regulate lateral segregation of HRAS and promote MAPK signaling (9, 10). Indeed, ectopic GAL1 overexpression or suppression increases or abrogates GTP-bound HRAS nanoclustering, respectively (11). Thus, GAL1 plays a key role in maintaining HRAS in the active state by mediating translocation of HRAS to the Lo/Ld border upon GTP loading.

We recently showed that activated HRAS which harbors the targeting domain of RRAS, a non-mitogenic paralog (HRAS-tR), is sequestered within the L_o domain. This sequestration of HRAS from the L_o/L_d border resulted in attenuated RAF/MEK/ERK activation, while retaining PI3K signaling to AKT. Whereas L_o sequestration blocked HRAS-induced cell proliferation and transformation, HRAS-tR-transformed cells were competent for tumor initiation and progression in allograft mouse models, indicating a need for further investigation (12).

Whether RAS-driven tumor maintenance requires continued expression of mutant RAS (so-called oncogene addiction) remains unclear (13). RAS has long been a target for pharmacological inhibition in cancer therapies. However, effective small-molecule inhibitors for direct RAS inhibition has proven to be challenging due to difficulty in locating targetable binding pockets on the surface. Moreover, targeting RAS by blunting post-translational events such as farnesyl-transferase inhibitors showed promising initial preclinical results but ultimately led to poor clinical significance (14). This has prompted efforts at targeting RAS effectors, most notably in either the RAF/MEK/ERK or PI3K/AKT/mTOR pathways. MAPK-based inhibitors have encountered obstacles in reducing tumor burden, either as a result of paradoxical feedback loops which increase signaling, such as in the case of the RAF homology B (BRAF) inhibitor sorafenib, or innate/acquired resistance [for review see (15)]. A number of inhibitors targeting the PI3K/AKT/mTOR axis have been under evaluation, with

varying efficiency. Collectively, monotherapies have shown suboptimal efficacy, which has led to recent efforts at combination therapies targeting both the MAPK and PI3K pathways. Herein, we investigated disrupting the Lo/Ld border plasma membrane microdomain localization of HRAS through GAL1 inhibition using OTX008, in combination with rapamycin, a potent mTOR inhibitor, as a novel approach to combat HRAS-driven tumorigenesis.

Materials and Methods

Antibodies, reagents and cDNAs. phospho-ERK (pp44/42 MAPK, ERK T202/Y204), and phospho-S6 ribosomal protein (pS6) (240/244) antibodies were from Cell Signaling Technology (Danvers, MA, USA). Caveolin-1 (CAV1) antibody was from BD Biosciences (Franklin Lakes, NJ, USA). Fluorophore-conjugated secondary antibodies were from LI-COR Biosciences (Lincoln, NE, USA) and Jackson Immunochemicals (West Grove, PA, USA). PI3K inhibitor LY294002 was from LC laboratories (Woburn, MA, USA). Rapamycin was from Santa Cruz Biotechnology (Santa Cruz, CA, USA). OTX008 was from Axon Medchem (Groningen, the Netherlands). MEK inhibitor U0126 was from Alfa Aesar (Ward Hill, MA, USA). Prolong Gold antifade mounting reagent was from Life Technologies (Carlsbad, CA, USA). GFP-RAS constructs were made as described elsewhere (12, 16). GFP-HRAS-(G12V) (Addgene plasmid 18666) was a gift from K. Svoboda.

Cell culture. Stable Green fluorescent protein (GFP)-RAS transfectants were generated on a NIH3T3 fibroblast background by transfection and single-cell flow-activated cell sorting for GFP-expressing cells. Cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Mediatech, Inc., Manassas, VA, USA) supplemented with 10% bovine calf serum, 4 mM L-glutamine, 4,500 mg/ml glucose, 50 U/ml penicillin, 50 μ g/ml streptomycin sulfate and 1% nonessential amino acids (Sigma-Aldrich, St. Louis, MO, USA) at 37°C in 5% CO₂. For cell culture inhibitor studies, cells were serum starved by culturing in DMEM-0.2% serum for 72 h, and treated for 16 h with either vehicle (dimethyl sulfoxide), 20 μ M LY294002, or 30 μ M U0126, prior to lysis and western blotting.

Western blotting. For plasma membrane microdomain partitioning studies, cells were grown in DMEM containing 10% serum. For signaling studies, cells were serum starved by culturing in DMEM/0.2% serum for 72 h. Cells were then rinsed twice in phosphate-buffered saline (PBS), and cell lysates were harvested by scraping in lysis buffer [10 mM Tris-Cl, pH 7.5, 100 mM NaCl, 2 mM MgOAc, 0.5% Nonidet P-40, 10 μ M GTP, 1 mM Na₃VO₄, 20 μ M β -glycerophosphate, 1 mM NaF], plus a cocktail of protease inhibitors (Roche, Indianapolis, IN, USA). Insoluble material was removed by centrifugation. Fractions of the lysates were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), followed by western blotting with antibodies to pS6, CAV1, or GFP.

Tumorigenicity in nude mice. Female NU(NCR)-Foxn1^{nu} mice were from Charles River (Wilmington, MA, USA) at 6 to 7 weeks of age. Cells for allografts were harvested and suspended in Hanks balanced salt solution (Thermo Scientific) at a density of 5×10^6 /ml, and 200 μ l of the cell suspension was injected subcutaneously into

Table I. Summary of Harvey RAS (*HRAS*) mutants and effects of anticancer therapeutics on signal transduction and tumorigenesis in athymic mice.

RAS variant	Drug	pS6	MAPK (ppERK)	Tumorigenesis
HRAS	Vehicle	+++	+++	+++
	Rapamycin	–	+	+
	OTX008	+++	+	+
	Rapamycin/OTX008	–	+	–
HRAS-tR	Vehicle	+	++	+++
	Rapamycin	–	+	–

HRAS-tr: HRAS harboring the RRAS trafficking domain; pS6: phospho-S6 ribosomal protein; MAPK (ppERK): mitogen-activated protein kinase, doubly phosphorylated extracellular regulated kinase. – Weak or undetectable activity, + weak activity, +++ very strong activity.

both flanks. Beginning when tumors reached a minimum size of 50 mm³, either 5 mg/kg rapamycin every 24 h, 5 mg/kg OTX008 every 48 hours, combined treatment with rapamycin every 24 h and OTX008 every 48 hours, or vehicle alone every 24 h, was administered. Vehicle-treated mice were given 200 µl of vehicle (5% polyethylene glycol 400, 5% Tween-80 in aqueous solution). Tumor dimensions were measured by observers blinded to the oncogenic RAS transgene and treatment, using calipers, and tumor volume was calculated using the formula: Volume=long axis × short axis² × 0.52 (17). Mice were euthanized at 20 days post tumor cell injection, and resected tumors were fixed in 4% paraformaldehyde, and stored in PBS with 0.02% sodium azide for immunohistochemistry or flash frozen for sucrose-gradient cell fractionation. All animal experiments followed protocols approved by the Institutional Animal Care and Use Committee at Temple University, (protocol approval number 4266).

Sucrose-gradient cell fractionation. Resected tumors were dounce homogenized, suspended in 0.5 M Na₂CO₃ (pH 11.0, with protease inhibitors), and fractionated as described elsewhere (7). Briefly, cells were passed 15 times through a 23-gauge needle, sonicated, and centrifuged at 26,034 × *g* in a SW41TI rotor (Beckman Coulter, Indianapolis, IN, USA) in a 10–45% (w/v) step sucrose gradient. Fractions were collected from the top sucrose layer, and equivalent total protein fractions were separated by SDS-PAGE and examined by western blotting with antibodies to CAV1 and GFP.

Immunohistochemistry. Orthotopic tumors from 6- to 8-week-old athymic nude mice were harvested, fixed in 4% paraformaldehyde, embedded in paraffin, and sectioned into 5-µm horizontal sections. Sections were dried 2–4 h in a 60°C oven after collection onto charged slides. Sections were deparaffinized and rehydrated through xylene/graded ethanol to distilled deoxygenated water. Sections were rinsed in distilled deoxygenated water, and then incubated with 0.4% pepsin for 20 min at 37°C. Primary antibodies to p-ERK and pS6 were added at a dilution of 1:200 in the blocking serum overnight at 4°C. The next day, sections were washed in Tris-buffered saline (TBS), then anti-rabbit Cy3 fluorophore-conjugated secondary antibodies were applied at a dilution of 1:2,000 for 1 h at room temperature. Fluorescent-stained sections were washed with TBS, and then incubated with 4',6-diamidino-2-phenylindole (DAPI), mounted under coverslips with Prolong Gold, and observed using a Nikon-Eclipse E800 epifluorescent microscope (Nikon, Melville, NY, USA) (20× objective lens; n.a.=0.75) connected to a

RETIGA EXi camera (Q Imaging, Surrey, BC, Canada). Post-acquisition image processing was performed using Adobe Photoshop and ImageJ. Operations included brightness/contrast adjustment to all pixels in the images, and grouping of images. Quantitation of image intensities was measured using ImageJ.

Results

Both the MAPK and PI3K pathways regulate mTORC1 signaling. We considered whether mTORC1 pathway represents a point of convergence for HRAS signaling. We examined phosphorylation of the mTORC1 downstream target ribosomal protein S6, in cells transformed with HRAS or Lo-sequestered HRAS harboring the RRAS trafficking domain (tR) (HRAS-tR) (12). HRAS induced robust S6 phosphorylation (pS6) at serine 240/244 in cells maintained in low-serum growth conditions, and addition of the PI3K inhibitor, LY294002, ablated HRAS-induced pS6, as expected. However, treatment of HRAS cells with the MEK inhibitor, U0126, also diminished pS6. Interestingly, HRAS-tR cells, in which HRAS-induced ERK phosphorylation is inhibited (12), also showed attenuated pS6 in growth medium with low serum, comparable to HRAS-expressing cells treated with U0126 (Figure 1a). Thus, HRAS Lo sequestration and inhibition of MEK block HRAS-induced mTORC1 signaling.

Due to diminished basal mTORC1 signaling in HRAS-tR cells, we considered whether tumor growth by these cells would be sensitized to treatment with an mTOR inhibitor. We employed a tumor allograft model in which athymic mice were injected s.c. in each flank with a bolus of cells expressing either a GFP-HRAS variant or with GFP vector control, and upon initial detection, tumor volumes were measured daily using calipers. GFP cells produced no detectable tumors. Beginning when tumors reached a minimum size of 50 mm³, mice were treated with either mTOR inhibitor rapamycin or with vehicle. HRAS and HRAS-tR cells each supported robust tumor growth (Figure 1b), as we have observed previously. Rapamycin treatment

Figure 1. Harvey RAS (HRAS)-trafficking domain (tR) attenuates mammalian target of rapamycin complex 1 (mTORC1) signaling, and sensitizes tumors to mTOR inhibition. *a*: Cells transformed with the indicated green fluorescent protein (GFP)-HRAS variants were serum-starved and treated with either vehicle (dimethyl sulfoxide, DMSO) or with 20 μ M phosphatidylinositol 3-kinase inhibitor LY294002 or 30 μ M mitogen-activated protein kinase kinase inhibitor U0126 overnight prior to lysis and western blotting. HRAS-tR, HRAS harboring the RRAS tR: GFP-HRAS (amino acids 1-174)-RRAS(amino acids 204-218) (12). Western blot antibodies were against phospho-S6 (pS6) (240/244), and caveolin-1 (CAV1) (total). *b*: Cells stably expressing the indicated RAS variants were injected as a bolus allograft into the flanks of athymic mice. Upon detection, tumor dimensions were measured every 24 h for volume calculation. Rapamycin was administered at 5 mg/kg daily starting at day 1. Results are shown as means $\pm$ s.e.m. Significantly different at * p <0.05; ** p <0.02. n =5.

resulted in attenuated tumor growth with HRAS-transformed allografts compared to vehicle control; however, HRAS-tR-driven tumors were more sensitive to rapamycin treatment, with minimal tumor growth over the 20-day experimental time frame (Figure 1b).

Resected tumors were fixed, sectioned and stained by immunohistochemistry, which confirmed inhibition of pS6 in HRAS tumor cells with rapamycin treatment. pS6 was diminished in HRAS-tR tumors compared to HRAS, similar to effects in serum-starved cells (Figure 2).

Figure 2. Rapamycin and Harvey RAS (*HRAS*) lipid ordered (L_o) sequestration inhibit S6 and ERK phosphorylation in *HRAS*-driven tumors. *a*: Representative images of *HRAS* and *HRAS*-tR tumor sections. Tumors from mice treated with either vehicle or rapamycin were resected, and subjected to immunohistochemistry with antibodies to phospho-S6 (pS6) and doubly phosphorylated ERK (ppERK). 4',6-Diamidino-2-phenylindole (DAPI) images were taken from the same section as ppERK, to indicate cell densities. *b*: Average mean fluorescence intensities of images as shown in (a). Mean grey values in unaltered images were calculated using ImageJ software. n.s.: Not significantly different; *significantly different at $p < 0.001$, $n = 4$.

a

b

c

Figure 3. Rapamycin and OTX008 combinatorial treatment yield additive antitumor effects in Harvey RAS (HRAS)-driven tumors. *a*: Sucrose-gradient fractionation of tumor lysates, probed by western blotting with antibodies to green fluorescent protein (GFP)-HRAS (GFP) and caveolin-1 (CAV1), from tumors in mice treated with vehicle or OTX008. *b*: Tumors were seeded in mice which were then subjected to drug treatments as described in the Materials and Methods Section, and tumor volumes were measured daily. Results are shown as means $\pm$ s.e.m. n.s., Not significantly different; significantly different at * p <0.05; ** p <0.01. n =6. *c*: Histograms represent quantification of staining intensities in images from sections of resected tumors stained with the indicated antibodies. *Significantly different at p <10⁻⁵.

Figure 4. Harvey RAS (*HRAS*) signaling and tumorigenesis in response to inhibition of galectin-1 and mammalian target of rapamycin (*mTOR*). Schematic of predicted effects of dual targeting in *HRAS*-driven cancer. Galectin-1 (*GAL1*) inhibition using OTX008 results in *HRAS* mistargeting to the plasma membrane and disruption of mitogen-activated protein kinase (MAPK) mitogenic signaling. Rapamycin treatment potently reduces *mTOR* survival signaling. Combinatorial use of these inhibitors results in an additive effect over each monotherapy. These findings support combinatorial anticancer therapy targeting *GAL1* and *mTOR* as a novel approach to inhibiting tumor growth in *HRAS* mutant cancer.

In order to address the effect of rapamycin treatment on *HRAS*/MAPK signaling, we assessed phosphorylation of ERK (ppERK) in *HRAS* cell tumors by immunohistochemistry. Surprisingly, we observed a marked reduction in ppERK in *HRAS* cell tumors from mice subjected to

rapamycin treatment. However, rapamycin treatment had no effect on the already limited ppERK in *HRAS*-tR cell tumors (Figure 2). Thus, genetically enforced Lo sequestration attenuates *HRAS*-induced *mTORC1* signaling, and sensitizes *HRAS*-driven tumors to rapamycin treatment.

We considered whether pharmacological inhibition of GAL1 would alter HRAS plasma membrane microdomain localization, and thereby disrupt oncogenic HRAS signaling in a manner similar to genetic modulation of the HRAS targeting domain (*i.e.* HRAS-tR). To address this, we utilized OTX008, a small-molecule inhibitor of GAL1 which has been demonstrated to be an anticancer agent (18, 19). We assessed membrane microdomain distribution of HRAS in allograft tumors resected from mice following treatment with either OTX008 or vehicle, and found that OTX008 treatment resulted in enrichment of HRAS in L_o domains, similar to HRAS-tR, as indicated by co-sedimentation with L_o marker CAV1 (Figure 3a).

In order to investigate a more translational approach, we assessed the effects of combined treatment using the mTOR inhibitor rapamycin, with GAL1 inhibitor OTX008, on HRAS-induced tumor growth. We observed a marked inhibition of tumor growth with each treatment individually (Figure 3b). However, delivery of both inhibitors provided an additive effect on limiting tumor volume, resulting in nearly undetectable tumor growth by HRAS-transformed cells (Figure 3b). This striking result indicates that these inhibitors target independent HRAS oncogenic pathways, and that combined mTOR and GAL1 inhibition results in cytostasis of HRAS-driven tumor cells. Staining of resected tumors verified that rapamycin inhibited mTORC1 signaling and OTX008 inhibited ppERK in HRAS tumors. Interestingly, rapamycin treatment also attenuated ERK phosphorylation in HRAS tumors, and this ERK-inhibitory effect was enhanced by combination with OTX008 treatment (Figure 3c). Similar to HRAS-tR, treatment of HRAS-driven tumors with OTX008 attenuated mTORC1 signaling as indicated by pS6 (Figure 3c). Thus, OTX008 treatment results in sequestration of GTP-HRAS within the L_o domain, inhibition of MAPK signaling, and sensitizes tumors driven by constitutively activated HRAS to rapamycin. Together, these findings support a novel strategy to combat HRAS-driven tumor progression, by combinatorial therapy with clinically viable pharmacological inhibitors. These results are summarized in Table 1.

Discussion

The results of this study support a novel therapeutic design for combating solid tumor progression in HRAS-driven oncogenesis. Whereas rapamycin treatment attenuated HRAS-driven tumor growth, genetic sequestration of HRAS in lipid rafts sensitized HRAS tumor cells to rapamycin. Treatment of HRAS cell tumor-bearing mice with GAL1 inhibitor OTX008 recapitulated the mistargeting effect of the Lo-sequestered HRAS variant, HRAS-tR. Previous studies have shown that OTX008 treatment reduced proliferation and invasion of cancer cells in culture (18), and our data

support this notion specifically by inhibition of HRAS-driven tumor growth *in vivo*. Moreover, combinatorial treatment with rapamycin and OTX008 ablated HRAS-driven tumor progression, thus yielding an additive effect over either monotherapy, and supporting a potential clinically viable approach to treat HRAS-mutant cancer through previously uncharacterized means.

The scaffold function of GAL1 supports retention of GTP-HRAS at the L_o/L_d border through mechanisms that are incompletely understood. Ectopic expression of GAL1 has been demonstrated to divert RAS signaling to the RAF/MEK/ERK effector pathway, at the expense of PI3K (20). Our data support this finding, as GAL1 inhibition by OTX008 treatment attenuated ERK activation in HRAS tumors. This effect may be attributed to the inability of lipid raft-sequestered HRAS to recruit and activate RAF1 kinase, similar to effects of the HRAS-tR variant (12). Genetically sequestering HRAS within the L_o domain reduced S6 phosphorylation, which was not observed under OTX008 treatment. While OTX008 treatment enriched HRAS-CAV1-positive fractions, it is unexpected that CAV1-positive fractions consistently spun at higher sucrose densities (Figure 3a). These observations suggest additional properties of OTX008 treatment, which need to be further explored. Thus, GAL1 inhibition blocks HRAS-induced ERK activation, potentially by destabilizing HRAS-GTP retention at the L_o/L_d border where RAF is recruited.

Development of anti-RAS therapeutic agents has turned towards targeting elements downstream of RAS, mainly either the PI3K or MAPK pathways (13-15). While several such approaches have been moderately effective, recent efforts have focused on preclinical evaluation of combination therapies. The present study demonstrates that GAL1 inhibition sensitizes HRAS-mutant tumors to rapamycin. Our results further indicate that HRAS oncogenic signaling may reflect cross-talk between the PI3K/mTOR and the RAF/MEK/ERK pathways, and that dual inhibition may ablate tumor progression by parallel blockade of these survival and proliferation pathways (Figure 4). Inhibition of GAL1 is currently under clinical evaluation, and our data support its function as a therapeutic target which may reduce progression of mutant HRAS-driven solid tumors. Moreover, preclinical and clinical trials support using mTOR inhibitors to combat RAS-mutant cancer (17, 21). The present study demonstrates the use of combining two clinically viable small-molecule inhibitors as a putative strategy for inhibiting mutant HRAS-driven tumor progression.

Novelty and Impact

The findings in this study support development of mTOR/galectin-1 dual inhibition as combinatorial anti-cancer therapy, as a novel approach to prevent tumor growth in HRAS-mutant cancers.

Conflicts of Interest

The Authors declare that there is no conflict of interest in regard to this study.

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