Disrupting BCR-ABL in Combination with Secondary Leukemia-Specific Pathways in CML Cells Leads to Enhanced Apoptosis and Decreased Proliferation

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Supporting Information

ABSTRACT: Chronic myeloid leukemia (CML) is a myeloproliferative disorder caused by expression of the fusion gene BCR-ABL following a chromosomal translocation in the hematopoietic stem cell. Therapeutic management of CML uses tyrosine kinase inhibitors (TKIs), which block ABL-signaling and effectively kill peripheral cells with BCR-ABL. However, TKIs are not curative, and chronic use is required in order to treat CML. The primary failure for TKIs is through the development of a resistant population due to mutations in the TKI binding regions. This led us to develop the mutant coiled-coil, CCmut2, an alternative method for BCR-ABL signaling inhibition by targeting the N-terminal oligomerization domain of BCR, necessary for ABL activation. In this article, we explore additional pathways that are important for leukemic stem cell survival in K562 cells. Using a candidate-based approach, we test the combination of CCmut2 and inhibitors of unique secondary pathways in leukemic cells. Transformative potential was reduced following silencing of the leukemic stem cell factor Alox5 by RNA interference. Furthermore, blockade of the oncogenic protein MUC-1 by the novel peptide GO-210 yielded reductions in proliferation and increased cell death. Finally, we found that inhibiting macroautophagy using chloroquine in addition to blocking BCR-ABL signaling with the CCmut2 was most effective in limiting cell survival and proliferation. This study has elucidated possible combination therapies for CML using novel blockade of BCR-ABL and secondary leukemia-specific pathways.

KEYWORDS: CML, coiled-coil, CCmut2, zileuton, GO-210, chloroquine, combination therapy, K562, BCR-ABL

INTRODUCTION

Chronic myeloid leukemia (CML) manifests following a reciprocal chromosomal translocation between the breakpoint cluster region (BCR) gene and the Abelson tyrosine kinase (ABL) gene [t(9;22)(q34;q11)] in the hematopoietic stem cell.1,8 Upon expression of the BCR-ABL fusion protein (a constitutively active tyrosine kinase), a leukemic stem cell (LSC) is generated, driving LSC self-renewal and expansion of BCR-ABL-expressing lineages including myeloid and lymphoid blasts.9,10 Tyrosine kinase inhibitors (TKIs) are competitive inhibitors for the ATP binding site of ABL and make up the therapeutic arsenal for disease management.3 We have previously described a unique interfering peptide, CCmut2, able to disrupt BCR-ABL homo-oligomers.4 Moreover, oligomerization is necessary for ABL activation.11 Interestingly, oligomeric disruption of trans-auto phosphorylation by CCmut2 exacts its activity via the coiled-coil domain in BCR, leading to an overall similar effect seen with TKIs: reduced phosphorylation of ABL and downstream targets STAT5 and Crk-L, induction of apoptosis, and reduction in proliferation.4,12

Single-agent TKI therapy for CML has effectively limited disease progression for the majority of patients.3 However, resistance to therapy and persistence of a subset of leukemic cells despite TKI activity3 demonstrate the necessity for multiple-agent therapy, especially to address the LSC population.10,14 Previous reports have demonstrated enhanced cytotoxicity when using a TKI in combination with a second agent targeting a BCR-ABL independent pathway,6,7,15,16 two of which have moved into clinical trials (NCT01130688; NCT01227135). Though these are promising developments to circumvent molecular failure, the TKI component will likely continue to have problems with resistance.2,17,18 The CCmut2 may be less prone to mutational resistance selection, mainly due to the highly specific and selective nature of a large interaction domain.4 This draws a parallel similar to differences in specificity between small molecules versus antibodies for cancer therapy.19 Therefore, a multiple-agent therapeutic approach involving the CCmut2 may be superior to TKI single-agent therapy (Figure 1A).

We were interested in discovering whether enhanced apoptotic activation or reduction in proliferation could be achieved by combining CCmut2 with secondary agents having independent mechanisms of action.20 Here, we detail the results
of this peptide (delivered as a gene and transcribed \textit{in vitro}) in combination with additional small molecule or biologic agents. Secondary target candidates were selected based on previous reports of drugs known to be effective when used in combination with imatinib, and those drugs were found to be effective only against leukemic (vs normal hematopoietic) cells. Additional criteria led to the selection of key proteins involved in CML progression, namely, ATG7, MUC-1, and Alox5. These targets were chosen because they do not cause loss of hematopoietic stem cell function. RNA interference (RNAi) or molecular disruption of these pathways was investigated.

The selection of and rationale for pathways and molecular agents for combination with the CCmut2 are as follows: (1) ATG7 and chloroquine (CQ). Macroautophagy (referred to as autophagy from this point on) is a cellular process activated in starvation conditions to improve recycling of cell components and enhance cell survival. This pathway becomes important in cancer as a mechanism for cell escape elimination when treated with anticancer agents. ATG7 is necessary for autophagy, and its inhibition blocks the formation of the autophagosome, an early step in the autophagy process, while CQ inhibits lysosomal acidification, which eliminates the breakdown of products contained in the autophagosome blocking cellular autophagy in the later stages. Specifically in CML, a
combination of imatinib and CQ enhance leukemic cell killing.\textsuperscript{7} For this reason, we were interested in blocking autophagy in combination with CCmut\textsuperscript{2}. (2) Alox5 and zileuton (zil). The arachidonate 5-lipoxygenase (Alox5) gene product 5-lipoxygenase (S-LO) is responsible for leukotriene synthesis from arachidonic acid (AA); reports indicate increased AA in cancer cells in general and in CML cells specifically.\textsuperscript{21,26} Kufe and colleagues have reported the association of the cytoplasmic portion of MUC-1 (MUC-1C) with BCR-ABL, enhancing the oncogenic cytoplasmic signaling of BCR-ABL. Furthermore, the use of GO-201, a specific inhibitor of MUC-1C with imatinib, has shown reduction in proliferation and induced differentiation in CML cells.\textsuperscript{5,16} These interactions are depicted in Figure 1B.

In this article, these agents in combination with CCmut\textsuperscript{2} were found to improve therapeutic potency in K562 cells. Transformative ability was most reduced by inhibiting protein expression of Alox5 using RNAi in combination with the CCmut\textsuperscript{2}. Reduction in proliferative capacity resulted largely due to CCmut\textsuperscript{2} alone but was further decreased by GO-201. Finally, increased caspase activity was seen with CQ and CCmut\textsuperscript{2}, while combinations of either GO-201 or CQ and CCmut\textsuperscript{2} enhanced the apoptotic and necrotic cell population as visualized by Annexin-V and 7-AAD staining.

\section*{MATERIALS AND METHODS}

\textbf{Constructs.} RNAi constructs were targeted against human Atg7, Alox5, MUC-1, or luciferase control. Target sequences for Atg7 or MUC-1 were derived from previous reports,\textsuperscript{6,7} while the Alox5 (NM_000698) RNAi sequence was designed using BLOCK-IT RNAi Designer (Life Technologies, Grand Island, NY). RNAi sequences are as follows: Alox5 (5′-ggaatagctcgcgcaatttg-3′); Atg7 (5′-cagttgtagatcataacgatg-3′); MUC-1C (5′-aagttgtagccgcaattctga-3′).\textsuperscript{6} Oligonucleotides encoding short hairpins against the following transcripts were synthesized at the University of Utah core facilities: Alox5 top (5′-gageggagacttcgcgcaattctgaagctgagcagactccttttggaga-3′) and bottom (5′-ggcggctgccaaagggatacctgcaaccttgacatctttg-3′); Atg7 top (5′-gatcctgctatcattttcatcactaacaagctga-3′) and bottom (5′-ggttgagatttagctacgctttggagtttttgtgaacctggtc-3′); MUC-1C (5′-aagttgtagccgcaattctga-3′).\textsuperscript{6} Oligonucleotides encoding short hairpins against the following transcripts were synthesized at the University of Utah core facilities: Alox5 top (5′-gageggagacttcgcgcaattctgaagctgagcagactccttttggaga-3′) and bottom (5′-ggcggctgccaaagggatacctgcaaccttgacatctttg-3′); Atg7 top (5′-gatcctgctatcattttcatcactaacaagctga-3′) and bottom (5′-ggttgagatttagctacgctttggagtttttgtgaacctggtc-3′); MUC-1C (5′-aagttgtagccgcaattctga-3′).\textsuperscript{6}

\textbf{Colonization Assay.} pGSH1 constructs expressing shRNA sequences against Atg7, MUC-1, or Alox5 were transfected and cultured 4 days to ensure knockdown. One day following transfection, gentamicin reagent (Life Technologies) was added at a concentration of 500 μg/mL in complete RPMI. On day 4, a second construct was transfected (pEGFP-C1; −CC, or −CCmut\textsuperscript{2}). Dual-transfected cells were resuspended in Iscove’s Modified Dulbecco’s Medium containing 2% FBS (Stem Cell Technologies, Vancouver, BC, Canada), and 1,000 cells were seeded in Methocult H4230 methylcellulose medium (Stem Cell Technologies). Imatinib mesylate (IM #CT-IM001, Chemie-Tek, Indianapolis, IN) was added to untransfected K562 cells in Methocult at the time of seeding. Transformation potential was assessed 7 days after seeding cells by counting colonies in 200 μm\textsuperscript{2}.

\textbf{Drug Treatments.} In all cases, small molecule or peptide-based inhibitors were added to transfected cells 6 h after transfections unless otherwise noted. GO-201 (Sigma-Aldrich, #G79293) is a well-described peptide inhibitor of MUC-1C\textsuperscript{27} and was used at a final concentration of 5 μM in 1× PBS (Life Technologies, #14190-144). Zileuton (Sigma-Aldrich, #Z4277) is a small molecule inhibitor active against 5-lipoxygenase (the protein product of Alox5) and was used at a final concentration of 20 μM dissolved in DMSO. Chloroquine (Sigma-Aldrich, #C6628) is an inhibitor of lysosomal acidification (autophagosome activation) and was used at a final concentration of 10 μM.

\textbf{Cell Proliferation.} K562 cells were transfected with EGFP, CC, or CCmut\textsuperscript{2}, followed by drug treatment 6 h later. Trypan blue exclusion\textsuperscript{8} was assessed 48 h after transfection to determine cell proliferation/viability.

\textbf{Caspase 3/7 Assay.} Cells were transfected as indicated above (in Cell Lines and Transfections) followed by drug treatment 6 h later. Forty-eight hours following transfection, cells were counted, pelleted, and frozen at −80 °C overnight. Cells were resuspended, lysed, and processed according to the Caspase Glo 3/7 manufacturer’s instructions (Promega, Madison, WI). Luminescence was measured after 1 h at 26 °C.

CO\textsubscript{2}. DNA constructs were transfected with the Ammax nucleofection system (Lonza Bio, Basel, Switzerland), using 6 μg of DNA in 100 μL of Solution V with 2 million cells, and then returned to complete RPMI medium.

\textbf{Western Blotting.} Cells were counted on a hemocytometer, pelleted, and frozen at −80 °C overnight. Cell pellets were resuspended in RIPA lysis and extraction buffer (Thermo Scientific/Pierce Protein Biology Products, #89900, Kalamazoo, MI). A BCA Protein assay was performed (Thermo Scientific, #23225), and 10 μg of protein was loaded in each lane of a denaturing gel. Standard Western blotting procedures were used.\textsuperscript{4} Antibodies used to detect Atg7 (Sigma-Aldrich, #A2856, St. Louis, MO), LC3A/B (Sigma-Aldrich, #L8918), MUC-1C (Thermo Scientific, #MUC-1 Ab-5/HM-1630), Alox5 (Abcam, #ab115764, Cambridge, MA), actin (Abcam, #ab1801), and eIF4E (Cell Signaling Technology, #C461H6, Danvers, MA) primary antibodies were used. HRP conjugated secondary antibodies were anti-Armenian hamster (Abcam, #ab5745) or anti-rabbit (Cell Signaling Technology, #7074). Quantification of bands using relative densitometry was completed using AlphaView SA (Protein Simple, v3.0, Santa Clara, CA). For LC3I/I ratios, background corrected sum values for each band were calculated as a ratio to eIF4E control. Then LC3-II percent control was divided by LC3-I percent control. This gives a relative ratio of LC3-II to LC3-I conversion.

\textbf{Colony Forming Assay.} pGSH1 constructs expressing shRNA sequences against Atg7, MUC-1, or Alox5 were transfected and cultured 4 days to ensure knockdown. One day following transfection, gentamicin reagent (Life Technologies) was added at a concentration of 500 μg/mL in complete RPMI. On day 4, a second construct was transfected (pEGFP-C1; −CC, or −CCmut\textsuperscript{2}). Dual-transfected cells were resuspended in Iscove’s Modified Dulbecco’s Medium containing 2% FBS (Stem Cell Technologies, Vancouver, BC, Canada), and 1,000 cells were seeded in Methocult H4230 methylcellulose medium (Stem Cell Technologies). Imatinib mesylate (IM #CT-IM001, Chemie-Tek, Indianapolis, IN) was added to untransfected K562 cells in Methocult at the time of seeding. Transformation potential was assessed 7 days after seeding cells by counting colonies in 200 μm\textsuperscript{2}.
°C on a SpectraMax M2 plate reader (Molecular Devices, Sunnyvale, CA).

**Annexin-V/7-AAD Assay.** Cells were transfected and treated as described above (in Cell Lines and Transfections) followed by drug treatment 6 h later. At 48, 72, or 96 h, cells were resuspended in 0.5 mL of Annexin-V binding buffer (Life Technologies, #V13246) stained with 1 nM 7-AAD (Life Technologies, #A1310) and a 1:20 dilution of Annexin-V-APC (Life Technologies, #A35110). Samples were sorted using a BD FACSCanto II flow cytometer according to GFP positivity (10,000 GFP events were collected). Cells were then sorted according to apoptotic (Annexin-V) and necrotic (7-AAD) markers. Data was further analyzed using FlowJo flow cytometry analysis software (Tree Star Inc., Ashland, OR).

**Statistics.** Data are expressed as the means ± SEM from at least 3 independent experiments. Significance of differences between groups was assessed in GraphPad Prism 5 (GraphPad Software, La Jolla, CA) using either a Student’s t test, two-way ANOVA with a Bonferroni post-test, or one-way ANOVA with Bonferroni post-test. A p-value of <0.05 was considered significant.

**RESULTS**

**BCR-Based Inhibition of BCR-ABL with Alox5 Knockdown Reduces the Transformation Potential of K562 Cells.** 5-Lipoxygenase (5-LO), the protein product of Alox5, mediates processes such as inflammation and oxidative stress through leukotriene synthesis. Because of this, 5-LO antagonists are an important therapy for inflammatory diseases and...
have also been suggested for cancer therapy.\textsuperscript{28} Reports of potential antiproliferative effects in hematologic malignancies from the loss of Alox5 or 5-LO inhibitors surfaced in the 1980s\textsuperscript{29,30} and recently bolstered in a CML in vivo model by data from Chen and colleagues.\textsuperscript{5,13,31}

To determine the contribution of several pathways to transformative ability (measured by colony forming cells), selected pathways were disrupted by knockdown of key genes regulating each pathway using shRNA expressing constructs. Western blotting for protein products of Atg7, MUC-1, and Alox5 demonstrated successful knock down of all targets (Figure 2A, second lane of each pair) when compared to that in control shRNA against luciferase control (Figure 2A, shLuc, first lane of each pair). These data are quantified using band densitometry and expressed as percent shLuc (Figure 2B). These constructs were then used in combination with GFP control, wild-type coiled-coil (CC), or mutant coiled-coil (CC\textsuperscript{mut2})\textsuperscript{4} in a week long transformation study. Transient expression of shAlox5 and CC or CC\textsuperscript{mut2} resulted in significant reduction of colonies (Figure 3, shAlox5 group, far right bars) compared to that in GFP control and shAlox5 dual expression (Figure 3, shAlox5 group, third to right bar, gray).

**Chloroquine Combinations Block an Upregulated Autophagy Pathway Following BCR-ABL Inhibition.**

Autophagy is a degradative process used by cells to break down intracellular material via lysosomes. Autophagy can promote or suppress oncogenesis depending on the cellular context;\textsuperscript{22} however, induction of autophagy provides a survival mechanism in BCR-ABL cells treated with imatinib (cells undergoing stress; see Figure 1B).\textsuperscript{7,32} Though previous reports indicated enhanced autophagy following the introduction of kinase inhibitors,\textsuperscript{7,32,35} this pathway has not been previously investigated following the expression of the CC\textsuperscript{mut2}. Additionally, since no reduction in transformative ability was observed following the knockdown of Atg7, we proceeded to investigate whether autophagy is activated following the transfection of GFP, CC, or CC\textsuperscript{mut2}. The conversion of microtubule associated protein light chain 3 (LC3) from LC3-I to LC3-II was used to monitor autophagy. This is the case when comparing lanes 3 and 4 or lanes 7 and 8. Because cellular levels of LC3-II are indicative of the number of autophagosomes (e.g., an increase in autophagy),\textsuperscript{7,34} increases in LC3-II protein expression were measured by immunoblotting. When no autophagy occurs, a more prominent LC3-I band will be visible, indicating the presence of the precursor and cytoplasmic LC3-I (i.e., LC3/I >1.0).\textsuperscript{34,35} This is visible in Figure 4, lanes 1–2 and 5–6. When autophagy occurs, and inhibitors are added to eliminate LC3-II breakdown via the lysosome, LC3-II becomes the prominent band (i.e., LC3II/I >1.0).\textsuperscript{34,35}

LC3-II/LC3-I ratios\textsuperscript{7,35} were calculated following exposure to 5 \(\mu\)M imatinib (Figure 4, lanes 3 and 4) or transfections and with or without CQ at 24 h (Figure 4, lanes 5–8). The data represented in Figure 4 demonstrate little to no activation of autophagy in untreated or GFP transfected K562 cells (Figure 4, lanes 1–2, 5–6). Autophagy is activated by inhibition of BCR-ABL using either BCR or ABL inhibitors at 24 h (Figure 4, lanes 3–4; 7–8). Similar trends were seen at 12 h (data not shown).

**CQ and GO-201 Further Diminish the Proliferative Capacity of K562 Cells beyond the Reductions Seen Using CC\textsuperscript{mut2} Alone.** MUC-1 is a membrane-bound glycosylated phosphoprotein whose normal function protects the body from chemicals/bacteria through its polarized expression on epithelial cell surfaces.\textsuperscript{21} The cytoplasmic domain of MUC-1, called MUC-1C, interacts with the BCR portion of BCR-ABL to promote continued oncogenic signaling.\textsuperscript{6} A peptide inhibitor of MUC-1C has been shown to downregulate BCR-ABL and inhibit cell growth,\textsuperscript{16} indicating its value as a therapeutic target.

We previously demonstrated\textsuperscript{4} decreased proliferation following the transient expression of CC\textsuperscript{mut2}, and these data are represented here (Figure 5, vehicle treated). To determine the effects of selected pathways in combination with BCR-mediated inhibition of ABL by the mutant coiled-coil, additional experiments were carried out as before\textsuperscript{4} with drugs added 6 h after transfection. Untreated groups were scaled to previous data and compared to treated cells at 48 h. A significant reduction was seen across all samples when treated with the CC\textsuperscript{mut2} (Figure 5, white bars), consistent with previous work,\textsuperscript{4} and further reductions were seen in the CQ and GO-201 treated groups when transfected with CC\textsuperscript{mut2} (CQ and GO-201 groups, white bars). In fact, GO-201 treatment reduces proliferation independent of the transfected group but further diminishes reduction in proliferation most in combination with the mutant coiled-coil (GO-201 group, white bars).

**CQ Enhances the Activity of Effector Caspase 3/7 in Concert with CC\textsuperscript{mut2}.** We next investigated whether increases in apoptotic signaling could be enhanced using the transient transfection of constructs with drug combinations at 48 h. Early apoptotic events can be indicated by executioner caspase 3 or 7 cleavage products. These products were measured using a luminescent DEVD substrate.\textsuperscript{36} The only combination which resulted in a significant (3 fold) increase in caspase 3/7 activity was the CC\textsuperscript{mut2} with the CQ treated group (Figure 6; compare GFP and CC\textsuperscript{mut2} bars in the CQ group). Other combinations did not significantly enhance caspase 3/7 activity.

**Increased Apoptotic and Necrotic Activity Are Observed Following CQ Combinations.** In addition to effector caspase activity, later stage apoptosis and necrosis were measured using cell permeable reagents demonstrating apoptotic (phosphatidylserine externalization recognized by Annexin-V) or necrotic (nuclear membrane permeability by DNA intercalation of 7-AAD) events. At the earliest time measured, CQ and GO-201 increased cell death response (Figure 7A and D), though GO-201 affected only the CC\textsuperscript{mut2} transfected population, and was not significantly different from the vehicle treated group (Figure 7D). CQ has a broader impact potentiating the CC transfected population specifically (Figure 7A). Seventy-two hours after initial transfection, the singular impact of the CC\textsuperscript{mut2} transfected cells becomes clear as significant increases are seen in the apoptotic population (Figure 7B). The GO-201 apoptotic increase seen at 48 h
DISCUSSION

In this study, we report that drug and transfection combinations can enhance elimination of K562 CML cells. Using a novel genetic therapy (CCmut2), we demonstrated enhanced apoptotic cell death and reduced proliferation ability of CCmut2 expressing K562 cells consistent with previous reports. We then evaluated the transformative and proliferative capacity of cells using a combination of constructs or drugs focused on inhibiting BCR-ABL signaling and other leukemia-specific pathways. Though we did not see a significant reduction in transformative ability with the transfection of a single plasmid alone, the combination of constructs targeting Alox5 knockdown with CCmut2 elucidated the importance of Alox5 in long-term cell survival and colony forming ability (Figure 3). Previous studies involving Alox5 in CML do not address the transformative ability of K562 cells but focus more on stem-like cells in vivo models. Though other assays did not indicate a robust role for Alox5 in apoptosis or proliferation, the fact that Alox5 could be an important target in blast-phase CML cells in addition to a primordial CML stem cell is an interesting observation.

We also demonstrated further reduction in proliferative capacity resulting from the combination of GO-201 or CQ and CCmut2 transfections (Figure 5). Additionally, effector caspase 3 and 7 activation increased following the combination with CQ at 48 h (Figure 6). Finally, combining CQ and CCmut2 appear to enhance apoptosis/necrosis when both exist in the cell long enough to have an effect individually (Figure 7). Interestingly, increases in apoptosis and necrosis were primarily due to the small molecule drug (CQ) or peptide therapeutic (GO-201), then later affected by the transfection (Figure 7B–C and E–F). This indicates a temporal shift in the activity and effect of the therapeutic, which may become important in future studies. Together, these data suggest further increases are achievable using a second agent in addition to the therapeutic interfering peptide CCmut2. Specifically, combinations with CCmut2 and CQ appear to have a broad effect on proliferation and apoptosis, followed by GO-201. In fact, the hydroxychloroquine derivative of CQ is currently being investigated as an adjuvant to IM therapy for CML in a clinical study (NCT01227135). Future studies will evaluate the most potent combinations in primary patient samples, including stem-like CML cells. Importantly, this article demonstrates the efficacy of a combination based approach using small molecule or peptide-based inhibitors to target both the causative oncogenic BCR-ABL but also key alternative pathways in a blast-crisis CML cell line.

Figure 5. K562 cell proliferation as indicated by trypan blue dye exclusion reveals that transfected cells subsequently treated with drugs can further depress the proliferative capacity of K562 cells. CCmut2 has a significant effect alone, compared to that of the GFP control. Drug addition does not significantly affect GFP-treated cells, with the exception of GO-201. CC + drug and CCmut2 + drug have further reductions in proliferation compared to that of vehicle treated cells. GO-201 combined with CCmut2 has a potent reduction in proliferation compared to that of other drugs at 48 h. Two-way ANOVA and the Bonferroni post-test have the following significance levels: *p < 0.05, **p < 0.01, ***p < 0.001, n ≥ 3. ψ indicates at least p < 0.05 following a one-way ANOVA comparing the drug treated sample to the control (vehicle treated) in matched transfected groups, Bonferroni post-test.

Figure 6. Apoptosis induction measured by activated caspase 3 or 7 is increased in CQ treated cells when comparing GFP control and CCmut2. Data is expressed as fold vehicle control. Two-way ANOVA with Bonferroni post-tests. n = 5; *p < 0.05.
ASSOCIATED CONTENT

Supporting Information
Statistical details for 2-way ANOVA. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes
The authors declare no competing financial interest.

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