Down-regulation of Myeloid Cell Leukemia-1 through Inhibiting Erk/Pin 1 Pathway by Sorafenib Facilitates Chemosensitization in Breast Cancer

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Abstract

Myeloid cell leukemia-1 (Mcl-1), a Bcl-2-like antiapoptotic protein, plays a role in cell immortalization and chemoresistance in a number of human malignancies. A peptidyl-prolyl cis/trans isomerase, Pin1 is involved in many cellular events, such as cell cycle progression, cell proliferation, and differentiation through isomerizing phosphorylated substrates. It has been reported that down-regulation of Pin1 induces apoptosis, and that Erk phosphorylates and up-regulates Mcl-1; however, the underlying mechanisms for the two phenomena are not clear yet. Here, we showed that Pin1 stabilizes Mcl-1, which is required for Mcl-1 phosphorylation by Erk. First, we found expression of Mcl-1 and Pin1 were positively correlated and associated with poor survival in human breast cancer. We then showed that Erk could phosphorylate Mcl-1 at two consensus residues, Thr 92 and 163, which is required for the association of Mcl-1 and Pin1, resulting in stabilization of Mcl-1. Moreover, Pin1 is also required for the up-regulation of Mcl-1 by Erk activation. Based on this newly identified mechanism of Mcl-1 stabilization, two strategies were used to overcome Mcl-1-mediated chemoresistance: inhibiting Erk by Sorafenib, an approved clinical anticancer drug, or knocking down Pin1 by using a siRNA technique. In conclusion, the current report not only unravels a novel mechanism to link Erk/Pin1 pathway and chemoresistance: inhibiting Erk by Sorafenib, an approved MAPK signaling or angiogenesis (8, 9). Thus far, the molecular mechanisms involving Sorafenib-induced apoptosis are not well-characterized.

Myeloid cell leukemia-1 (Mcl-1), a Bcl-2-like antiapoptotic protein containing three BH domains (BH1-3) without a defined BH4 domain at the NH2 terminus, was originally identified in differentiating myeloid cells (10). Mcl-1 also has a transmembrane domain at the COOH terminus as a localization signal to translocate to various intracellular membranes, especially to the outer mitochondrial membrane (11). Mcl-1 can be rapidly degraded by certain death-inducing signals, but it is able to be readily induced by diverse survival cytokines such as epidermal growth factor, vascular endothelial growth factor, granulocyte-macrophage colony-stimulating factor, and interleukin 3 through phosphatidylinositol-3-OH kinase/Akt, MEK/MAPK, or Janus-activated kinase/STAT signaling cascades (12, 13). The rapid induction and degradation of Mcl-1 suggests that Mcl-1 can serve as a sensor of acute environmental changes to balance between cell survival and death, which is an essential factor for embryogenesis and for maintenance of the development of both B and T lymphocytes (14, 15). Furthermore, increased Mcl-1 expression may enhance the general well-being of cells, not only by promoting short-term viability in a wide range of cells but also by contributing to long-term immortalization and tumorigenesis in certain cells (16–18). Meanwhile, it is well-documented that overexpression of Mcl-1 plays a role in chemoresistance in a number of human

Introduction

The constitutive activation of certain cellular signaling pathways contribute to tumor development and resistance to chemotherapy (1, 2). Upon growth factor stimulation, RAS-RAF-extracellular signal-regulated kinase (ERK)/Mitogen-activated protein kinase (MAPK) triggers a series of cascade events that govern cell transformation, differentiation, proliferation, and survival (3, 4). There are ~30% of tumors that harbor active mutants of the RAS oncoprotein, and a downstream effector of RAS, RAF, is also frequently mutated in tumors (5, 6). Together, the active RAS-RAF-MAPK/ERK kinase (MEK) pathway inevitably accounts for a hallmark of malignant phenotypes: abnormal cell growth, invasion, and angiogenesis. Therefore, interruption of the RAS-RAF-MEK cascade has become a primary target for cancer therapy. Small molecule inhibitors targeting RAF and MEK kinase have shown promising results in cancer treatment. For instance, Sorafenib (BAY 43-9006), a multikinase inhibitor, was approved by the Food and Drug Administration (FDA) for treating patients with renal cell carcinoma (7). Sorafenib was initially identified as a RAF kinase inhibitor, and later, it was found to be able to inhibit the MAPK pathway in multiple cancer cell lines. In human xenograft models including ovarian, colon, lung, pancreatic, and melanoma, Sorafenib inhibits tumor growth through inhibition of either MAPK signaling or angiogenesis (8, 9). Thus far, the molecular mechanisms involving Sorafenib-induced apoptosis are not well-characterized.

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).
malignancies, such as breast cancer, leukemia, melanoma, pancreatic cancer, hepatocellular carcinoma, and others (13, 19). Recently, GSK-3β, a multifunctional serine/threonine protein kinase, was identified as an upstream regulator, which phosphorylates Mcl-1 and then recruits E3 ligase β-Trcp to degrade Mcl-1, leading to apoptosis and tumor suppression. Through this mechanism, GSK-3β enhances chemosensitization (20, 21). However, it is still not clearly understood how Mcl-1 is stabilized in cancer cells.

Pin1, a recently identified peptidyl-prolyl cis/trans isomerase (PPIase), has two domains: a PPIase domain at its COOH terminus responsible for isomerization and a WW domain at the NH2 terminus, which functions as a binding element specific for pSer/Thr-Pro motifs (22–25). Through these two domains, Pin1 binds to and isomerizes specific pSer/Thr-Pro motifs and catalytically induces conformational changes after phosphorylation. Such conformational changes can have profound effects on the function of many Pin1 substrates, such as p53, cyclin D1, C-jun, nuclear factor-κB, C-myc, E2F, β-catenin, and APP, thereby playing an important role in many cellular events, such as cell cycle progression, transcriptional regulation, RNA processing, and cell proliferation and differentiation (26–32). It has been reported that down-regulation of Pin1 induces apoptosis (33–35), but the mechanism is not clearly understood. It is also known that Erk phosphorylates Mcl-1 and up-regulates its expression (36), and that the phosphorylation site of Erk is pSer/Thr-Pro, which is also the consensus motif recognized by Pin1. In the current study, we showed that Pin1 stabilizes the antia apoptotic protein, Mcl-1, which is activated by Erk/MAPK pathway.

Materials and Methods

Constructs and reagents. Pin1, Pin1-W34A, and Pin1-R68,69A were kindly provided by Dr. K.P. Lu (Department of Medicine, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA). pHA-hMcl-1 was kindly provided by H-F. Yang-Yen (Institute of Molecular Biology, Academia Sinica, Taipei, Taiwan). The full-length hMcl-1 cDNA was subcloned into vectors, pCMV5-MYC and pGEX-6P-1. Using the QuickChange multiplex-directed mutagenesis kit (Stratagene), all Mcl-1 mutants were generated according to the manufacturer’s protocol, and were further verified by automated sequencing. U0126, an MEK inhibitor, was purchased from Cell Signaling. MG132 (a proteasome inhibitor) and 12-O-tetradecanoylphorbol-13-acetate (TPA) were purchased from Sigma.

Cell culture, transfection, proliferation, and apoptosis assays. Breast cancer cell lines MDA-MB-231, MDA-MB-453, MCF-7, BT20, T47D, BT474, HBL100, and ZR75-1; ovarian cancer cell lines Ovar920, Ovar3, and 2774; pancreatic cancer cell AsPC1, PANC1, Colo357, Colo535, and HPAC; colon cancer cell lines SW480, Caco2, RKO, and DLD1; lung cancer cell lines A549, H1435, and H1975; hepatoma cell line Hep3B; and cervical cancer cell line HeLa were bought from American Type Culture Collection. Cells were grown in DMEM/F12 medium supplemented with 10% fetal bovine serum. Transient transfections with DNA were performed with an optimal ratio of DNA to liposome based on prior experiments (21). siRNA-mediated down-regulation of Pin1 was performed by transfecting SMART pool–specific or nonspecific control pool double-stranded RNA oligonucleotides (Dharmacon) using Lipofectamine 2000 (Invitrogen). The percentage of surviving cells upon chemo-drug treatment was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The apoptotic cells were assessed by a flow cytometry assay (FACS) as previously described (20). Briefly, trypsinized cells were washed with PBS and then fixed with 70% ethanol overnight at −20°C. Before FACS analysis, cells were washed with PBS, and then fluorochrome solution (50 μg/mL propidium iodide plus 25 μg/mL RNAse in PBS) was added. Each experiment was performed in triplicate, and error bars represent mean ± SE.

Immunoblotting, immunoprecipitation, and in vitro kinase assay. Immunoblotting and immunoprecipitation (IP) were performed as previously described (37), with the following antibodies: mouse and rabbit anti-Mcl-1 (BD Transduction Labs and Biolegend); mouse anti–GSK-3β (BD

Figure 1. Expression of Mcl-1 correlates with Pin1, which is associated with poor survival in 101 human breast cancer specimens. A, consecutive tissues from human primary breast tumor specimens were immunostained with antibodies specific to Pin1 and Mcl-1, respectively. Case 1 is a representative specimen with high expression of Pin1 and Mcl-1; case 2 is a specimen with low or no expression of Pin1 and Mcl-1. B, relationship between expression of Mcl-1 and Pin1 in breast cancer specimens. C, Kaplan-Meier overall survival curves for breast cancer patients with correlated expression of Mcl-1 and Pin1.
Transduction Labs); HA-tag, Myc-tag, and Tubulin (Sigma); phospho-Erk and Erk (Upstate Biotechnology); and rabbit anti-Pin1 (Santa Cruz Biotechnology). For quantification of the Western analysis, the density of each band was quantified by Quantity One software; the background of each selected area (band) was automatically calculated and then deducted by the software. For in vitro kinase assays, purified glutathione S-transferase (GST)-Mcl-1 protein was incubated with activated Erk1 (Upstate) in the presence of 50 mmol/L ATP in kinase buffer for 30 min at 30°C. Reaction products were resolved by SDS-PAGE and then visualized by autoradiography.

Immunohistochemical staining. For immunohistochemical staining of human breast cancer tissue samples, each sample was stained with specific antibodies as indicated and scored by an H-score method that combines the values of immunoreaction intensity and the percentage of tumor cell staining. Briefly, the immunoreactivity of Pin1 and Mcl-1 were ranked into 2 groups according to the percentage of the positive tumor cells: positive (+, >10%), and negative (− and +, <10%). One hundred and one surgically resected human breast cancer specimens were collected from the Department of Pathology, Shanghai East Breast Disease Hospital, P. R. China.

Statistical analysis. The linear regression analysis was used to analyze the correlation between Pin1 expression and level of Mcl-1 in multiple cell lines. The χ² test was used to analyze the relation between Mcl-1 level with Pin1 in 101 primary breast cancer tissues. Kaplan-Meier curves for the overall survival of 101 primary breast cancer patients were plotted and compared. Log-rank test was used to evaluate the difference in expression between three groups of Mcl-1 and Pin1 as indicated with regard to overall survival. Statistical analysis and graphs were performed with SPSS software, and the level of significance was set at 0.05.

Results

Expression of Mcl-1 and Pin1 is positively correlated and associated with poor survival in human breast cancer. To investigate the pathological relevance of the relationship between Pin1 and Mcl-1 expression in vitro, we analyzed these 2 proteins in 101 human breast cancer tissue samples. Immunohistochemical staining showed that there was a positive correlation between the levels of Mcl-1 and Pin1 in human breast cancer samples (P = 0.043; Fig. 1A and B). We then compared the expression of Mcl-1 and Pin1 in tumor tissues with the patients’ survival follow-up. The results indicated that the patients’ survival was decreased significantly with the coexpression of Mcl-1 and Pin1, whereas negative Mcl-1 and Pin1 expression was significantly associated with an increase of overall survival (Fig. 1C). Thus, the expression level of Mcl-1 and Pin1 may be a potential candidate for a predictive marker of poor prognosis in breast cancer patients.

Pin1 stabilizes Mcl-1. To validate the relevance of the relationship between Pin1 and Mcl-1 expression observed in human breast cancer samples, we compared the expression of the two proteins in a panel of 24 cancer cell lines of breast, ovarian, pancreas, colon, and lung. As expected, Mcl-1 expression was significantly correlated with Pin1 expression (Fig. 2A). To further investigate whether Pin1 may affect Mcl-1 level, we first examined the Mcl-1 level in MEF wild-type (WT) cell and Pin1 knockout MEF cells.
Figure 3. Erk phosphorylates Mcl-1 at two residues. A, mass spectral analysis of Mcl-1 phosphorylation by Erk. Purified GST-Mcl-1 protein was incubated with active Erk1 kinase in the presence of 50 mmol/L ATP in a kinase buffer for 30 min at 30°C. Reaction products were subjected to SDS-PAGE and then stained with Coomassie blue, and the phosphorylated samples were analyzed by Mass Spectrometry. B, different mutants of GST-Mcl-1 protein as indicated were incubated with active Erk1 kinase, the kinase assay was performed as described in Materials and Methods, and then the reaction samples were subjected to SDS-PAGE, and 32P-labeled proteins were visualized by autoradiography.
(Pin⁻/⁻ MEF) cell. The results showed that Mcl-1 level was decreased in Pin⁻/⁻ MEF cells (Fig. 2B) and was recovered by introducing WT Pin1 expression into Pin⁻/⁻ MEF cell. However, dominant-negative Pin1 (R68,69APin1), which lost its PPIase function, did not increase Mcl-1 expression compared with WT Pin1 (Fig. 2C), indicating that Pin1 may stabilize Mcl-1. As GSK-3β is known to degrade Mcl-1, we cotransfected GSK-3β with either WT Pin1 or R68,69APin1 in 293T cells and showed that only the WT Pin1 counteracted GSK-3β-mediated Mcl-1 degradation to further support the notion that Pin1 stabilizes Mcl-1 (Fig. 2D). Additional studies confirmed that Pin1 inhibits GSK-3β–induced ubiquitination of Mcl-1 (Supplementary Fig. S1).

Pin1 associates with and stabilizes Mcl-1, which depends on Mcl-1 phosphorylation at two residues by Erk. Because Erk has been shown to up-regulate Mcl-1, and the phosphorylation site of Erk is pSer/Thr-Pro motif and is also recognized by Pin1, we investigated whether Erk may play a role in the Pin1-mediated stabilization of Mcl-1. First, the results of Mass Spectrometry analysis indicated that Erk phosphorylated Mcl-1 not only at residue Thr 163 but also at residue Thr 92 (Fig. 3D). To further validate the mass spectrometry data, we then constructed mutants in which the two potential threonine phosphorylation sites of Mcl-1 were changed to alanine. We found that the double mutation of Mcl-1-92/163AA could completely abrogate the phosphorylation

Figure 4. Pin1 associates with and stabilizes Mcl-1, which depends on Mcl-1 phosphorylation by Erk. A, Mcl-1 (Myc-tag) was transfected into 293T cells with WT Pin1 or WW domain mutant Pin1-W34A (HA-tag), and then Mcl-1 or Pin1 were IP from transfected 293T cell lysates (1,000 μg per lane) and subjected to Western blotting. B, Mcl-1 (Myc-tag) was transfected into 293T cells with WT Pin1 or Pin1-92/163AA mutant (HA-tag), and then Pin1 was IP from transfected 293T cell lysates (1,000 μg per lane) and subjected to Western blotting. C, HeLa cell (left) and MCF7 cell (right) were treated with mitogen activator TPA alone or with MAPK inhibitor U0126 for 4 h, and then endogenous Mcl-1 was IP from treated cell lysates and subjected to Western blotting. D, Mcl-1-WT, Mcl-1-92/163AA, or Mcl-1-92/163DD mutant was transfected into 293T cells, and cells were then treated with cycloheximide (20 mmol/L) for the indicated times. Cell lysates were analyzed by Western blotting against Myc-Mcl-1. Equal amounts of protein were subjected to Western blot analyses, as determined by comparing amounts of tubulin (top). Densitometry results for Mcl-1-WT, Mcl-1-92/163AA, or Mcl-1-92/163DD after cycloheximide treatment were plotted, and the half-lives of Mcl-1-WT, Mcl-1-92/163AA, and Mcl-1-92/163DD were determined (bottom).
of Mcl-1 by Erk (Fig. 3B). Between the two single mutations, 92A and 163A, Mcl-1-163A exhibited better inhibition of phosphorylation by Erk, indicating that 163Thr could be the major phosphorylation site of Mcl-1 by Erk (Fig. 3B).

Next, we tested the association between Mcl-1 and Pin1, and the results of IP showed that WT Pin1 but not the WW domain mutant Pin1-W34A is able to bind with Mcl-1 (Fig. 4A). The mutant of Mcl-1 92/163AA, which cannot be phosphorylated by Erk, dramatically decreased its association with Pin1. However, the mutant of Mcl-1-64A, of which Thr 64 is recently identified as a CDK phosphorylation site to stabilize Mcl-1 (38), still strongly associated with Pin1 (Fig. 4B), suggesting that Mcl-1 phosphorylation at 92/163 residues by Erk may be required for association of Mcl-1 and Pin1. Furthermore, the results are supported by the fact that Erk activation by TPA, a mitogen activator, enhanced the association of Mcl-1 and Pin1 and inhibition of Erk activation by U0126, a MAPK inhibitor, significantly decreased their binding (Fig. 4C). Because the two phosphorylation sites of Mcl-1 are required for the association of Mcl-1 and Pin1, we then investigated whether the two sites are required for Mcl-1 stabilization. We showed that Mcl-1-92/163DD, a phosphorylation-mimicking mutant, was more stabilized than WT Mcl-1 and Mcl-1-92/163AA, a nonphosphorylation mutant (Supplementary Fig. S2). Mcl-1-92/163AA, a nonphosphorylation mutant, was rapidly degraded; however, Mcl-1-92/163DD, a phosphorylation-mimicking mutant, exhibited longer stability by nearly 2-fold compared with WT Mcl-1 (8 versus 4 h; Fig. 4D).

**Down-regulation of Pin1 induces apoptosis and chemosensitization.** Because phosphorylation of Mcl-1 by Erk is required for the association and stabilization of Mcl-1 by Pin1, we investigated whether Pin1 is required for Erk-induced Mcl-1 up-regulation. SiRNA against Pin1 was performed to down-regulate Pin1. As expected, down-regulation of Pin1 blocked TPA-induced Mcl-1 up-regulation (Fig. 5A). Because down-regulation of Pin1 decreased Mcl-1 expression, and Mcl-1 is known to induce antiapoptosis and chemoresistance, we next examined whether down-regulation of Pin1 may render apoptosis and chemosensitization. In MCF7 cells, SiRNA against Pin1 led to a moderate amount apoptosis compared with SiRNA control (11% versus 4%), which was blocked by cotransfection with Mcl-1 (Fig. 5B). Furthermore, SiRNA against Pin1 significantly sensitized Taxol, and 5-fluorouracil (5-Fu) caused-cell death by almost 2-fold compared with control Si-RNA, which was also overridden by overexpression of Mcl-1 (Fig. 5C), indicating that down-regulation of Pin1 facilitates chemosensitization through down-regulation of Mcl-1.

**Sorafenib sensitizes chemotherapy through down-regulation Mcl-1.** Phosphorylation by Erk is required for Pin1-mediated Mcl-1 stabilization; therefore, we next investigated whether inhibition of the Erk pathway could overcome Mcl-1-mediated chemoresistance. We found that Sorafenib, a RAF/MAPK pathway inhibitor that is recently approved by the FDA, suppressed Mcl-1 expression in a dose-dependent manner in two breast cancer cell lines, MCF7 and MDA-MB435 (Fig. 6A). We then combined Sorafenib and Taxol or 5-Fu to treat MCF7 cells and found that it dramatically sensitized the tumor cell to the chemodrug Taxol or 5-Fu in a dose-dependent manner (Fig. 6B). The sensitized killing effects were much more significant when Mcl-1 expression was inhibited by Sorafenib at 5 and 10 μmol/L (*, P < 0.05). We further investigated whether overexpression of Mcl-1 could override Sorafenib-caused chemosensitization. The results showed that transfection of Mcl-1 blocked Taxol and Sorafenib-caused cell death, and cotransfection of GSK-3β, which is known to degrade Mcl-1 cells, were sensitized to Taxol and Sorafenib-caused cell death again. However, transfection of Mcl-1 92/163DD, a phosphorylation-mimicking mutant, or transfection of Mcl-1 plus Pin1, was still resistant to Taxol plus Sorafenib–caused cell death, even
under the treatment of GSK-3β (Fig. 6C). Taken together, our results indicate that Sorafenib circumvents Mcl-1–caused chemoresistance, which suggests that the combination chemotherapy of Taxol and Sorafenib may be a promising treatment for overcoming breast cancer chemoresistance in clinic.

Discussion

Dual regulation of Mcl-1 by Erk/Pin1 and GSK-3β/β-Trcp pathways. As a member of the antiapoptotic Bcl-2 family, Mcl-1 promotes cell viability similar to Bcl-2 and Bcl-XL, but in comparison, Mcl-1 is rapid-response gene with short half-life. Because no mutation was found in the full-length of Mcl-1, upstream regulating signals seem to play an important role in Mcl-1 stabilization and degradation. However, the upstream regulators are not fully identified. Recently, CDK was found to phosphorylate Mcl-1 at Thr 64 residue and then stabilize it (38). We also identified that GSK-3β is a upstream regulator, which phosphorylates Mcl-1 at Ser 155, Ser 159, and Thr163 residues and then recruits E3 ligase β-Trcp to degrade Mcl-1 (20, 21). Erk is known to up-regulate Mcl-1, but its mechanism is not clearly understood. In the current study, Mcl-1 was phosphorylated by Erk at two consensus motifs, 92TP and 163TP, which can be recognized by the isomerase Pin1, possibly causing its conformation change to stabilize Mcl-1. We observed that Thr163 was both recognized and phosphorylated by GSK-3β and Erk, which, interestingly, resulted in the opposite fate for Mcl-1, i.e., phosphorylation of Thr 163 by GSK-3β causes degradation of Mcl-1, but phosphorylation by Erk stabilizes it. The phenomenon indicated that phosphorylation of Thr163 is a triggering or priming event that allows subsequent phosphorylations by GSK-3β (Ser 155, Ser 159) or by Erk (Thr92) to decide the fate of Mcl-1. We hypothesize that conformational change of Mcl-1 by Pin1 may interfere with its association with GSK-3β and β-Trcp, which may block its degradation by them. The protein structure modulation change of Mcl-1 by Pin1 after phosphorylation by Erk and the crosstalk between Erk/Pin1 and GSK-3β/β-Trcp on the regulation of Mcl-1 will be further studied. Generally, our current results, together with previous findings, provide a plausible mechanism (Supplementary Fig. S3) for how the fate of Mcl-1 is dictated by Erk/Pin1 and GSK-3β/β-Trcp pathways.

Sorafenib can overcome Mcl-1–caused chemoresistance. Mcl-1 was originally identified to play a role in the development...
of human myeloid leukemia; subsequently, its overexpression is often found in multiple types of solid tumor, such as hepatoma, ovarian cancer, pancreatic cancer, and others. Other than its role as an oncogenic factor to promote tumorigenesis (16–18), its high expression was also thought of as a contributing factor to chemoresistance (13, 19). For example, Mcl-1 is highly expressed in over 60% of human breast cancer patient samples and plays a role in chemoresistance in breast cancer (20, 21). In the current study, negative expression of Pin1 and Mcl-1 were both significantly correlated with patients’ survival in 101 human breast cancer samples. In contrast, positive expression of both Pin1 and Mcl-1 correlated with patients’ survival in 101 human breast cancer samples. In the current study, we identified a novel underlying mechanism for Erk-induced Mcl-1 up-regulation, namely, the phosphorylation of Mcl-1 by Erk to be primed and stabilized by Pin1. Based on the above findings, we developed two strategies to overcome Mcl-1-mediated chemoresistance, i.e., either inhibiting Erk/MAPK pathway (Sorafenib, an approved clinical anticancer drug) or knocking down Pin1 by SiRNA. The results showed that they both down-regulate Mcl-1. Furthermore, under treatment with the two methods, the breast cancer cell line MCF7 was sensitized to Taxol. In conclusion, this newly identified mechanism provides a scientific basis for a plausible combination of chemotherapeutics in breast cancer, Taxol plus Sorafenib, to overcome chemoresistance.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Acknowledgments

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