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Author(s)
Ohta, K; Okoshi, R; Wakabayashi, M; Ishikawa, A; Sato, Y; Kizaki, H

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Geldanamycin, a heat-shock protein 90-binding agent, induces thymocyte apoptosis through destabilization of Lck in presence of 12-O-tetradecanoylphorbol 13-acetate

Kazumasa OHTA, Rintarou OKOSHI, Maiko WAKABAYASHI, Ayako ISHIKAWA, Yutaka SATO and Harutoshi KIZAKI
Department of Biochemistry, Tokyo Dental College, 1-2-2, Masago, Mihama-ku, Chiba 261-8502, Japan
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ABSTRACT
Geldanamycin, a heat-shock protein 90 (Hsp90)-binding agent, modulates various cellular activities. The present study found that, although geldanamycin by itself had no effect on thymocyte viability, it induced apoptosis in thymocytes with a reduction of the mitochondrial transmembrane potential (ΔΨm) in the presence of 12-O-tetradecanoylphorbol 13-acetate (TPA), an activator of protein kinase C (PKC). This apoptosis depended on transcription and translation, and on activation of caspase-8 and -3. Geldanamycin treatment in the presence of TPA also enhanced destabilization of Lck. This destabilization was independent of transcription and translation. It was inhibited, however, by conventional PKC inhibitors, preventing apoptosis. Proteasome inhibitor affected neither the degradation of Lck nor DNA fragmentation, although they inhibited reduction of ΔΨm. These results suggest that the ubiquitin-proteasome system is not involved in Lck destabilization, and that ΔΨm reduction is not directly related to the progression of apoptosis. Furthermore, inhibition of Lck in the presence of TPA induced apoptosis in thymocytes. Our findings suggest that Hsp90 modulates thymocyte apoptosis in concert with PKC through the destabilization of Lck and in a caspase-8- and -3-dependent manner.

In addition to being a chaperone protein, cytosolic heat shock protein 90 (Hsp90) plays an important role in maintaining the activity of numerous signaling proteins, including steroid hormone receptors and signal transduction proteins (27, 38, 39). Hsp90 binding-agents such as benzoquinoid ansamycins, geldanamycin and herbimycin A, and a structurally unrelated compound, radicicol, are known to interact with various target molecules and modulate cellular activities by inactivation, destabilization, or eventual degradation of Hsp90 client proteins, resulting in the utilization for chemotherapy (21, 31).

Thymocyte apoptosis is involved in clonal deletion of self-reactive cells within the thymus during development, and is physiologically regulated by cross-talk among intracellular signaling molecules through stimulation of T cell receptors and associated molecules such as CD3 and CD28, glucocorticoids, and cytokines (35, 36). Glucocorticoid is an important regulator of thymocyte apoptosis and works by binding to its cognate receptor which is associated with Hsp90 (26). We and others have shown that Hsp90-binding agents, geldanamycin, herbimycin A, and radicicol, inhibited glucocorticoid-induced thymocyte apoptosis by inhibiting interaction between glucocorticoid receptors and Hsp90 (23, 26).

Protein kinase C (PKC) is also involved in apoptosis in a variety of cells, including thymocytes, although its role varies depending on its isofrom and situation (10, 15). 12-O-Tetradecanoylphorbol 13-acetate (TPA) is a powerful PKC activator that has
been commonly reported to inhibit apoptosis induced by receptor activation (29), growth factor deprivation (2) and different cytotoxic agents (16). On the contrary, TPA has been reported to potentiate apoptosis induction (5, 7). Action of TPA on apoptosis remains controversial.

In this study, we showed that treatment of thymocytes with both Hsp90-binding agents and TPA, a phorbol ester capable of activating PKC, together induced caspase pathway-dependent apoptosis which did not rely on reduction of the mitochondrial transmembrane potential (ΔΨm). This treatment was accompanied by a destabilization of Lck that depended on neither the proteasome-ubiquitin system nor on caspase activation. Inhibitors of conventional PKC (cPKC) prevented destabilization of Lck, which resulted in inhibition of apoptosis. Inhibition of Lck by PP2, a potent Src family tyrosine kinase inhibitor, in the presence of TPA also induced apoptosis in thymocytes.

MATERIALS AND METHODS

Materials. Geldanamycin, radicicol, dexamethasone, TPA, 4-α-phorbol 12-myristate 13 acetate (4-α-PMA), propidium iodide (PI), 3,3’-dihexyloxycarbocyanine iodide (DiOC₆(3)), E64, Nα-p-tosyl-L-lysine chloromethyl ketone hydrochloride (TLCK), actinomycin D, and cycloheximide were purchased from Sigma-Aldrich (St. Louis, MO, USA). Gö6976, Ro-31-8425, rottlerin, and PP2 were obtained from Merck KGaA (Darmstadt, Germany). Z-VAD-fmk, Z-DEVD-fmk, Z-IETD-fmk, and Z-LEHD-fmk were obtained from R&D Systems (Minneapolis, MN, USA). Z-Ile-Glu(OBu)₄-Ala-Leu-H (PSI) was obtained from Peptide Institute (Osaka, Japan). Anti-poly (ADP-ribose) polymerase (PARP) (H-250) and anti-Lck (3A5), Anti-Bid (mouse specific), and anti-actin (A-2066) antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA), Cell Signalling Technology (Beverly, MA, USA), and Sigma-Aldrich, respectively. All other chemicals used were of reagent grade.

Thymocyte culture. Thymocyte suspensions (4–8 × 10⁶ cells/mL) were prepared from the thymus of male, 5- to 6-week-old BALB/c mice in RPMI 1640 media supplemented with 10% fetal bovine serum, 50 μM 2-mercaptoethanol, 10 mM HEPES, and antibiotics (24).

Assay of DNA fragmentation. DNA fragmentation was approximated according to the method of Nicoletti et al. (22) as the ratio of hypoploid cells by PI staining and flow cytometry using FACSCalibur (BD, San Jose, CA, USA). It was then further determined by fragmented DNA assay (24). The percentage of DNA fragmented was defined as the ratio of DNA in the detergent-soluble supernatant (fragmented DNA) obtained by centrifugation at 27,000 × g to that in the lysate (total DNA).

Assay of ΔΨm. After incubation, the thymocytes were collected and the ΔΨm was analyzed by DiOC₆(3) staining and flow cytometry using FACS-Calibur (33).

Western blotting. Cells were collected, washed with ice-cold phosphate-buffered saline and then sonicated in the buffer (10 mM Tris-HCl buffer, pH 7.4, containing 10 mM NaCl, 3 mM MgCl₂, 0.5% Nonidet P-40, and complete protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany)) at 20 W for 1 min and incubated for at 4°C 30 min. Proteins were separated on 10% SDS-polyacrylamide gel and transferred to Immobilon P membrane (Millipore, Bedford, MA, USA). Membrane was blocked with 5% skimmed milk and incubated with anti-PARP (1: 2000), anti-Bid (1: 1000), anti-Lck (1: 1000), or anti-actin (1: 10000) antibody and detected with horseradish peroxidase-conjugated anti-rabbit/mouse IgG antibody using the ECL plus system (GE Healthcare, Buckinghamshire, UK).

Assay of caspase activity. Cells were treated with geldanamycin and TPA, and then collected by centrifugation at the appropriate times. After homogenization in the cell lysis buffer supplied, the activities of caspase-3, -8, and -9 were assessed using caspase-3/CPP32, caspase-8/FLICE, and caspase-9/Mch6 fluorometric protease assay kits, respectively (Medical & Biological Laboratories, Nagoya, Japan) according to the manufacturer’s instructions.

RESULTS

Geldanamycin plus TPA induces thymocyte apoptosis

Although geldanamycin inhibited dexamethasone-induced thymocyte apoptosis by interfering with Hsp90 (23, 26), geldanamycin alone had no effect on induction of apoptosis, at least with up to 6 h incubation (Fig. 1A). TPA, an activator of PKC, alone did not induce significant DNA fragmentation at 6 h, although TPA induced apoptosis in thymocytes after 24 h incubation (12). In this study, however, geldan-
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Geldanamycin induced significant DNA fragmentation dose- and time-dependently in the presence of TPA (Fig. 1A and 1B). Induction of DNA fragmentation by TPA plus geldanamycin was associated with a reduction of ΔΨm (Fig. 1C). 4-α-PMA was incapable of activating PKC and enhanced neither DNA fragmentation nor ΔΨm reduction, indicating that activation of PKC was involved in the enhancement of geldanamycin-induced apoptosis. Radicicol, a structurally unrelated Hsp90-binding agent, also enhanced DNA fragmentation with ΔΨm reduction in the presence of TPA in the same way as geldanamycin (Fig. 1B and 1C). It has been reported that TPA plus herbimycin A, an analogous compound to geldanamycin, induced transcription- and translation-dependent apoptosis in thymocytes (1). Apoptosis induced by geldanamycin plus TPA was inhibited by actinomycin D and cycloheximide (data not shown). These results suggest that geldanamycin plus TPA-induced apoptosis was also dependent on transcription and translation.

Apoptosis induced by geldanamycin plus TPA depends on caspase pathways

To analyze the involvement of caspase pathways in ΔΨm reduction and DNA fragmentation induced by geldanamycin plus TPA, we assayed the activities of caspase-3, -8 and -9, and examined the effects of Z-VAD-fmk, a pan-caspase inhibitor, and Z-DEVD-fmk, Z-IETD-fmk, and Z-LEHD-fmk, a specific
inhibitor of caspase-3, caspase-8, and caspase-9, respectively, on DNA fragmentation and ΔΨm. Activation of caspase-3, -8, and -9 was detected at 3 h incubation (Fig. 2). The amount of Bid, a substrate for caspase-8, decreased at 4 h, presumably by cleavage of activated caspase-8 (Fig. 2B, inset). Activation of caspase-3 was confirmed by cleavage of PARP (Fig. 2A, inset). None of the caspase inhibitors had an effect on DNA fragmentation in the presence or absence of either geldanamycin or TPA.

DNA fragmentation induced by geldanamycin plus TPA was inhibited by the pan-caspase, caspase-3, or -8 inhibitor, but only partially by the caspase-9 inhibitor (Fig. 3A). DNA fragmentation, therefore, was mainly dependent on activation of initiator caspase-8 and effector caspase-3.

Neither caspase inhibitor affected reduction of ΔΨm in the absence of either geldanamycin or TPA. ΔΨm reduction induced by geldanamycin plus TPA was inhibited by the pan-caspase inhibitor, but not by the inhibitors specific to caspase-3, -8, or -9 (Fig. 3B). These results suggest that ΔΨm reduction is probably due to activation of caspase(s) sensitive to a pan-caspase inhibitor, rather than caspase-3, -8, or -9 inhibitors. However, DNA fragmentation, but not ΔΨm reduction, was inhibited by the caspase-8 inhibitor, suggesting that ΔΨm reduction is not a prerequisite of DNA fragmentation. This result

**Fig. 2** Activity of caspases induced by geldanamycin plus TPA. Cells were incubated with (∘, ●) or without (□, ▲) 200 nM geldanamycin (GA, G) in the absence (○, □) or presence (●, ▲) of 10 nM TPA (T) for the indicated times. Caspase activities were measured using DEVD-AFC, IETD-AFC, or LEHD-AFC as substrate for caspase-3 (A), caspase-8 (B), or caspase-9 (C), respectively. Whole cell extracts from 4 h treatment were analyzed by Western blotting using anti-PARP (inserted in panel A) for caspase 3 activation or anti-Bid (inserted in panel B) for caspase 8 activation. Actin was analyzed as a control for protein loading.

**Fig. 3** Inhibition of geldanamycin plus TPA-induced DNA fragmentation and ΔΨm reduction by caspase inhibitors. Cells were incubated in the presence of 200 nM geldanamycin (GA) plus 10 nM TPA with 100 µM Z-VAD-fmk, 100 µM Z-DEVD-fmk, or 100 µM Z-LEHD-fmk for 4 h and analyzed for DNA fragmentation (A) or for 6 h and analyzed for ΔΨm (B). Data are shown as mean ± S.D. from three experiments.
agrees with the finding that inhibition of DNA fragmentation by the caspase-9 inhibitor was incomplete (Fig. 3A). It is notable that the activation of caspase-9 depends on the mitochondrial pathway with ΔΨm reduction (4).

**Lck is destabilized by geldanamycin and TPA**

Hsp90 is known to modulate the stability of non-receptor type tyrosine kinases in T cells (37), resulting in dysfunction of those kinases. Lck is a member of the Src family of tyrosine kinases, which are involved in T cell activation together with T cell receptors (32), and also in thymocyte development (19). Therefore, we examined the effect of geldanamycin and TPA on Lck stability by Western blotting. Lck level showed only a slight decrease by geldanamycin treatment (Fig. 4A). A band with retarded mobility was yielded by treatment with TPA alone. The slower form of the molecule was of about 61 kDa, and Lck may be phosphorylated by TPA-activated PKCs (9, 34). Geldanamycin plus TPA enhanced destabilization of Lck molecules with both 56 kDa and 61 kDa (Fig. 4A). Decrease in Lck level was specific to the treatment with geldanamycin plus TPA, and was not secondary to apoptosis, since it was not detected in dexamethasone-treated thymocytes, which yielded DNA fragmentation similar to that induced by geldanamycin plus TPA (Fig. 4A). Destabilization of Lck was not dependent on transcription and translation, since it was not prevented by actinomycin D or cycloheximide (Fig. 4B).

**PKC inhibitors prevent Lck destabilization and apoptosis**

As mentioned above, the PKC activator TPA yielded a slower band of Lck by Western blot analysis. It has been suggested to be a phosphorylated form of Lck (9, 34). Phosphorylation of Lck by TPA was followed by destabilization in the presence of geldanamycin, which was independent of transcription and translation. Therefore, we examined the effect of inhibitors of PKC on DNA fragmentation and Lck destabilization. Thymocytes were pretreated with the cPKC inhibitors Gö6976 and Ro-31-8425, and a novel PKC (nPKC)-specific inhibitor, rottlerin, for 30 min prior to the addition of TPA and geldanamycin. Treatment with the inhibitors alone had no effect on DNA fragmentation. Both the cPKC and nPKC inhibitors inhibited DNA fragmentation, although Gö6976 was less effective (Fig. 5A).

Both cPKC inhibitors, Gö6976 and Ro-31-8425,
inhibited the formation of slower bands of Lck in Western blot analysis, and destabilization of Lck was prevented (Fig. 5B). Inhibition of phosphorylation and destabilization of Lck was accompanied by a reduction of DNA fragmentation. These results suggest that cPKC is involved in the acceleration of apoptosis induced by geldanamycin plus TPA. Rottlerin did not inhibit destabilization of Lck, although it did reduce the formation of a slower band (Fig. 5B). However, DNA fragmentation was inhibited by rottlerin (Fig. 5A).

**Ubiquitin-proteasome system is not involved in destabilization of Lck**

Geldanamycin prevents the ATP-dependent release from Hsp90 of client proteins undergoing refolding, leading to their degradation, presumably by the ubiquitin-proteasome system (17, 18). To examine proteasomal involvement in Lck stability and apoptosis, the effect of proteasome inhibitor PSI was examined in thymocytes. The thymocytes were preincubated with PSI prior to treatment with geldanamycin plus TPA. A decrease in Lck was not prevented (Fig. 6A). In addition, DNA fragmentation was not prevented by PSI (Fig. 6B), indicating that the effecter caspase, caspase-3, was activated even in the presence of PSI. Furthermore, Lck was not degraded by pan-caspase inhibitor (Fig. 6A). However, ΔΨm reduction was inhibited by PSI (Fig. 6B), in agreement with previous data showing that ΔΨm reduction was not a prerequisite to the progress of apoptosis (Fig. 3).

Because inhibition of proteasome did not unequivocally address the degradation of Lck, another potential mechanism of degradation was investigated by using a lysosomal cysteine protease inhibitor, E64, and serine protease inhibitor, TLCK. E64 prevented neither Lck degradation nor DNA fragmentation in geldanamycin plus TPA-treated cells (Fig. 6). TLCK did not inhibit the degradation of Lck, although it inhibited DNA fragmentation (Fig. 6). It has been reported that TLCK inhibited both etoposide- and dexamethasone-induced DNA fragmentation (6).

**Inhibitor of Src family tyrosine kinases enhances DNA fragmentation in the presence of TPA**

Destabilization of the Lck molecule indicated a decrease in its tyrosine kinase activity. To determine whether the tyrosine kinase inhibitor could induce apoptosis in concert with TPA, and whether it facilitated the degradation of Lck, we examined the effects of PP2, an inhibitor of tyrosine kinase with a high affinity for Lck, on apoptosis and degradation of Lck. PP2 itself had almost no effect on DNA fragmentation or ΔΨm reduction (Fig. 7A). When thymocytes were incubated with PP2 plus TPA, enhanced DNA fragmentation with ΔΨm reduction were observed (Fig. 7A). PP2 had no effect on Lck degradation, either in the presence or absence of TPA (Fig. 7B). These results indicate that the inhibition of Lck activity is associated with the progression of apoptosis in concert with PKC.
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DISCUSSION

In the present study, we showed that the treatment of mostly non-proliferating thymocytes with geldanamycin plus TPA induced apoptosis dependent on caspase pathways, although neither geldanamycin nor TPA alone induced apoptosis (Figs. 1–3). Treatment of geldanamycin plus TPA was accompanied by a rapid destabilization of Lck (Fig. 4) that depended neither on the proteasome-ubiquitin system nor on caspase activation (Fig. 6). Destabilization of Lck was prevented by inhibitors of cPKCs, which was followed by inhibition of apoptosis (Fig. 5).

Lck is a Src family tyrosine kinase involved in T cell differentiation, survival, and activation (40). Lck plays a particularly important role in T cell proliferation after T cell stimulation. Geldanamycin is particularly toxic to rapidly proliferating cells, including activated splenic T cells (37) and malignant cells. Its effect on malignant cells is known to be tumoricidal (27). Bijlmakers et al. (3) reported that levels of the constitutive active form of Lck were reduced to a greater degree by geldanamycin than those of wild type Lck. In proliferating T cells, geldanamycin alone induced destabilization of newly-synthesized Lck molecules, although it also slowly depleted matured molecules (37). Proliferation signals, as well as activation of PKC by TPA, induced phosphorylation of Lck (20). The present study showed that TPA treatment rapidly converted Lck to its phosphorylated form, resulting in destabilization by geldanamycin (Fig. 5). Indeed, inhibition of cPKCs inhibited destabilization, which was then followed by inhibition of apoptosis (Fig. 5). Therefore, phosphorylation of Lck by PKC may render the molecule sensitive to Hsp90-binding agents. Inhibition of phosphorylation by nPKCs inhibitor did not protect against destabilization of Lck, although DNA fragmentation was inhibited (Fig. 5). Rottlerin also inhibited glucocorticoid-induced DNA fragmentation (28), suggesting that nPKC is involved in certain later and common step(s) to inhibit apoptosis induced by a variety of stimuli. Constitutive active Lck has been shown to be more sensitive to geldanamycin than wild type Lck (3), and it has been demonstrated that destabilization of Lck is probably involved in the induction of apoptosis. This suggests that inhibition of Lck tyrosine kinase activity induces apoptosis. Indeed, inhibition of its kinase activity by PP2 resulted in profound apoptosis. PP2 may more potently inhibit the activity of phosphorylated Lck. The majority of thymocytes are non-proliferating, and Lck plays an important role in thymocyte differentiation (19, 40). In lck knockout mouse, a profound reduction of thymus size with a decreased number of immature thymocytes has been observed to be accompanied by disdifferentiation of thymocytes (20). The activation state of the PKC pathway in proliferating cells, as well as cancer cells, may partly explain how those cells become sensitive to geldanamycin-treatment, resulting in inhibition of cell proliferation or tumoricidal activity.
Hsp90-binding agents have been known to accelerate the degradation of their client proteins, including Lck, via the ubiquitin-proteasome system. However, here, proteasome inhibitor did not prevent the Lck destabilization induced by geldanamycin plus TPA (Fig. 6). In addition, lysosomal enzymes were not involved in this destabilization, as E64 did not prevent degradation (Fig. 6). It remains to be clarified which enzyme(s) are involved in Lck destabilization and degradation.

The proapoptotic effect of geldanamycin plus TPA on thymocytes is not only attributable to loss of Lck, as many other signaling proteins such as Raf1 have been shown to be vulnerable to geldanamycin treatment (30). The Ras/Raf-1 signaling pathway plays an important role in proliferation, cell differentiation, growth arrest, and death (13). Upon activation of the Ras/Raf-1 pathway, a cascade of downstream reactions is initiated, ultimately leading to activation of ERKs, which act as a survival factor. Hsp90 has been found in association with many components and regulators of this pathway, including Ras and Raf-1 (30). When we investigated the phosphorylation level of ERKs by Western blotting, geldanamycin did not affect the basal level of ERKs. TPA enhanced phosphorylation of ERKs, and geldanamycin inhibited TPA-induced phosphorylation, but at a similar level to that in the control and geldanamycin-treated cells (data not shown). Therefore, ERK level, namely the Ras/Raf-1 pathway perturbed by geldanamycin, may not be involved in the induction of apoptosis by geldanamycin plus TPA.

Geldanamycin plus TPA induced activation of caspase-8 (Fig. 2B). Cellular FLICE/caspase-8-inhibitory protein (cFLIP) is a potent negative regulator of death receptor-induced apoptosis, as receptor-induced apoptosis is associated with activation of caspase-8. Geldanamycin interfered with tumor necrosis factor (TNF)-induced NF-κB activation and inhibited TNF-induced upregulation of cFLIP (14). In addition, geldanamycin has been known to inhibit IκB kinase activity induced by viral FLIP, resulting in primary effusion lymphoma cell death (8). These results suggest that Hsp90 interacts with cFLIP. However, in this study, Western blot analysis revealed no alteration in level of cFLIP after treatment with geldanamycin plus TPA (data not shown). Geldanamycin alone failed to activate caspase-8 (Fig. 2B). These findings indicate that geldanamycin plus TPA did not affect cFLIP regulation.

Hsp90 has also been shown to be involved in the inhibition of apoptosis by suppressing apoptosome formation with cytochrome c and Apaf-1 (25). Here, we observed activation of caspase-9, indicating apoptosome formation (Fig. 2C). However, the inhibitor of caspase-9 was less effective in preventing DNA fragmentation (Fig. 3). In addition, DNA fragmentation was inhibited without a reduction of ΔΨm in the presence of the caspase-8 inhibitor (Fig. 3). It is unlikely that Hsp90 interacts with apoptosome in the induction of apoptosis by geldanamycin plus TPA in thymocytes. On the other hand, PSI inhibited ΔΨm reduction, but not DNA fragmentation (Fig. 6B). It has been reported that proteasome was activated at a premitochondrial step in thymocyte apoptosis induced by dexamethasone or etoposide, and that the inhibition of proteasome activity inhibited both ΔΨm reduction and DNA fragmentation (11). The present results suggest that ΔΨm reduction does not correlate with apoptosis, and that its reduction is not a prerequisite to the progression of apoptosis. Therefore, the ΔΨm reduction observed here in the presence of geldanamycin plus TPA was probably secondary to the activation of caspase-8 and/or caspase-3, and not directly involved in the progression of apoptosis. This may reflect an amplifying cascade rather than the initiation of apoptosis.

Although Lck activity was inhibited by PP2 in the absence of TPA, apoptosis was not induced. However, in the presence of TPA, PP2 induced apoptosis dose-dependently, even in the absence of geldanamycin (Fig. 7A). The phosphorylation of Lck induces its conformational change, and may result in the conversion of the Lck molecule to be sensitive to PP2. PP2 binds tightly and selectively to LCK by making additional contacts in a deep, hydrophobic pocket near the ATP-binding cleft (41). Geldanamycin specifically inhibits the essential ATPase activity of Hsp90 (21).

The present study showed that the treatment of thymocytes with both Hsp90-binding agents and TPA induced apoptosis dependent on caspase pathways, but not on a reduction of ΔΨm. The treatment was accompanied by destabilization of Lck. However, it remains to be clarified why the destabilization of Lck triggered transcription- and translation-dependent apoptosis, and also what proteolytic mechanism is involved in the destabilization and degradation of Lck.

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