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Cycloalkyl-substituted aryl chloroethylureas inhibiting cell cycle progression in G_0/G_1 phase and thioredoxin-1 nuclear translocation

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ABSTRACT

1-(2-Chloroethyl)-3-(4-cyclohexylphenyl)urea (cHCEU) has been shown to abrogate the presence of thioredoxin-1 into the nucleus through its selective covalent alkylation. In the present letter we have evaluated the structure–activity relationships of the substituents at positions 3 and 4 of the phenyl ring of cHCEU derivatives on cell cycle progression and thioredoxin-1 nuclear translocation. Active CEU derivatives exhibited GI_{50} ranging from 1.9 to 49 μM on breast carcinoma MCF-7, skin melanoma M21, and colon carcinoma HT-29 cells. On one hand, compounds **1**, **2**, **9c**, **10c**, **13**, and **14** arrested the cell cycle in G_2/M phase while CEUs **3**, **4**, **5c**, **6c**, **11c**, and **12c** blocked the cell division in G_0/G_1 phase. On the other hand, CEUs **2**–**4**, **5c**, **7c**, **8c**, **11c**, and **12c** abrogated the translocation of thioredoxin-1 while the other CEU derivatives were inactive in that respect. Our results suggest that CEU substituted on the phenyl ring at position 3 or 4 by lower cycloalkyl or cycloalkoxy groups arrest cell progression in G_0/G_1 phase through mechanism of action different from their antimicrotubule counterparts, presumably via thioredoxin-1 alkylation and modulation of its activity. The mechanism of action of these new molecules is still undetermined. However, the significant accumulation of cells in G_0/G_1 phase suggests that these molecules may act similarly to known chemopreventive agents against cancers. In addition, the inhibition of Trx-1 nuclear localization also suggests the abrogation of an important chemoresistance mechanism towards a variety of chemotherapeutic agents.

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In the past two decades, we have focused our research program on the development of new 'soft' alkylating agents designated as aryl chloroethylureas (CEU).[1–10] CEU derivatives such as 4-*tert*-butyl-3-[(2-chloroethyl)]ureido]benzene (*t*BCEU, **13**) and its bioisostere 4-iodo-3-[(2-chloroethyl)]ureido]benzene (ICEU, **14**) were shown to bind irreversibly to the colchicine-binding site (C-BS) on β_{II}-tubulin (Fig. 1).[3–5] The covalent binding of *t*BCEU, ICEU, and several other CEU derivatives to the C-BS led to the arrest of the cell division in G_2/M phase, cytoskeleton disruption, and apoptosis.[3–5,10] Recently, ICEU was shown to be specifically biodistributed into the gastrointestinal tract and to inhibit efficiently mouse colon carcinoma (CT-26) tumors grafted onto mice.[11,12] Screening assays were performed to evaluate the antiproliferative properties of closely related CEU derivatives and their effects on cell division, and on C-BS alkylation. Our results showed that 4-cyclohexyl-[3-(2-chloroethyl)ureido]benzene (cHCEU, **4**)[7,13,14] inhibits the cell division in G_0/G_1 phase instead of G_2/M phase and that cHCEU does not bind covalently to β_{II}-tubulin when tested at concentrations inhibiting tumor cell growth by 50%.[12,13] The use of [^{14}C-carbonyl]-cHCEU clearly showed that the drug was not binding to C-BS[13] but instead to a few other proteins, notably, the mitochondrial voltage-dependent anion channel isoform-1(VDAC-1),[13] and more recently, the thioredoxin isoform-1 (Trx-1).[14] Noteworthy, cHCEU binding to Trx-1 was shown to inhibit its presence into the cell nucleus.

Trx-1 is a 12 kDa protein located in the cytoplasm and translocated into the nucleus under a number of physiological and stress conditions.[15–17] Trx-1 is an electron donor for Trx peroxidase, thioredoxin reductase, and ribonucleotide reductase.[16,17] Through its unique redox-mediating properties, Trx-1 controls the activity of several proteins such as AP-1 and NFκB, which are essential for cell cycle progression.[15–17] It was shown that Trx-1 could control cell cycle movement through G_1, S, and G_2/M phases in cells stimulated to exit quiescence and to enter G_1 phase. The intranuclear Trx-1 controls the activity of the AP-1 c-fos/c-jun heterodimer by reducing the nuclear redox factor, Ref-1/Hap-1.[14–20] AP-1 is important for the control of G_0/G_1

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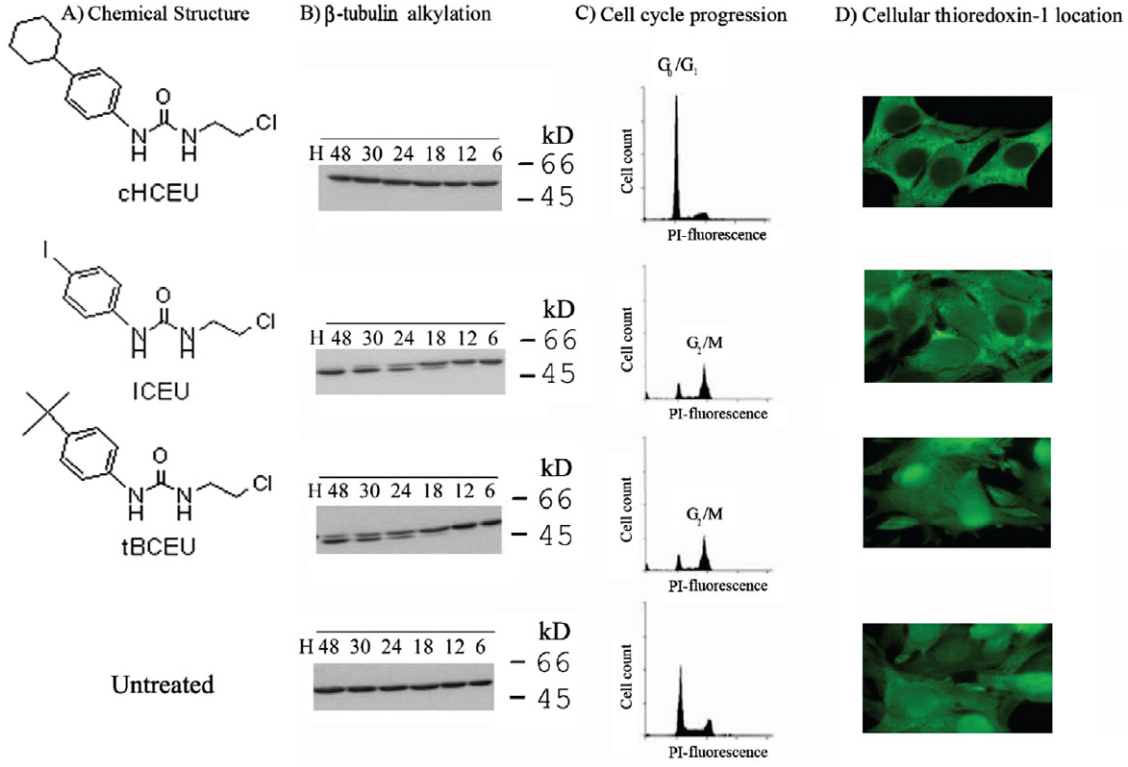


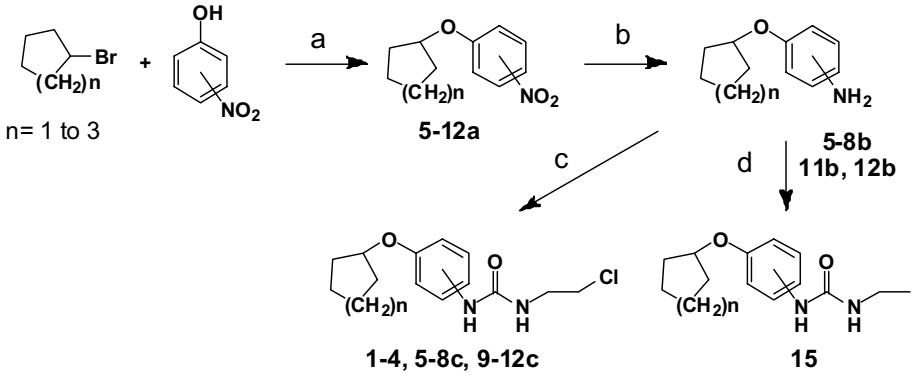
Figure 1. (A) Chemical structures of 1-(2-chloroethyl)-3-(4-cyclohexylphenyl)urea (cHCEU), 4-*tert*-butyl-[3-(2-chloroethyl)ureido]benzene (*t*BCEU) and 4-iodo-3-[(2-chloroethyl)]ureido]benzene (ICEU). (B) Alkylation of tubulin isoform-β_{II}, which was performed as reported earlier.[3–5,13,14] The alkylated β_{II}-tubulin by-product appears as a higher molecular weight band reacting with the monoclonal anti-β_{II}-tubulin antibody. A supplementary band indicates that tubulin isoform-β_{II} is alkylated by the drug. (C) Cell cycle progression was evaluated by flow cytometry using propidium iodide staining.[3–5,13,14] (D) Intracellular localization of thioredoxin-1.

transition involving AP-1-responsive cyclin D1 gene. In addition, Trx-1 is also required to maintain the deoxyribonucleotide pool during the S phase of the cell division.[12,21] Of interest, Trx-1 is overexpressed in cancers such as colon, pancreas, and breast and correlates with poor prognosis.[17–20] Increased Trx expression protects cells from apoptosis induced by a variety of agents, such as etoposide, doxorubicin, docetaxel, and cisplatin.[21–24] Trx-1 is therefore a recognized target for the development of new antineoplastic and chemopreventive agents and in that context several molecules derived from alkyl 2-amidazolyl disulfides, quinols, napthoquinone spiroketals, and selenium derivatives have been developed already.[25–29] However, it seems that so far only 1-methylpropyl 2-imidazolyl disulfide (PX-12®) is currently undergoing clinical trials.[30]

In the present study, we have studied the effect of various substituents of the aromatic ring of a series of cHCEU derivatives and their efficacy to block nuclear translocation of Trx-1, cell cycle progression, and tumor cell proliferation.

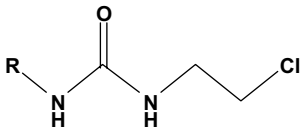
Scheme 1 illustrates the synthesis of CEU and EU derivatives using the nucleophilic addition of either 2-chloroethylisocyanate or ethylisocyanate to the corresponding anilines.[6–10] Anilines substituted by a cycloalkyloxy moiety were prepared using a Williamson-type reaction followed by the reduction of the aromatic nitro group with $SnCl_2$.[11,31]

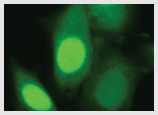
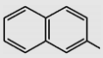
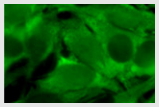
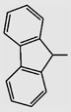
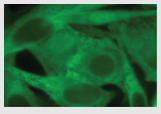
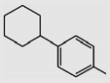
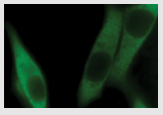
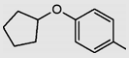
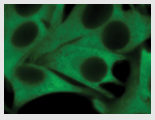
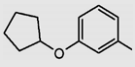
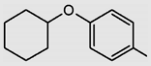
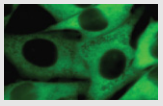
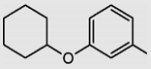
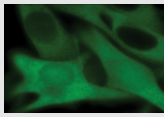
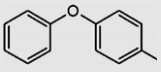
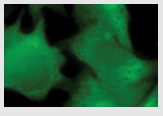
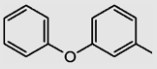
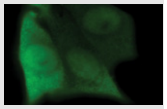
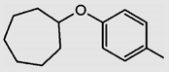
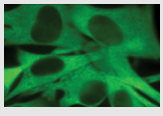
Tumor cell growth inhibition activity of CEU derivatives and antineoplastics such as PX-12, cisplatin, colchicine, and paclitaxel was evaluated on three human cell lines, namely, MCF-7 breast carcinoma, M21 skin melanoma, and HT-29 colon carcinoma cells. Cell growth inhibition was assessed according to the NCI/NIH Developmental Therapeutics Program.[33] The results are summarized in Table 1 and expressed as the concentration of drug inhibiting the cell growth by 50% (GI_{50}).



Scheme 1. Synthesis of aryl chloroethylureas. Reagents: (a) NaOH, DMF, reflux; (b) $SnCl_2{\cdot}2H_2O$, EtOH, reflux; (c) 2-chloroethylisocyanate or; (d) ethylisocyanate, CH_2Cl_2.

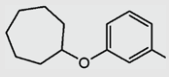
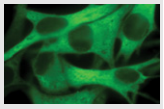
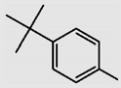
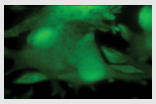
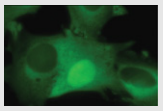
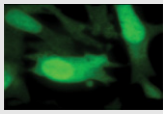
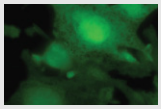
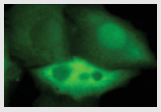
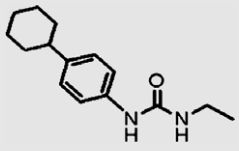
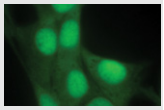
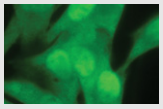
Table 1
Antiproliferative activity of compounds **1**–**4**, **5**–**12c**, and **13**–**15**; PX-12; cisplatin; and paclitaxel was performed on MCF-7, M21, and HT-29 cells, flow cytometry analysis was realized using MCF-7 cells, and immunocytochemistry was performed on M21 cells using a monoclonal anti-thioredoxin-1 antibody



Compound	R	GI_{50} (μM)			FACS (%) MCF-7			Trx-1 location M21
		HT-29	M21	MCF-7	G_0/G_1	S	G_2/M	
1[6,7,10]		3.6	8.0	14.1	31	29	40	
2[6,7]		1.9	4.1	5.6	17	38	45	
3[10]		19	27	22	63	29	8	
4 (cHCEU)[7]		12.6	21	21	71	16	13	
5c		31	38	49	72	19	9	
6c		9.9	22	35	64	24	12	
7c		23	36	45	57	29	14	
8c		7.9	20	25	59	33	8	
9c		2.4	5.2	9.1	15	20	65	
10c		5.6	16	24	44	26	30	
11c		12.5	18	19	75	19	6	

(*continued on next page*)

Table 1 (*continued*)

Compound	R	GI_{50} (μM)			FACS (%) MCF-7			Trx-1 location M21
		HT-29	M21	MCF-7	G_0/G_1	S	G_2/M	
12c		10.7	20	21	72	22	6	
13[6,7,10] (*t*BCEU)		2.3	4.3	6.2	17	24	58	
14[6,7,10] (ICEU)		1.9	3.9	6.0	25	33	42	
PX-12[a]		25	8.3	8.3	29	13	58	
Cisplatin		10.1	12.9	6.4	53	27	20	
Colchicine		0.004	0.015	0.009	19	19	62	
Paclitaxel		0.015	0.037	0.054	9	11	80	
15		n.e.	n.e.	n.e.	55	23	22	
DMSO		n.e.	n.e.	n.e.	59	22	19	

For FACS and immunocytochemistry analysis, exponentially growing MCF-7 or M21 cells were incubated for 16 h, in absence or in presence of CEUs (**1**, **9c**, **13**, **14** at 25 μM; **2**–**4**, **5**–**8c**, **10**–**12c**, **15** at 50 μM), cisplatin (30 μM), colchicine (0.1 μM), paclitaxel (0.1 μM), PX-12 (25 μM). The concentration of DMSO was maintained at 0.1%.

[a] Prepared according to Kirkpatrick.[32]

The results show that the antiproliferative activity of the new compounds is related, notably, to the presence of an electrophilic group on the urea moiety of the molecules.[10] Accordingly, 4-cyclohexyl-[3-(ethyl)ureido]benzene **15**, an ethylurea derivative that is devoid of electrophilicity did not inhibit the proliferation of the cell lines tested in this study even at 100 μM. However, CEU derivatives **1**–**4**, **5**–**12c**, *t*BCEU, and ICEU displayed significant antiproliferative activity on all tumor cell lines tested, exhibiting GI_{50} values ranging from 2 to 49 μM. It is of interest to point out that the substitution at position 3 (**6c**, **8c**, **12c**) significantly improved the antiproliferative activity when compared to molecules substituted on position 4 (**4**, **5c**, **7c**, **11c**).

Previous studies have shown that prototypical CEU derivatives such as *t*BCEU and ICEU arrested the cell cycle in G_2/M phase; phenomenon most likely related to the covalent binding of the drugs to the C-BS of β_{II}-tubulin.[3–5,10] Unexpectedly, the prototypical compound **4** blocked the cell cycle in G_0/G_1 phase.[14] In that context, we have evaluated using flow cytometry analysis whether the new cHCEU derivatives inhibit the cell cycle in G_0/G_1 or in G_2/M phase. The experiments were conducted on MCF-7 cells using 50 μM for 2-times the GI_{50} of the drug for 24 h. Table 1 shows for each drug the percentage of the cell arrested in the various phases of the cell cycle. As reported previously, *t*BCEU, ICEU, and the Trx-1 thioalkylating agent PX-12 blocked the cell cycle progression in G_2/M phase (Table 1). Compounds **1**, **2**, **9c** and **10c** also induce the accumulation of cells in G_2/M phase. All other CEU derivatives (**3**, **4**, **5c**, **6c**, **11c**, **12c**) clearly arrested the cell cycle in G_0/G_1 phase.

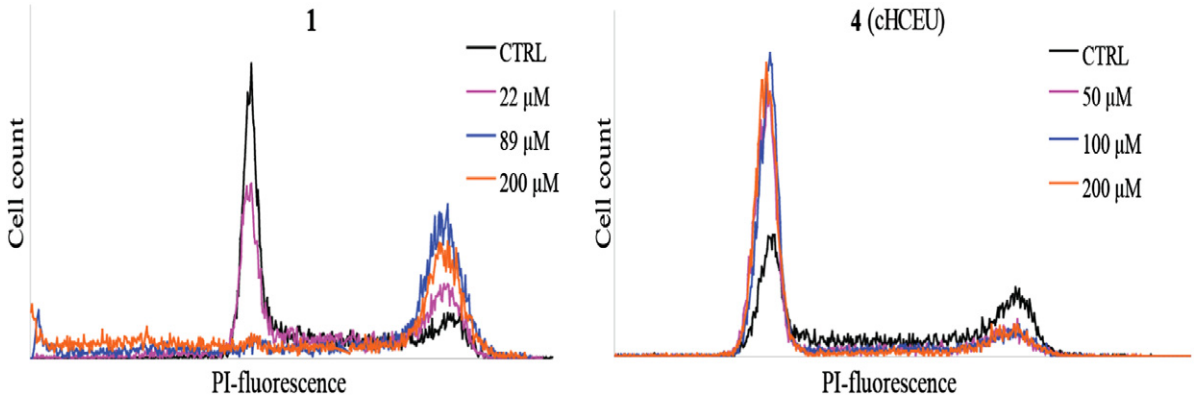


Figure 2. Effect of compounds **1** and **4** on cell cycle progression. Exponentially growing MCF-7 cells were incubated for 24 h in absence or presence at 1-, 2-, and 4-times their respective GI_{50}. The concentration of DMSO used as an excipient was maintained at 0.1%. The cell cycle was evaluated by flow cytometry using propidium iodide staining.

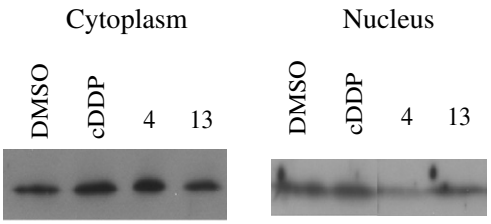


Figure 3. Effect of compounds **4**, **13**, and cisplatin on cytosolic and nuclear fractions of thioredoxin-1. M21 cells were treated for 24 h with 25 μM of cisplatin (cDDP) and compound **13**, or 50 μM of compound **4**, prior to cell fractionation. The resulting nuclear and cytoplasmic proteins extracts were next separated on SDS–PAGE 15% and then analyzed by Western blot using a polyclonal anti-Trx-1. Results are representative of two separate experiments.

Figure 2 depicts the cell cycle analysis of compounds **1** and **4** that are targeting β-tubulin and Trx-1, respectively. The experiments were performed on MCF-7 tumor cells using 1-, 2-, and 4-times their respective GI_{50} for 24 h and they clearly show that **1** and **4** block the cell cycle progression in G_2/M and G_0/G_1 phase, respectively.

As shown in Table 1, compound **4** abrogated the presence of Trx-1 into the nucleus in contrast to DMSO used as an excipient. That unexpected and important pharmacological phenomena prompted us to assess the effect of the new cHCEU derivatives on the cellular location of Trx-1 using immunocytochemistry analysis. The results shown in Table 1 illustrate that the oxophenyl derivatives **9c**, and **10c** do not significantly affect the intracellular location of Trx-1. In contrast, the alicyclic compounds **4**, **5c**, **7c**, **11c**, and **12c** abrogated the presence of Trx-1 into the nucleus. The naphthyl and the fluorenyl compounds **2** and **3**, together with compounds **6c** and **8c**, and **14** exhibited a mitigated effect on nuclear Trx-1 and unexpectedly, compound **1** seems to increase the presence of Trx-1 into the nucleus as observed with cisplatin, which is known to trigger such a phenomenon.[21,34] Interestingly, alicyclic compounds substituted at position 4 (e.g., **5c** and **7c**) were more potent to inhibit Trx-1 nuclear translocation than their 3-substituted counterparts (e.g., **6c** and **8c**). Nuclear extraction and Western blot analysis (Fig. 3) confirmed the results obtained using immunocytochemistry analysis and clearly show that the Trx-1 nuclear translocation is repressed by compound **4** but not by the antimicrotubule compound **13**. In contrast to DMSO, **1**, cisplatin, and PX-12 increased Trx-1 localization into the nucleus. Trx-1 translocation into the nucleus is associated to stress following H_2O_2 exposition, UV radiation, cisplatin treatment, and hypoxia.[16,21,28] Interestingly, the extended nuclear translocation of Trx-1 following cisplatin treatment is part of the mechanism of chemoresistance to that drug, probably through p53, an anti-apoptotic gene, up-regulation.[21,35] Trx-1 confined into the nucleus compartment has been shown to induce cell transcription and proliferation to promote cell survival[16,21,28] through a signaling pathway involving the reduction of Ref-1 and the induction of AP-1 activity.[15–17] The latter is a powerful transcription factor promoting cell growth and survival and controlling also the G_0/G_1 transition via the AP-1-responsive cyclin D1.[15–17,35]

This study was designed to assess, at least partly, the importance of the nature and the position of the substituting moieties on the aromatic ring of cHCEU derivatives on cell cycle progression, the presence of Trx-1 into the nucleus, and cell proliferation. Interestingly, all new CEU derivatives of the prototypical compound **4** synthesized in this study exhibited antiproliferative activity between 2 and 49 μM. However, these results should be interpreted cautiously since the arrest of the cell cycle in G_0/G_1 transition phase suggests that the cells are entering into a 'quiescent-like' state where the cells do not divide actively and are partly protected against cytotoxic agents, while drug arresting the cell cycle in G_2/M phase may normally initiate the apoptotic program.[36–38] Of note, our study shows that alicyclic substitution on position 4 of the aliphatic ring with cycloalkyl or a cycloalkyloxy moiety increases the efficacy of CEUs to inhibit Trx-1 nuclear translocation. The present study expands the family of CEU that inhibit the cell cycle in G_0/G_1 phase, a unique property that has been observed so far only with compound **4**. Similarly to cHCEU, the new inhibitors of the cell cycle progression in G_0/G_1 phase do not inhibit cell growth through alkylation of the C-BS on β_{II}-tubulin. The precise mechanisms of action underlying both the accumulation of the cells in G_0/G_1 phase and the Trx-1 nuclear translocation inhibition in response to cHCEU and several of its derivatives are still under investigation. However, they suggest that these new compounds might act as modulators of chemoresistance to a variety of anticancer drugs such as cisplatin and 5,12 anthracyclinediones.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2008.05.028.

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