# 1-Chloro-2,4-Dinitrobenzene Stimulates the Estrogenic Activity in MCF-7 Cells

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The estrogenic effect of chemicals, including 1-chloro-2,4-dinitrobenzene (CDNB), in the combination with 17$\beta$-estradiol ($E_2$) was screened by a reporter gene assay using breast cancer cells (MCF-7). It was found that CDNB stimulated $E_2$-induced transcriptional activity of estrogen receptor (ER) and down-regulated both ER protein and mRNA. However, CDNB alone and CDNB metabolite(s) showed no estrogenic activity and no binding activity to ER, suggesting an indirect pathway other than ER. CDNB gave no transcriptional activity for an aryl hydrocarbon receptor (AhR), suggesting no possibility of a pathway through cross-talk between AhR and ER. On the other hand, CDNB enhanced the mitogen activated protein kinase (MAPK) pathway, suggesting estrogenic action *via* MAPK. These results indicated that CDNB possessed estrogen-like activity in the transcription and regulation of ER though a different pathway from $E_2$.

**Key words** —— 1-chloro-2,4-dinitrobenzene, metabolite, aryl hydrocarbon receptor, mitogen activated protein kinase, MCF-7 cell

## INTRODUCTION

Estrogen plays important roles in the function, growth and differentiation of mammary glands, the uterus, and ovaries. It also affects other tissues, including bone, liver, the cardiovascular system, and brain. Estrogen acts primarily through the estrogen receptor (ER), which is a member of the nuclear hormone receptor superfamily and a ligand-dependent transcription factor.[1] ER reacts specifically with estrogen, and is transported as a dimer into the site of the estrogen response element (ERE) in the nucleus. The estrogen-ER complex recruits coactivators and general transcription factors, and then regulates the expression of target genes.[2,3]

However, certain chemicals, so-called endocrine disruptors (EDs), affect the endocrine system containing estrogen. EDs are defined as xenobiotics that interfere with the function of natural hormones in terms of production, release, transport, metabolism, receptor binding, or excretion in the body, resulting in disturbing the maintenance of homeostasis and the regulation of developmental processes.[4] It is important and urgent task in the risk assessment of ED to screen and list the positive chemicals from a huge number of chemicals using *in vivo* and *in vitro* tests and many have been reported. For instance, bisphenol A and nonylphenol mimic estrogens *via* binding to ER.[5,6] However, most of these tests have been performed on single pure chemicals; nevertheless, the exposure occurs actually as mixtures of chemicals and not a single chemical. At least, the chemicals should express an effect in the presence of natural hormones such as 17$\beta$-estradiol ($E_2$).

Therefore, we studied the effect of chemicals on the estrogenic activity in the coexistence of $E_2$, expecting to find new chemicals with inhibitory or stimulative activity for $E_2$. In our previous paper, the yeast two-hybrid assay revealed inhibitory chemicals including hexachlorophene, pentachlorophenol, and vitamin K3 (menadione; K3).[7] In this paper, we examined the estrogenic activity in the presence of $E_2$ on chemicals negative in the yeast two-hybrid assay using an MCF-7 reporter gene assay, and found that 1-chloro-2,4-dinitrobenzene (CDNB) showed an enhancing effect by activation of mitogen activated protein kinase (MAPK), not ER.

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## MATERIALS AND METHODS

**Chemicals** —— CDNB was purchased from Nacalai Tesque Co. (Kyoto, Japan), $E_2$ and $\beta$-napthoflavone were from Wako Pure Chemical Industries, Ltd. (Osaka, Japan), and MG-132 was obtained from Calbiochem (Germany). 4-Hydroxytamoxifen (4-OHT) was a gift from Dr. Hayakawa, K. (Kanazawa University). The other chemicals were of the highest grade commercially available, and were used without further purification. These chemicals were prepared as solutions in dimethylsulfoxide (DMSO).

**Cells** —— MCF-7 cells were grown for routine maintenance in Eagle's Minimal Essential Medium (EMEM) with phenol red (Nissui Pharmaceuticals Co. Ltd., Tokyo, Japan), supplemented with 10 mM non-essential amino acids (Nacalai Tesque Co., Tokyo, Japan) and 10% heat-inactivated fetal bovine serum (FBS). Cells were maintained in a humidified environment at 37°C with 5% $CO_2$ in air.

**Reporter Gene Assay** —— For the reporter gene assay, $1.5 \times 10^5$ cells were seeded into 35 mm dishes in phenol red-free EMEM containing 10 mM non-essential amino acids and 10% charcoal-dextran treated FBS. The following day, cells were transently transfected with 1 $\mu$g of plasmid (400 ng of reporter plasmid with 4xERE-TATA-luciferase and 100 ng of pSV40-$\beta$-gal to measure the transfection efficiency, and 500 ng of pBluescript) using the Fugene6 transfection reagent (Roche Diagnostics Co., Indianapolis, U.S.A.) according to the manufacturer's protocol. The cells were incubated for 24 hr after transfection, and exposed to chemicals with and without $E_2$ in fresh medum. The test chemicals were dissolved in DMSO (final concentration in the culture medium did not exceed 0.1%). After incubation for 24 hr, cells were washed two times with PBS, lyzed and assayed for luciferase activity using a luminometer (Lumat LB9501, Berthold GmbH & Co., Germany). Luciferase activity was presented after normalization to $\beta$-galactosidase activity.

**Northern Blots** —— MCF-7 cells were cultivated in 100 mm tissue culture plates for 48 hr, and then treated for 24 hr with DMSO, 1 nM $E_2$, 10 $\mu$M CDNB or CDNB with $E_2$. Twenty micrograms of total RNA per treatment group was separated by electrophoresis on 1% agarose gels, transferred onto nylon membrane (HybondN+, Amersham Pharmacia Biotech, England), bound to the membrane by UV crosslinking, and dried at 80°C for 2 hr. The membrane was then prehybridized in a solution containing 1% bovine serum albumin (BSA) (Sigma, U.S.A.), 7% sodium dodecyl sulfate (SDS) and 0.5 M sodium phosphate buffer (pH 7.2) for 18 hr to 24 hr at 60°C and hybridizied in the same buffer for 24 hr with the [$\alpha$-$^{32}$P]dCTP cDNA probes. Levels of specific mRNA transcripts were standardized as a value relative to G3PDH mRNA in the same sample and band intensities were determined on BAS images (Fujifilm, Tokyo, Japan).

**Protein Isolation and Western Blots** —— MCF-7 cells, after cultivation in 35 mm dishes for 48 hr, were treated with chemicals for the indicated times. After treatment, the cells were washed twice with PBS and then lysed in 70 $\mu$l of lysis buffer containing 8 M urea, 1% NP-40, and 2% 2-mercaptoethanol. After removing the cell debris, the supernatants were used for protein concentration assays. The proteins were boiled for 2 min, applied to a 10% SDS-polyacrylamide gel and transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore immobilon transfer membrane, U.S.A.). The membrane was blocked using 3% skim-milk (Yukijirushi, Japan) or 2.5% BSA overnight at 4°C and probed with primary antibodies; ER$\alpha$ (1 : 200 in 1% skim-milk, Santa Cruz Biotechnology Inc., U.S.A.), extracellular signal-regulated kinases 1 (ERK1) (1 : 1000 in 1.25% BSA, BD Biosceinces, U.S.A.), or phospho-p44/p42 MAP kinase (1 : 1000, Cell signaling technology). Following incubation with a peroxidase-conjugated secondary antibody, immunoglobulins were visualized using the ECL detection system (Amersham Pharmacia Biotech, U.S.A.).

**ER Competitive Binding Assay** —— The binding of the chemicals to ER$\alpha$ was determined using a fluorescence polarization assay, an FP Screen-for-Competitors Kit (ER$\alpha$, high sensitivity; PanVera, Madison, U.S.A.). Briefly, 1 $\mu$l of each chemical solution was added to 49 $\mu$l of screening buffer in tubes and mixed well by shaking. Then, 50 $\mu$l of ER$\alpha$-fluorescence estrogen (ES1) complex solution was added to the tube, incubated at room temperature for 1 hr and the fluorescence was determined using BEACON2000 (PanVera). DMSO instead of the chemical solution was used as a negative control (0% inhibition), and 10 $\mu$l of ES1 (50 nM) instead of ER$\alpha$-ES1 complex as a positive control (100% inhibition). Curve fitting was performed by GraphPad Prism 2.01 software to obtain $IC_{50}$.

**CDNB Metabolism** —— CDNB was treated with rat liver S-9 mixture (Oriental Yeast Co. Ltd., Japan) for 1 hr at 37°C, according to the manufacturer's protocol. The rat liver S-9 mixture contained 20 $\mu$l/ml

S-9, 0.8 mM NADPH, 0.8 mM NADH, 1.0 mM glucose-6-phosphate (G-6-P), 0.4 u/ml G-6-P dehydrogenase, 20 mM $Na_2HPO_4$, 20 mM $NaH_2PO_4$, 6.6 mM KCl, and 1.6 mM $MgCl_2$.

**Yeast Assay for Aryl Hydrocarbon Receptor (AhR) Ligand Activity** —— The yeast transformed with the aryl hydrocarbon receptor/aryl hydrocarbon receptor nuclear translocator (AhR/ARNT) plasmid and XRE plasmid was used as described by Miller.[8] The AhR ligand activity was determined essentially according to the method of Adachi *et al.*[9] The yeast strain YCM3 was grown for 5 hr at 30°C in synthetic dropout (SD) medium lacking tryptophan. Test chemicals (2 $\mu$l in DMSO), 5 $\mu$l of culture, and 200 $\mu$l of SD medium containing 2% galactosidase were mixed and incubated overnight at 30°C. The cell densities were determined by reading the OD at 595 nm. After 10 $\mu$l of the reaction mixture was added to 140 $\mu$l of Z-buffer, $\beta$-galactosidase activity was determined by incubation with o-nitrophenol-$\beta$-D-galactopyranoside for 60 min at 37°C. The absorbance of the $\beta$-galactosidase assay was read at 415 nm.

## RESULTS

### Enhancement of $E_2$-Induced Transcription by CDNB in MCF-7 Cells

The effect of CDNB on $E_2$-induced transcriptional activity was determined by a reporter gene assay, using MCF-7 cells transfected with the ERE-TATA-luciferase plasmid. When CDNB was exposed to the cells for 24 hr with and without $E_2$, CDNB enhanced dose–dependently the transcriptional activity under the condition of 1 nM $E_2$, while CDNB alone did not show any estrogenic activity (Fig. 1).

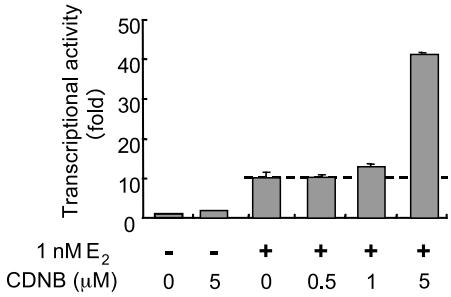


**Fig. 1.** Enhancement of $E_2$-Induced Transcriptional Activity by CDNB

MCF-7 cells were transiently transfected with ERE-TATA-luciferase plasmids as described in MATERIALS AND METHODS. The transcriptional activity was determined as luciferase activity using a reporter gene assay. Luciferase activity was normalized to $\beta$-galactosidase activity in each sample. Transcriptinal activity was expressed when the luciferase activity of DMSO was 1. Values represent the mean ± S.D. ($n$ = 3).

### No Binding Activity of CDNB to ER$\alpha$

CDNB showed no binding activity to ER$\alpha$ in the system where non-labeled $E_2$ and 4-OHT, a known antagonist, inhibited dose–dependently the binding of fluorescein-labeled estrogen to ER$\alpha$ (Fig. 2). This indicated that CDNB did not enhance the $E_2$ activity through ER$\alpha$.

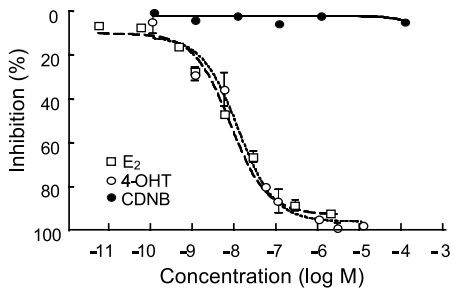


**Fig. 2.** Competitive Binding Assay of CDNB to ER

Binding of fluorescein-labeled estrogen to ER was inhibited competitively by increasing concentrations of $E_2$ and 4-OHT, but not CDNB. The ratio of displacement was measured using a luminescence meter. Values represent the mean ± S.D. ($n$ = 3). gene assay. Luciferase activity was normalized to $\beta$-galactosidase activity in each sample. Transcriptinal activity was expressed when the luciferase activity of DMSO was 1. Values represent the mean ± S.D. ($n$ = 3).

### Decrease in ER mRNA and Protein Levels by CDNB

The expression of ER mRNA was repressed by CDNB, as well as $E_2$, but not 4-OHT. Furthermore, CDNB with $E_2$ decreased more than $E_2$ alone (Fig. 3A). ER protein levels were also down-regulated by CDNB with $E_2$ more than CDNB or $E_2$ alone (Fig. 3B).

### No Effect of CDNB Metabolite on Transcriptional Activity of ER$\alpha$

It is known that certain chemicals acquire estrogenic activity after being metabolized. Here, it was expected to be the same in the case of CDNB, so the transcriptional activity of ER$\alpha$ was examined on metabolite(s) of CDNB, obtained after incubation of CDNB with a rat liver S-9 mixture at 37°C for 1 hr. Then, the activities of CDNB metabolite(s) were compared with and without $E_2$. CDNB metabolite(s) with $E_2$ did not enhance the transcriptional activity, unlike the CDNB case (Fig. 4). Furthermore, the CDNB metabolite(s) itself did not have the estro-

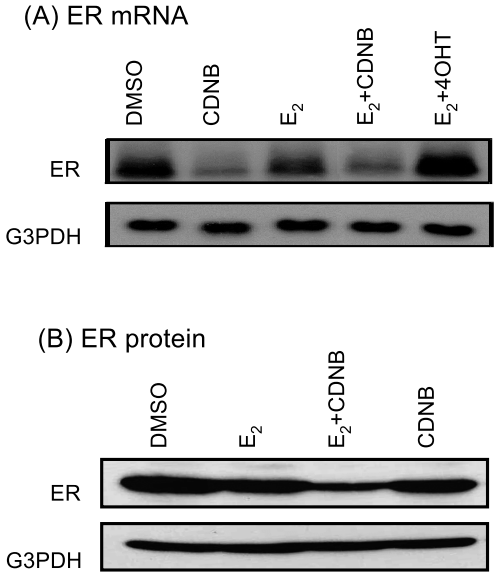


**Fig. 3.** Decrease in ER mRNA and Protein by CDNB

MCF-7 cells were exposed to DMSO, 1 nM $E_2$, 10 $\mu$M CDNB, or CDNB with $E_2$, or 4-OHT with $E_2$. ER mRNA (A) and ER protein (B) were detected by Northern blotting after 24 hr exposure and by Western blotting after 12 hr exposure, respectively. G3PDH was shown as a control of constant protein loading.

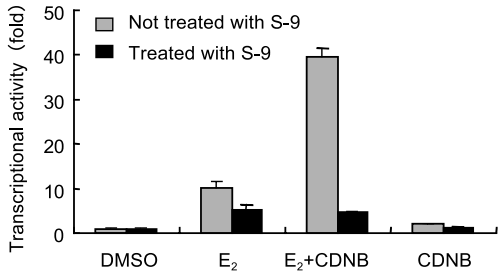


**Fig. 4.** Effect of CDNB Metabolite(s) on Transcriptional Activity

The transcriptional activity was determined by the reporter gene assay as described in MATERIALS AND METHODS. As CDNB metabolite(s), 5 mM CDNB treated with an S-9 mixture at 37°C for 1 hr was used, and MCF-7 cells were exposed to DMSO, 1 nM $E_2$M CDNB, 5 $\mu$M CDNB metabolite(s), CDNB with $E_2$, and CDNB metabolite(s) with $E_2$. Values represent the mean ± S.D. ($n = 3$).

genic activity, the same as CDNB. This result suggested that the enhancive effect of CDNB was not affected by the CDNB metabolite(s).

### No Effect of CDNB on AhR

AhR was reported to cross-talk with ER,[10] so the yeast AhR reporter assay was used to determine if the effect of CDNB was through AhR or not. However, CDNB did not have agonistic activity for AhR in a range from $10^{-9}$ to $10^{-6}$ M, while CDNB did not affect the activity of 10 $\mu$M $\beta$-napthoflavone (NF), a known agonist for AhR (Fig. 5).

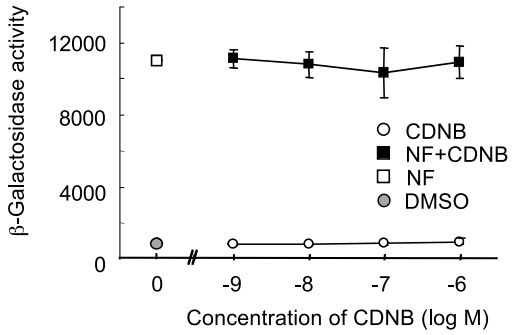


**Fig. 5.** Effect of CDNB on AhR Activity

$\beta$-Galactosidase activity was determined by the yeast assay as described in MATERIALS AND METHODS. Yeasts were exposed to DMSO, the given concentrations of CDNB, 100 nM NF, and concentrations of CDNB with 100 nM NF. Values represent the mean ± S.D. ($n = 3$).

### Induction of Phosphorylation of ERK by CDNB

Activated MAPK induces the phosphorylation of ER, enhances the transcriptional activity of $E_2$ and decreases the ER protein level.[11] It was investigated whether CDNB activated MAP kinase or not by detecting the phosphorylated ERK using Western blotting analysis. CDNB and CDNB with $E_2$, or $E_2$ alone induced the phosphorylation of ERK, whereas DMSO did not (Fig. 6A). The phosphorylation of ERK in cells treated with $E_2$ alone was weakly detected at 2 hr. In contrast, CDNB retained the ability to phosphorylate ERK from 1 hr to 8 hr. Furthermore, CDNB with $E_2$ strongly phosphorylated ERK from 1 hr and showed a peak at 2 hr. On the other hand, CDNB, CDNB with $E_2$, $E_2$ alone or DMSO did not change the expression of ERK (Fig. 6B).

These results suggested that CDNB activated MAPK to enhance $E_2$-induced transcriptional activity and to decrease the ER protein level.

## DISCUSSION

Estrogen produces physiological actions within a variety of target sites by activating ER.[12] For example, the growth of breast cancer cells is dependent on estrogen through ER.[13] In this study, ER$\alpha$-positive MCF-7 breast cancer cells were used to investigate estrogen-dependent responses and the cross-talk between ER and AhR, and the stimulative effect was found to be due to CDNB. In the

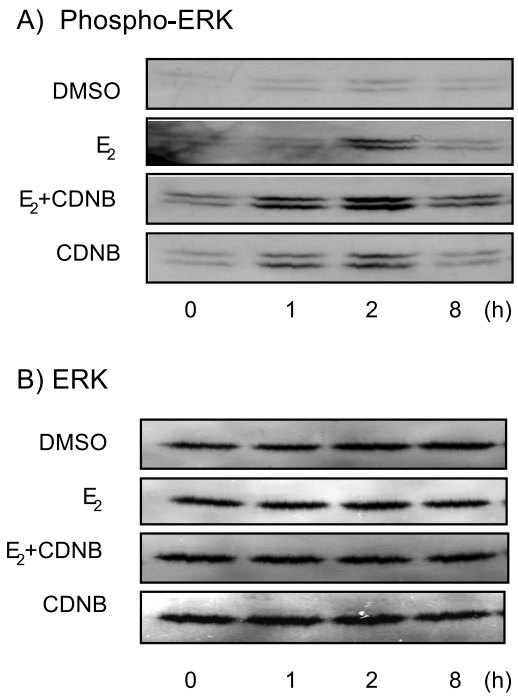


**Fig. 6.** Kinetics of ERK Phosphorylation

MCF-7 cells, cultivated for 4 days, were exposed for various periods of time to DMSO, 1 nM $E_2$, 10 $\mu$M CDNB, and $E_2$ with CDNB. Phospho-ERK (A) and ERK (B) were detected by Western blotting.

previous paper, we did screening using a yeast two-hybrid assay and found inhibitory compounds, but no enhancing compounds.[7] Here, we found why this occurred, that is, the yeast two-hybrid assay is based on the ligand-dependent interaction between ER and a coactivator.[14]

Certain chemicals existing in the environment have a harmful effect on the hormonal system of the human body. Many investigators have reported the risk analysis associated with these chemicals. However, the effect of the chemicals actually appears as mixture with estrogen in the body. In spite of this fact, the combined action has not been well studied *in vitro*. In this study, we showed the effect of CDNB on estrogenic activity with coexistence of $E_2$.

CDNB was evaluated as the stimulator of $E_2$ activity, although CDNB alone did not have any agonistic activity for ER in the MCF-7 cell reporter gene assay (Fig. 1). The enhancing effect of CDNB on $E_2$ activity was not induced through the binding of ER$\alpha$ (Fig. 2). However, Fig. 3 demonstrates that CDNB decreased the ER level as like $E_2$.[15]

To explain the mechanism for this action, we first investigated the metabolite of CDNB. Xenobiotics were metabolized in the body, and then several of their metabolites showed estrogenic action. For example, *trans*-stilbene and methoxychlor showed agonistic ER activity through metabolism.[16–20] However, the results showed that the metabolite of CDNB did not affect the estrogenic action (Fig. 4). Furthermore, 2,4-dinitrophenol, a possible CDNB metabolite, did not have the estrogenic activity in the reporter gene assay (data not shown). These results strongly suggested that the CDNB mechanism of action did not depend on ER.

ER-independent activation can also occurr in breast cancer cells. One example is the cross-talk between ER and AhR as reported recently.[21] Dioxins mimicked the effect of estrogen through a mechanism that involved the activation of ER by the transcriptionally active AhR-ARNT complex.[22] Also, a 90-kDa heat shock protein (HSP90) mediated cross-talk between the ER and AhR signal transduction pathway,[23] and the cross-talk between intracellular signaling pathways influenced ER transcriptional activity in a tissue and cell-specific manner.[24] Agonists for AhR inhibited ER signaling and the expression of target genes.[25,26] Furthermore, resveratrol, known as an agonist of ER,[27] inhibited the AhR action.[28–30] If CDNB is an antagonist for AhR, it would stimulate estrogenic action. However, CDNB did not show any agonistic or antagonistic activity for AhR (Fig. 5). The results suggested that the stimulation of $E_2$ activity by CDNB did not operate *via* AhR.

Another mechanism for activation of ER is *via* ER phosphorylation. It has been reported that MAPK phosphorylates the N-terminal domain of ER,[31] to enhance estrogen-induced transcriptional activity of ER[32] by affecting the recruitment of coactivator.[33] In addition, ER phosphorylation by MAPK is required for full activity of ER activation function 1 and enhanced transcriptional activity.[32] The major MAPK cascades involve ERK including Raf-1, c-Jun N-terminal kinase/stress activated protein kinase (JNK/SAPK), or p38 MAPK. Because the ERK cascade was relevant to breast cancer,[34] we focused on the action of CDNB on ERK. The result showed the activation of ERK by CDNB (Fig. 6). Our findings clarified that CDNB stimulated estrogenic action *via* MAPK. The activation of ERK, one of the MAPK pathways, by CDNB enhanced ER-dependent transcription (Fig. 1). Furthermore, the decrease in ER protein level by CDNB (Fig. 3B) supported the report that activation of MAPK reduced the ER expression.[35] Recently, it was reported that CDNB induced the activation of dendritic cells through MAPK.[36]

It was revealed from our results that CDNB pos-

sessed estrogen-like activity in the transcription and regulation of ER$\alpha$ in a different manner from $E_2$, *via* MAPK. This implies that various mechanisms should be taken into consideration, particularly when risks of chemicals with hormonal effects are evaluated by *in vitro* assays.

According to the chemical hazard data book,[37] CDNB is produced near 200 t in Japan as materials for dyes, pigments, UV absorbents, and others. There has no data of detection from water and sediment samples although it is hard-of-biodegradation and low-of-bioaccumulation. It shows high acute ecotoxcity, whereas comparatively weak acute toxicity to human. But it shows some effects on the motility of sperm and the development of embryo in animals. Although we cannot say about relationship of *in vitro* effect of CDNB with *in vivo* toxicity, it is concluded that *in vitro* studies provides important information for risk assessment of chemicals.

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