Induction of Myeloid Differentiation of HL-60 Cells with Naphthalene Sulfonamide Calmodulin Antagonists

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ABSTRACT

The naphthalene sulfonamide calmodulin antagonists, N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide and N-(4-aminobutyl)-5-chloro-2-naphthalenesulfonamide, both induce limited myeloid differentiation of the human promyelocytic cell line, HL-60. In addition, these inhibitors augment the differentiation observed when HL-60 cells are induced with retinoic acid, dimethyl sulfoxide, or dibutyryl cyclic adenosine monophosphate.

The dose-response curve for HL-60 differentiation was consistent with the published 50% inhibitory dose for inhibition of calmodulin-activated phosphodiesterase and with the calmodulin drug-binding potential of N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide and N-(4-aminobutyl)-5-chloro-2-naphthalenesulfonamide and their less active congeners, N-(6-aminohexyl)-1-naphthalenesulfonamide and N-(4-aminobutyl)-2-naphthalenesulfonamide. These effects, of the naphthalene sulfonamide calmodulin antagonists, are consistent with a regulatory role for calmodulin in cell differentiation, but parallel effects on protein kinase C cannot be excluded.

INTRODUCTION

Substantial evidence has been amassed to support the premise that calcium-signalling might play a vital role in the regulation of cellular differentiation (1, 2). Studies utilizing Ca^{2+} antagonists and ionophores have supported an obligatory role for this cation in both induction and commitment of erythroleukemia cells to differentiate (3-8).

In many instances, calcium exerts its regulatory role by activation of a family of related, high-affinity calcium binding proteins (i.e., calmodulin, troponin C, and parvalbumin) which are present in eukaryotes (9,10). Within this class of regulatory proteins, calmodulin appears to be the major intracellular receptor for Ca^{2+}. Upon binding Ca^{2+}, calmodulin regulates numerous cellular functions (i.e., phosphorylation, cell motility, phagocytosis, cell division, etc.) which are altered upon differentiation (9,10), suggesting that this protein may play a regulatory role in the differentiation process.

However, the divalent cation Ca^{2+} is also a direct cofactor for various non-calmodulin-mediated activities, including protein phosphorylation reactions catalyzed by protein kinase C (11-13). Since protein kinase C has recently been identified as an apparent mediator of macrophage maturation of HL-60 (16), the discrete roles of calmodulin and protein kinase C in this process must be defined.

This study examines the response of HL-60 cells to the naphthalene sulfonamide calmodulin antagonists W-7, W-13, and their less potent analogues W-5 and W-12. Although these drugs may also antagonize the action of protein kinase C (12, 17, 18). Here the myeloid differentiation of HL-60 cells induced by naphthalene sulfonamides is correlated with calmodulin and protein kinase C inhibition potential in vitro. The responses of HL-60 cells to combinations of naphthalene sulfonamides and retinoic acid, DMSO, or dbcAMP are also analyzed.

MATERIALS AND METHODS

Materials. The HL-60 cells used in this study were obtained from Dr. R. C. Gallo, NIH. The tissue culture medium (RPMI-1640), fetal calf serum, and penicillin-streptomycin were purchased from Grand Island Biological Co. N-2-hydroxylethylpiperazine-N-2-ethanesulfonic acid was obtained from Calbiochem-Behring Corp. Polystyrene tissue culture flasks were obtained from Corning. Retinoic acid and dbcAMP were purchased from Sigma, and DMSO was obtained from Fisher Scientific. Phorbol myristate acid and NBT were obtained from Sigma. The formyl peptide (N-formyl-Nle-Leu-Phenylalanyl-L-tyrosyl-L-lysine) was synthesized by Dr. S. Wilkinson (Wellcome Research Laboratories, Beckenham, England). [3H]deoxythymidine (specific activity, 77.2 Ci/mmol) was obtained from New England Nuclear. Bovine testes calmodulin was purified by phenethylamine-Sepharose 6B affinity-based procedures (19) coupled with the standard isolation procedures of Watterson et al. (20). The calmodulin antagonists, W-5, W-12, and W-13, were generous gifts from Dr. P. M. Conn, Pharmacology Department, University of Iowa, and Dr. M. Cormier, University of Georgia, and were also synthesized in our laboratory by R. Reynolds. The W-7 was a generous gift from Dr. S. Kennedy, Neurosciences Research Branch of the National Institute of Mental Health.

Tissue Culture. The cell line utilized for this study was HL-60, the human promyelocytic cell line originally isolated and characterized by Gallagher et al. (21). The cells were grown in suspension culture in RPMI-1640 supplemented with 10% fetal calf serum, 10 mm N-2-hydroxylethylpiperazine-N-2-ethanesulfonic acid, penicillin (50 units/ml), and streptomycin (50 µg/ml) between a density of 0.35 x 10⁶ and 1.0 x 10⁶ cells/ml. The cell line was determined to be free of Mycoplasma (Hoescht stain) and routinely carried in our laboratory for 4 mo, at which time it was replaced with a fresh vial of cells from frozen early passage stocks.

Evaluation of Differentiation. Two early markers of myeloid differentiation were used in these studies. These were assayed by measuring increased binding of a chemotactic peptide to HL-60 cells (22) and by monitoring elevated free radical production through reduction of NBT (23). Changes in cell number and viability were also evaluated.

The chemotactic receptor assay monitors the ability of HL-60 cells to bind the peptide, N-formyl-Nle-Leu-Phenylalanyl-L-tyrosyl-L-lysine. Following 30-min incubation at room temperature, the binding reaction was terminated by trapping the HL-60 cells on glass fiber filters with rapid filtration. The results are reported as fmol of peptide bound per 10⁶ cells.

The ability of cells to reduce NBT was assessed by the procedure of Collins, et al. (23). Briefly, the cells were harvested by centrifugation and resuspended in medium at 10⁶ cells/ml. Cells were then incubated

Received 2/5/85; revised 11/27/85; accepted 1/29/86.
1 Supported by a grant from the American Cancer Society CH-301 (M. L. V.) and a grant from the National Cancer Institute CA 35680 (J. N.)
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3 The abbreviations used are: W-7, N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide; W-12, N-(4-aminobutyl)-2-naphthalenesulfonamide; W-13, N-(4-aminobutyl)-5-chloro-2-naphthalenesulfonamide; W-5, N-(6-aminohexyl)-1-naphthalenesulfonamide; dbcAMP, dibutyryl cyclic adenosine monophosphate; DMSO, dimethyl sulfoxide; NBT, nitroblue tetrazolium dye; ID₅₀, 50% inhibitory dose.
at 37°C for 20 min with NBT (final concentration, 0.1%) and phorbol myristate acid (final concentration, 100 ng/ml). The percentage of cells containing intracellular blue-black formazan deposits was then determined after preparation of slides by cytocentrifugation. At least 300 cells were assessed in random cell fields for each determination.

Cell number was determined by hemocytometer; cell viability was assessed by direct counting of cells excluding trypan blue.

Preparation and Assay of Protein Kinase C. Cytosolic protein kinase C was purified through the Sephadex G-150 step as described previously (16). The effect of the naphthalene sulfonamides on protein kinase C activity was then evaluated by established procedures (16). Briefly, the reaction mixture (250 μl) contained 10 μM ATP (0.2-0.4 μCi [32P]-ATP), phosphatidylserine (20 μg/ml), diolein (2 μg/ml), 1 mM CaCl₂, 10 mM MgCl₂, calf thymus histone V-S (200 μg/ml) (H1), 0–250 μM naphthalene sulfonamide, and 50 μl HL-60 protein kinase C. The reaction was initiated by addition of the enzyme. After 10 min at 30°C, the reaction was stopped by the addition of 500 μg of bovine serum albumin and 1 ml of 25% trichloroacetic acid. The precipitated proteins were collected on Millipore HA filters and washed with trichloroacetic acid. Dried filters were then placed in scintillation vials containing 5 ml of Ultrafluor (National Diagnostics, Inc.) and quantified by scintillation counting spectroscopy.

Procedure Used for Handling Calmodulin Antagonists. Stock solutions (10 mM) of W-7, W-12, and W-13 were prepared in 95% ethanol. Working solutions (1–2 mM) of these drugs were obtained by dilution of the stock solutions into culture medium without fetal calf serum. A working solution (5 mM) of W-5 was prepared directly in culture medium without fetal calf serum and sterile filtered. Spectra of each stock solution from 370–250 nm were recorded to ensure consistent drug concentrations between experiments. Published extinction coefficients (26) were also used for quantitative preparation of drug stocks (W-5, E₂0₅0 = 6400; W-7, E₂0₅⁰ = 8060).

As described in the figure legends, HL-60 cultures were exposed to various concentrations of calmodulin antagonists for different time periods. Since these drugs are very lipid soluble (27), removal from the cell may be quite difficult (28). For this reason, the following procedure was adapted for removal of the calmodulin antagonists from cell cultures. The cells were pelleted from the drug-containing culture medium at room temperature. The cultures were then reconstituted by resuspending the cells (at a concentration equal to their original volume) in medium containing fetal calf serum, returned to the incubator at 37°C for 30 min to allow diffusion of drugs from the cell membrane, pelleted from the medium once more, and again resuspended in medium with fetal calf serum. After thus removing drugs, the cultures were returned to the incubator until completion of the experiment.

Quantitation of Calmodulin. For this analysis, 2 × 10⁷ cells were pelleted and resuspended in 1.0 ml of lysis buffer (100 mM sodium acetate-10 mM β-2-mercaptoethanol-10 mM ethyleneglycol bis(β-aminoethyl ether)-N,N',N'-tetraacetic acid-20 mM phenylmethylsulfonyl fluoride-1 % trasylol, pH 7.4). An aliquot was disrupted with a Branson sonifier (four 15-s bursts), the disrupted cells were sonicated (10 HIM)of W-7, W-12, and W-13 were prepared in 95% ethanol.

Cell number was determined by hemocytometer, cell viability was assessed by direct counting of cells excluding trypan blue.

RESULTS

A number of agents (retinoic acid, DMSO, dbcAMP, and various naphthalene sulfonamide derivatives) were used alone and in combination to induce differentiation of HL-60 cells. In Fig. 1, expression of differentiation markers caused by these inducers is compared. Differentiation was monitored by increased binding of the formyl peptide to HL-60 cells and by the enhanced ability of these cells to reduce NBT-tetrazolium dye.

Calmodulin antagonists have the ability to induce differentiation of HL-60 cells and to enhance the differentiation induced by retinoic acid, DMSO, or dbcAMP (31). As illustrated in Fig. 1, DMSO, retinoic acid, and the calmodulin antagonist, W-13 alone, were inducers of differentiation. In contrast to DMSO, the potent calmodulin antagonist W-13 and 30 nM retinoic acid alone induced only small increases in formyl peptide binding (DMSO-treated cultures, 115 fmol peptide per 10⁶ cells; retinoic acid-treated cultures, 60 fmol peptide per 10⁶ cells; W-13-treated cultures, 25–40 fmol peptide per 10⁶ cells; control cultures, 10–15 fmol peptide per 10⁶ cells). However, a 6-h exposure to W-13 (24–30 h following administration of DMSO or retinoic acid) enhanced formyl peptide binding 2- to 3-fold and also increased the percentage of NBT-positive cells in the treated cultures. Furthermore, addition of the calmodulin antagonist, W-13, to cells exposed to dbcAMP for 24 h did not increase receptor production but did augment the percentage of
It should be pointed out that, under different conditions, retinoic acid alone can more effectively induce differentiation of HL-60 cells (32). However, in the combined drug experiments described here, only 30 nM retinoic acid was used versus the optimal concentration of 1 μM, and retinoic acid was not continuously present (as required for maximum response) (32) but was removed 30 h following its addition to the cultures.

As in other cell systems (33, 34), addition of a calmodulin antagonist to HL-60 greatly inhibited cell division. Cultures were seeded at 0.35 × 10⁶ cells/ml. Control cultures reached 1.9–2.2 × 10⁶ cells/ml 4 days later, but cells treated with naphthalene sulfonamides for 24–30 h after initiation of the experiment only grew to 0.6–1.2 × 10⁶ cells/ml (see Fig. 1), suggesting that the inhibitor-treated cultures only went through one or two rounds of division. In a separate study, [³H]deoxythymidine incorporation into DNA was monitored in HL-60 cells immediately following a 6-h exposure to either retinoic acid, DMSO, or W-13. Cultures treated with the potent calmodulin antagonist were inhibited 80% (control cultures, 136,000 cpm/10⁶ cells, versus cultures treated with 30 μM W-13, 25,000 cpm/10⁶ cells), while cultures treated with DMSO or retinoic acid displayed no immediate reduction in DNA synthesis. DNA synthesis returned to normal levels 18 h after removal of W-13 from the cell cultures, although no cell division was evidenced by an increase in cell number until 66 h after drug removal. These studies indicate that the naphthalene sulfonamides, unlike retinoic acid and DMSO, have an immediate inhibitory effect on DNA synthesis and cell replication.

HL-60 differentiation is a general drug dose-dependent response to naphthalene sulfonamides. Fig. 2 more closely examines the ability of another naphthalene sulfonamide, W-7, to induce differentiation of HL-60 cells in a dose-dependent manner. Like W-13, W-7 alone was a weak inducer (Fig. 2A; however, in combination with retinoic acid, W-7 was quite effective (Fig. 2B). This combined drug regimen consistently yielded a response that was slightly more than additive. In both of these studies, enhancement of differentiation by W-7 could only be demonstrated over a very narrow range (20–35 μM). Cell viability did not significantly vary from control levels at these drug concentrations; however, higher concentrations of W-7 (50 μM) were extremely toxic. Furthermore, the toxicity of W-7 was not affected by the presence of retinoic acid.

In the previous experiments (Figs. 1 and 2), naphthalene sulfonamides were added to cells for 6 h (24–30 h) following addition of retinoic acid. Exposing cells to the naphthalene sulfonamides for longer periods of time (up to 24 h) resulted in a steady, but gradual enhancement of differentiation markers, with no effect on cell viability (data not shown). Furthermore, the 24- to 30-h time window did not appear to be critical for the enhancement of differentiation by the naphthalene sulfonamides. Fig. 3A suggested that exposure of cultures to W-13 anytime during the first 24 h following addition of retinoic acid resulted in a similarly enhanced differentiation response. Even a 12-h pretreatment of cultures with W-13 resulted in a less-marked, but still additive differentiation response. In addition, continued presence of retinoic acid (beyond the period of exposure to the naphthalene sulfonamide) did not lead to additional increases in differentiation markers (Fig. 3A and B), suggesting that commitment to differentiation is complete within 12 h when these drug combinations are used.

Fig. 3C depicts a separate study comparing the effective concentration range for induction of differentiation by W-13 treatment at different time intervals. This experiment also shows that exposure of HL-60 cells to W-13 from 0–6 h is equally, if not more, effective than treatment between 24 and 30 h following induction with retinoic acid.

Fig. 4 compares the ability of W-5, W-7, W-12, and W-13 to enhance retinoic acid-induced differentiation in HL-60 cells. The two potent calmodulin antagonists, W-7 and W-13, were much more effective inducers of differentiation than their less potent analogues, W-5 and W-12. Table 1 compares the concentration of these drugs required to elicit binding of 125 fmol of formyl peptide to HL-60 cells (approximately 50% of maximum response in our assay) with published values for inhibition of calmodulin-dependent phosphorylase activity and the ID₅₀ for displacement of [³H]W-7 from calmodulin (27). These experiments demonstrated a good correlation between the ability of these drugs to induce differentiation and their potential to bind to and inhibit calmodulin.

These calmodulin antagonists have also been reported to be inhibitors of protein kinase C (12, 17, 18). However, exposure of HL-60 protein kinase C to these antagonists resulted in no significant inhibition of this enzyme, as determined by the procedure described in "Materials and Methods." Concentrations as high as 250 μM W-5, 40 μM W-7, 150 μM W-12, and 50 μM W-13 displayed no inhibitory effects on the in vitro phosphorylation of histone H1.
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Fig. 3. Dependence of W-13 augmentation of retinoic acid (R.A.)-induced differentiation on time and W-13 concentration. In A and B, the time relationship, relative to retinoic acid exposure, for induction of differentiation by W-13 was explored.

In A, 25 μM W-13 was added for 12 h prior to addition of 30 nM retinoic acid (time 0) or at two subsequent time periods, 0-12 h and 12-24 h after retinoic acid addition. The drugs were removed from cells according to the procedure outlined in “Materials and Methods.” The W-13 exposure periods were followed by resuspension of the HL-60 cells in medium containing 30 nM retinoic acid.

In B, an identical procedure was followed, except that cultures were suspended in medium without retinoic acid subsequent to removal of W-13. Cells treated with W-13 from -12 to 0 h were never exposed to retinoic acid, while cells treated with W-13 from 0 to 12 h were exposed to retinoic acid for 12 h. Further, cells treated with W-13 from 12 to 24 h were exposed to retinoic acid from 0 to 24 h.

Four days following initiation of the experiment, the cells were harvested and assayed for fmol peptide bound per 10^6 cells.

In C, HL-60 cells were incubated in culture medium containing 30 nM retinoic acid for 30 h. Various concentrations of W-13 (between 0 and 35 μM) were added to these cultures for two different 6-h periods, 0-6 h (C) and 24-30 h (C). At 30 h the cells were washed free of all drugs by centrifugation (see “Materials and Methods”) and resuspended in culture medium without retinoic acid. Four days following induction of differentiation by retinoic acid, the cells were harvested and assayed for fmol peptide bound per 10^6 cells.

Fig. 4. Naphthalene sulfonamides as promoters of differentiation in HL-60 cells. HL-60 cells were exposed to 30 nM retinoic acid for 30 h. The indicated concentrations of calmodulin antagonists were then added to the cultures for 6 h, between 24 and 30 h following addition of retinoic acid. At 30 h the cells were washed free of all drugs by centrifugation (see “Materials and Methods”) and resuspended in culture medium. Four days following induction of differentiation by retinoic acid, the cells were harvested and assayed for fmol peptide bound per 10^6 cells. Dose-response curves for enhancement of differentiation by the calmodulin antagonists: W-7 (A); W-13 (C); W-5 (A); and W-12 (C) are depicted.

The ascending portion of each curve was calculated by linear regression, which resulted in correlation coefficients of 0.98, 0.97, 0.95, and 0.98, respectively, for W-7, W-13, W-12, and W-5.

Since a calmodulin response appeared to be consistent with the ability of these antagonists to promote differentiation of cells toward neutrophils, we investigated the possibility that a cell’s calmodulin content may change during this process (Fig. 5). These studies did not indicate that differentiation responses

are controlled through mediation of intracellular calmodulin levels. Although the levels of calmodulin per cell dropped significantly over the differentiation time course, the decrease in calmodulin was somewhat less than that seen for total cellular protein in these cultures. Therefore, the relative calmodulin concentration (calmodulin per mg of total soluble protein) remained quite constant throughout the differentiation process.

DISCUSSION

This study examined possible roles for calmodulin in the maturation of promyelocytes toward cells with granulocytic characteristics. Though not a perfect model, HL-60 cells provide a facile system for investigation of this differentiation process, since they can be stimulated to mature toward monocytes or granulocytes with a variety of agents, alone (32, 35-37) or in combination (37). In the experiments described above, naphthalene sulfonamide calmodulin antagonists were used alone and in combination with DMSO, retinoic acid, and dbcAMP, to induce differentiation of HL-60 cells toward gran-
ulocytes. The naphthalene sulfonamides were chosen for this study because of the availability of a series of congeners with different potentials for inhibition of calmodulin function. However, these drugs, like all calmodulin antagonists, have non-calmodulin-related side effects so that indirect drug studies cannot prove calmodulin is linked to control of differentiation (for a review, see Ref. 10). However, the fact that treatment with calmodulin antagonists leads to normal expression of differentiation markers establishes a strong rationale for further investigation of a role for this regulator in maintenance of the undifferentiated phenotype of HL-60.

In our studies, retinoic acid, DMSO, dbcAMP, and the naphthalene sulfonamides alone were capable of inducing differentiation of HL-60 cells. However, a combined treatment with these agents (i.e., DMSO and the naphthalene sulfonamides or retinoic acid with the naphthalene sulfonamides) resulted in a response that was only slightly more than additive, suggesting that these inducers may work at separate points in the differentiation process. In this regard, the naphthalene sulfonamides have been reported to be effective inhibitors of cell division in HL-60 (38) and other cell types (33, 34). These drugs appear to arrest DNA synthesis through interference with a calmodulin-dependent process at the G1-S boundary during cell cycle traverse (33, 34). Likewise, in our study, the naphthalene sulfonamides caused rapid cessation of DNA synthesis and cell growth, which was followed by an increased expression of differentiation markers. In contrast, treatment with DMSO and retinoic acid led to a gradual decrease in cell growth rate with concomitant increased expression of differentiated function. These contrasting cellular responses support the suggestion that these drugs act by different mechanisms to induce differentiation and may explain why they are more effective in combination than as single agents.

Changes in contractile protein composition of myeloid cells during differentiation have also been reported (39). Calmodulin antagonists may disrupt microtubule assembly and disassembly, which has been implicated as a calmodulin-regulated process (40, 41). Moreover, disruption of microfilaments by cytochalasin B and D resulted in slight enhancement of differentiation induced by 1,25-dihydroxyvitamin D. Further, small increases in NBT reduction were also observed when vitamin D-treated cells were exposed to a single concentration (20 μM) of W-7 or W-13. At the same time, however, overall protein phosphorylation of the putative protein kinase C target protein, M, 17,000 phosphoprotein, is reduced in HL-60 cells exposed to 35 μM W-13. At the same time, however, overall protein phosphorylation increases in the drug-treated cells (data not shown). Although this work is clearly beyond the scope of the present study, it does demonstrate the complexity of the intracellular response of cells to perturbation by the naphthalene sulfonamide antagonists.

Our most recent studies indicate that the levels of W-13 used in our experiments may antagonize protein kinase C activities as well as calmodulin activities. In preliminary studies of changes in protein phosphorylation patterns caused by naphthalene sulfonamides, cells were treated before and after induction of differentiation to monocytes by phorbol diesters. In spite of protein kinase C's apparent resistance to inhibition by naphthalene sulfonamides in an in vitro assay, the results of our recent in vivo analysis of drug-treated cells are consistent with interference of protein kinase C-dependent events. Consistent with previously reported effects of phenothiazines (43), phosphorylation of the putative protein kinase C target protein, M, 17,000 phosphoprotein, is reduced in HL-60 cells exposed to 35 μM W-13. At the same time, however, overall protein phosphorylation increases in the drug-treated cells (data not shown). Although this work is clearly beyond the scope of the present study, it does demonstrate the complexity of the intracellular response of cells to perturbation by the naphthalene sulfonamide antagonists.

Further, non-calmodulin-dependent effects of Ca2+ may be controlled by interference with calmodulin-dependent enzymes. For instance, the primary target of calmodulin inhibitors in HL-60 cells could be a calmodulin-dependent ATPase (44-46) similar to that identified in erythrocytes. Inhibition of this calmodulin-regulated enzyme may lead to higher intracellular levels of Ca2+ through interference with normal Ca2+ efflux mechanisms, as well as an increase in ATP pools. Other cellular enzymes might subsequently respond to these elevated Ca2+ and/or ATP levels.

The results presented in our studies would appear to be consistent with recent models, contrasting effects of differentiating agents. Previous studies have suggested that agents which cause differentiation of myeloblastic leukemia cells may be divided into two general categories: (a) those that block and sustain inhibition of DNA synthesis and (b) those that cause an immediate increase in differentiative functions with a concomitant decrease in DNA synthesis as maturation proceeds (36, 47). Proliferating HL-60 cells are poised at a decision point between cell division and differentiation. Inducers of differentiation may set in motion a series of events that redirect these cells from division to differentiation-oriented functions. This decision is invariably characterized by reduction in the rate of cell division and DNA synthesis and acquisition of new cellular capacities (differentiated functions).

Our studies suggest that the naphthalene sulfonamides can both trigger and augment the myeloid differentiation process. As discussed earlier, these drugs (which rapidly block DNA synthesis and cell division) augment effects of inducers like DMSO and retinoic acid (which cause a slow transition to a differentiated phenotype). These data indicate that these agents exert their effect through perturbation of different cellular mechanisms and suggest a biochemical rationale for new combination chemotherapy strategies.

REFERENCES


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