Relation of P-glycoprotein expression with spontaneous *in vitro* apoptosis in B-cell chronic lymphocytic leukemia*

K. JAMROZIAK[1], P. SMOLEWSKI[1], B. CEBULA[1], A. SZMIGIELSKA-KAPLON[1], Z. DARZYNKIEWICZ[2], T. ROBAK[1]

[1]Department of Hematology, e-mail: krzysztof.jamroziak@wp.pl, Medical University of Lodz, 93-510 Lodz, Poland; [2]Brander Cancer Research Institute, New York Medical College, Valhalla, New York 10595, USA

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Prolonged lifespan of monoclonal lymphocytes in B-cell lymphocytic leukemia (B-CLL) arises from their resistance to programmed cell death. In contrast, when cultured *in vitro,* B-CLL tumour cells rapidly undergo apoptosis. There is mounting evidence that P-glycoprotein (P-gp), an adenosine triphosphate-binding cassette (ABC) family transporter, plays a significant role in the regulation of apoptosis induced by various stimuli. Since P-gp is commonly expressed in B-CLL cells, we aimed to establish whether its expression level influences resistance to spontaneous apoptosis in B-CLL. For that purpose, P-gp expression by UIC2 antibody staining and P-gp activity by rhodamine 123 (Rh123) efflux in presence or absence of P-gp inhibitor verapamil were studied in peripheral blood lymphocytes obtained from 43 previously untreated B-CLL patients. Simultaneously, the percentage of cells undergoing spontaneous in vitro apoptosis (apoptotic index, AI) by means of activation of caspases and annexin-V-based assays was evaluated. The AI were higher in B-CLL cells than in normal peripheral blood mononuclear cells (medians of AI 27.7% vs 3.9%, p=0.0001 and 34.7% vs 7.4%, p=0.0038, in 24 and 48-hour culture respectively). The AI were also higher among female patients as compared to male patients (medians: 29.7 vs 19.2 p=0.048). Interestingly, we found moderate inverse correlation between P-gp protein expression and AI after 24-hour culture in analysed B-CLL samples (r= –0.36, p=0.019). Moreover, P-gp positive B-CLL samples expressed significantly higher AI than P-gp negative samples with an arbitrary cut-off at Kolmogorov-Smirnov statistics D-value 0.2 (medians of AI 18.4% vs 29.7%, p=0.026). Based on these results we suggest that P-gp expression has some protective effect on B-CLL cell survival *in vitro*. The difference in the rates of spontaneous apoptosis among male and female patients may contribute to gender-dependent variations in clinical outcome in B-CLL.

Key words: P-glycoprotein, apoptosis, B-cell chronic lymphocytic leukemia

The longevity of B-cell lymphocytic leukemia (B-CLL) cells is a result of aberrations in apoptotic cell death pathways, which are also a main obstacle to successful cytotoxic therapy. On the intracellular level this resistance to apoptosis is most frequently related to high bcl-2 protein expression or high bcl-2/bax ratio [16]. However, down-regulation of Mcl-1, another bcl-2 family member as well as X-linked inhibitor of apoptosis protein (XIAP) seem also to be an important factor in drug-induced apoptosis in B-CLL [20]. Moreover, constitutive activation of some signal transduction pathways e.g. phosphatidylinositol-3 kinase (PI-3K) pathway mediates additional survival signals in B-CLL [29]. Thus, the regulation of B-CLL cells survival is a complex phenomenon with several mechanisms being involved.

Interestingly, in contrast to their survival characteristics *in vivo*, B-CLL malignant cells rapidly undergo spontaneous apoptosis when cultured. The mechanism of this phenomenon is unclear. Recent report indicates that advanced-stage B-CLL cells are relatively resistant to spontaneous apoptosis, whereas high susceptibility to apoptosis is encountered in early-stage B-CLL [25]. Consequently, further studies on the mechanisms regulating spontaneous apoptosis in B-CLL could have potential prognostic and clinical implications.

P-glycoprotein (P-gp), a 170kD product of *MDR1* gene, belongs to adenosine triphosphate-binding cassette (ABC)

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family of membrane transporters [1]. Under physiological conditions P-gp protects the organism against toxic xenobiotics, while in tumors P-gp-dependent extrusion of antineoplastic agents is the major cause of the multi-drug resistance (MDR) phenotype [1]. However, P-gp has been recently shown to inhibit apoptosis induced by a variety of factors including: serum deprivation, FasL, tumor necrosis factor (TNF), ultraviolet and gamma irradiation [19]. SMYTH et al, showed that protection by P-gp is probably associated with factors involved in caspase-dependent pathway of apoptosis [34]. Thus, there is strong evidence for the involvement of P-gp in regulation of apoptosis.

Interestingly, P-gp expression was associated with resistance to *in vitro* spontaneous apoptosis in acute myeloid leukemia (AML) [26]. P-gp is a well established negative prognostic factor in AML and recently the same correlation was shown for the rate of spontaneous apoptosis associated with high bcl-2/bax ratio in this malignancy [11]. Several studies proved that majority of B-CLL clones intrinsically express relatively high levels of P-gp [9, 13, 32, 35, 36]. However, to our best knowledge, this has not been investigated in the context of spontaneous apoptosis yet.

Therefore, the aim of this study was to reveal whether there is a relationship between expression and activity of P-gp and incidence of spontaneous apoptosis in B-CLL. For that purpose these parameters were measured in parallel in circulating lymphocytes obtained from a population of untreated B-CLL patients.

Material and methods

Cell lines. The human colon carcinoma SW 620 and its multidrug-resistant derivative SW 620/Adr300 cell lines [23], kindly provided by Dr R.W. Robey and Dr S.E. Bates, Developmental Therapeutics Dept., National Cancer Institute, National Institute of Health, Bethesda, MD, USA, were used as reference for testing P-gp expression and activity. Both cell lines were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum (FCS) with addition of streptomycin at 50 μl/ml and penicillin at 50 IU/ml (Life Technologies) at 37 ˚C, in an atmosphere of 5% CO_2. The media of SW 620/Adr300 cell line was supplemented with 300 nM of Adriamycin (Sigma). Cells were grown without Adriamycin for four weeks prior to analysis.

Human subjects. The study was performed on peripheral blood samples obtained from 43 patients with B-CLL (24 males and 19 females) with median age of 68 years (range 40–81). The diagnosis was established according to standard morphologic and immunophenotypic criteria. Clinical staging was performed according to Rai and Binet classifications. Exclusively patients, who have never received any cytotoxic treatment for B-CLL were enrolled to the study.

Table 1. Clinical characteristics of 43 B-CLL patients included in the study

Parameter	Median (range)
Age (years)	68.0 (40.0–81.0)
Sex	
male	24
female	19
White cell count (G/l)	47.6 (16.8–190.0)
Erythrocytes (T/l)	4.2 (2.6–5.2)
Hemoglobin (g/dl)	12.7 (6.6–16.6)
Platelets (G/l)	163.0 (36.0–268.0)
Clinical stage according to Rai	
0	17
I	12
II	5
III	5
IV	4
Clinical stage according to Binet	
A	25
B	9
C	9
Status of disease	
stable	34
progressive	9
Duration of follow-up (months)	20.5 (3.0–196.0)

In nine patients the disease was defined as stable, whereas 34 had progressive B-CLL (including lymphocytosis doubling time <one year, massive splenomegaly, bulky adenopathy, recurrent disease-related infections, weight loss >10% in a six month period, temperature 38 ˚C related to disease, extreme fatigue, III and IV Rai stage). The informed consent according to institutional guidelines was obtained from every patient. Detailed clinical characteristics of patients included in the study is presented in Table 1. Additionally, peripheral blood samples were taken from six volunteers (five females, one male) with median age of 35 (range 23–50) as normal controls.

Isolation and culture of peripheral blood mononuclear cells. Peripheral blood mononuclear cells (PBMC) were isolated from fresh EDTA-whole peripheral blood samples of patients and controls by Ficoll gradient density separation and washed twice in phosphate-buffered saline (PBS). After isolation, PBMC from 10 (23%) B-CLL patients were verified by dual-color flow cytometry and found to contain $89.1 \pm 8.8\%$ (mean $\pm$ SD) CD5+/CD19+ cells. Freshly isolated PBMC were transferred to RPMI 1640 medium supplemented with 10% FCS with addition of streptomycin at 50 μl/ml and penicillin at 50 IU/ml (Life Technologies). The cells were incubated in wells, each sample in total volume of

300 μl, in concentration 1x10^6 cells per ml. The cultures were maintained up to 48 hours in a 5% CO_2, fully humidified atmosphere, at 37 ˚C.

Expression and activity of P-glycoprotein. According to standard recommendations for evaluating of P-gp status we used two-parameter technique measuring protein expression by specific monoclonal antibody staining and P-gp activity as ability to efflux fluorescent compound [2]. P-gp expression and activity was assessed on freshly isolated PBMC by means of flow cytometry. The concentration of isolated cells was set to 2x10^6 cells/ml in RPMI 1640 medium.

For measurement of P-gp expression cells were incubated at room temperature for 15 minutes, in separate tubes, with phycoerythritin (PE)-conjugated UIC2 monoclonal antibody (Immunotech), directed against extracellular epitope of P-gp, at concentration of 2 μg/ml or with isotype control PE-conjugated IgG2a monoclonal antibody (Dako) at concentration of 2 μg/ml. Results were detected as differences in mean fluorescence intensity (MFI) between UIC2 and IgG2a-labeled samples.

The evaluation of P-gp activity was based on fluorescent P-gp substrate rhodamine 123 (Rh123) (Sigma) efflux in presence or absence of a P-gp inhibitor verapamil (Sigma). Two separate tubes were used for each experiment; one containing Rh123 at concentration of 200 ng/ml and another one containing mixture of Rh123 at concentration of 200 ng/ml and verapamil at concentration of 10 μM. Initially, cells were loaded with Rh123 for 30 minutes, at 37 ˚C in atmosphere of 5% CO_2. Subsequently, samples were washed twice in RPMI and left to efflux for 60 minutes at 37 ˚C at 5% CO_2; to the sample previously containing verapamil, this inhibitor was added at the same concentration. Results were calculated as a difference in MFI of Rh123 between sample incubated with and without verapamil.

The results for P-gp expression and activity were expressed as D-values derived from Kolmogorov-Smirnov (K-S) statistics. When MFI of examined sample was lower than that of appropriate control, i.e. sample with IgG2a or Rh-123 without verapamil (for measurements of expression or activity, respectively) D-value was evaluated as 0. Since the threshold of P-gp positivity is controversial we preferably analyzed the P-gp expression and activity as continuous variables. However, in some analysis, an arbitrary cut-off level was set at D0.2.

Evaluation of apoptosis. Apoptosis of cultured B-CLL cells and control cells was measured at two time-points: after 24 and after 48 hours of incubation using two sensitive flow cytometric assays. The percentage of positive cells in each of those methods was denominated as apoptotic index (AI).

FAM-VAD-FMK (FLICA) binding assay: The FLICA assay is based on detection of binding of the fluorochrome-labeled inhibitors of caspases [33]. In this study we used the pan-caspase FAM-VAD-FMK inhibitor (FLICA-VAD), obtained from Immunochemistry Technologies, Bloomington, MN, USA. This reagent is commercially available as a CaspaTag^{TM} Fluorescein Caspase Activity kit. Staining was performed as described before [33]. Data were collected by flow cytometry.

Phosphatidylserine exposure and cell membrane permeability: The annexin-V/propidium iodide (PI) assay was used as a marker of cell viability and apoptosis. The method has been described earlier in detail [21]. In brief, the cells after incubation were washed twice with cold PBS. Than 100 μl of binding buffer, containing 2 μl FITC conjugated annexin V and 10 μg/ml PI were added and the sample was incubated in dark for 15 minutes. The cellular fluorescence was measured by flow cytometry.

Flow cytometric data collection and analysis. All analyses were carried out using a FacsCalibur flow cytometer (Becton Dickinson) equipped in 488 nm argon laser and standard set of emission filters and fluorescence detectors. At least 10 000 events per sample were collected in each measurement. Data were analyzed using CellQuestPro (Becton Dickinson) computer software.

Statistical analysis. Statistical analysis was performed using STATISTICA version 5.1 (StatSoft, Inc.) statistical software package. In order to test correlations between variables Spearman's correlation coefficient was calculated. To evaluate differences among subgroups non-parametric Man-Whitney U-test and ANOVA Kruskall-Wallis test were applied. All p-values are two sided and $p<0.05$ was considered significant.

Results

Expression and activity of P-glycoprotein. Initially, the reference cell lines were analyzed in respect of P-gp expression and activity. In four independent experiments P-gp expression given as K-S statistics D-values for UIC2 vs. IgG2a isotype-matched control staining was 0.51 ± 0.16 for SW 620 cells as compared to 0.92 ± 0.1 for SW 620/Adr300 cells. Consistently with this up-regulation of P-gp protein expression in drug-selected cell line, P-gp activity analyzed as verapamil-modulated Rh123 efflux was $D=0.98 \pm 0.01$ in SW 620/Adr300 cell line and only $D=0.27 \pm 0.11$ in SW 620 cells.

Subsequently, the PBMC samples from 43 B-CLL patients were evaluated. P-gp expression varied importantly among patients with median at D=0.165 (range 0.08–0.85). There were 18 (42%) P-gp expressing patients with cut-off value set at D=0.2. No correlations were found between P-gp expression and laboratory or clinical parameters in analyzed B-CLL population (data not shown).

Distinct variations were observed among B-CLL samples analyzed for P-gp activity. D-value ranged from 0 to 0.67 with median at 0.15. When cut-off value was set at D=0.2

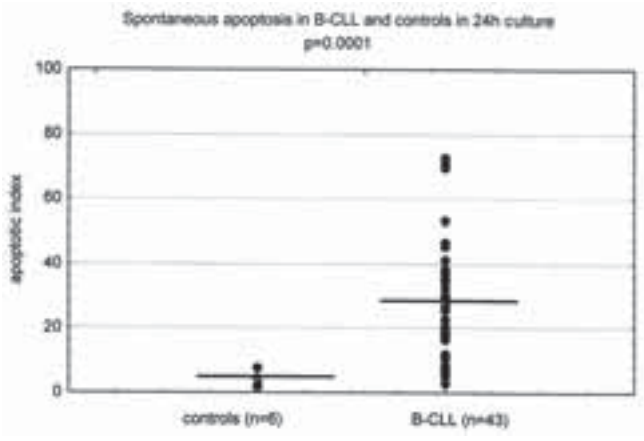

Figure 1. Comparison of rates of spontaneous apoptosis expressed as apoptotic index between cultured *in vitro* peripheral blood mononuclear cells isolated from B-CLL patients and healthy control subjects.

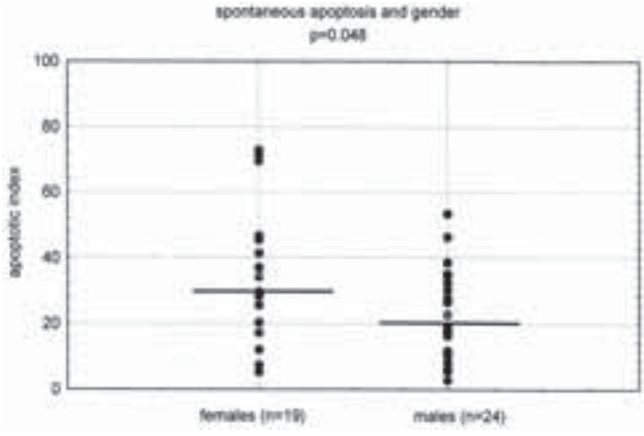

Figure 2. Difference in the rate of *in vitro* spontaneous apoptosis between male and female B-CLL patients.

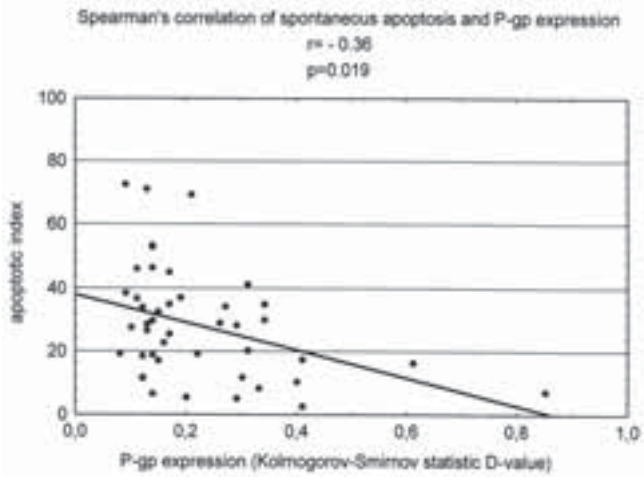

Figure 3. Relation of spontaneous apoptosis level and protein expression of P-glycoprotein in 24-hour *in vitro* culture of peripheral blood mononuclear cells isolated from 43 B-CLL patients.

there were 18 (41%) P-gp-positive samples. Interestingly, P-gp protein expression did not correlate significantly with P-gp activity ($p>0.05$). A weak correlation between total white cell blood count and P-gp activity was observed ($r=0.30$, $p=0.049$). No other correlations were found between P-gp activity and basic hematological data, or with clinical parameters of the patients.

Evaluation of spontaneous apoptosis. The spontaneous *in vitro* apoptosis was measured by FLICA assay in all 43 patients after 24 hours of culture and in 14 of these patients also at second time-point after 48 hours of culture. Simultaneously, in 20 patients spontaneous apoptosis was assessed by annexin-V/ PI assay. PBMN cells isolated from six controls were evaluated at 24- and 48-hour culture by both methods. As was previously described [37], the results of these two techniques showed significant correlation ($r=0.84$, $p=0.0001$). Therefore, statistical analysis was performed based on the frequency of FLICA positive cells.

Among analyzed B-CLL samples the AI varied distinctly. Median AI was 27.7% (range 2.9–72.8) and 34.7% (range 11.8–69.6) after 24 and 48 hours of incubation, respectively. The medians of AI detected in normal PBMN cells were 3.9% (range 1.6–7.4) in 24-hour culture and 7.4 (range 1.8–29.3) in 48-hour culture. The AI at both time points were significantly higher in B-CLL than in normal subjects, $p=0.0001$ and $p=0.0038$ in 24 and 48-hour culture, respectively (Fig. 1). Interestingly apoptosis after 24 hour culture was more prominent among female B-CLL patients than in males (Fig. 2). Median AI among female patients ($n=19$) was 29.7, while only 19.2 among male patients ($n=24$), $p=0.048$. This finding could not be attributed to distribution of patients with early or advanced B-CLL clinical stage, which was comparable in both gender groups (data not shown). No other significant associations were shown between AI and clinical or laboratory patient characteristics.

P-gp status and spontaneous apoptosis. Moderate, although significant inverse correlation was found between percentage of apoptotic cells and the P-gp expression after 24-hour culture ($r=-0.36$, $p=0.019$) (Fig. 3). Moreover, when the cut-off was set at $D=0.2$, AI in P-gp positive B-CLL samples (median 18.4%) was significantly higher than in P-gp negative samples (median 29.7%) in 24 hour culture, $p=0.026$. No significant associations were found for P-gp expression and AI of 48-hour culture or when P-gp activity data was analyzed.

Discussion

B-CLL cells are resistant to apoptosis *in vivo*, but undergo rapid spontaneous apoptosis *in vitro*. Here we found, that

rate of *in vitro* spontaneous apoptosis was significantly higher in B-CLL lymphocytes as compared to normal PBMN cells (medians of AI 27.7% vs. 3.9%, p=0.0001 and 34.7% vs. 7.4%, p=0.0038, in 24 and 48-hour culture respectively). Theoretically, this high susceptibility to spontaneous apoptosis of cultured B-CLL cells may be a consequence of deficiency in culture of either an established or yet undefined survival factor. Several cytokines were shown to promote cultured B-CLL cells survival e.g. interleukin (IL)-2, IL-4, IL-13 [10, 17, 20]. Moreover, the interactions of malignant cells with different subset of leukocytes including recently described blood-derived nurse-like cells as well as with stromal cells were proposed to enhance their resistance to apoptosis [6, 22]. Furthermore, homotypic interactions in B-CLL clone seem to be also of importance [28].

In this study, we aimed to explore another potential mechanism that may be associated with resistance to apoptosis of B-CLL cells, that is expression of P-gp. We found that P-gp was expressed in 42% of the tested subjects and verapamil modulated efflux was present in 41% patients, that is in accordance with proportions found by others (40–100%) (10–14). However, the necessity of using arbitrary cut-offs to distinguish P-gp positive samples as well as differences in applied methods limit the value of such comparisons [2]. In our study P-gp protein expression did not correlate with the results of the functional test. The presence of such discordance was previously reported in AML [24]. This could be partially explained by relatively high expression in B-CLL of other ABC transporters with verapamil-modulated affinity to Rh123 e.g. multidrug resistance associated protein-1 (MRP1) [5], and according to our observations also breast cancer resistance protein (ABCG2) [18].

The most interesting finding of this study was significant inverse correlation between P-gp expression and the frequency of apoptotic B-CLL cells (r= –0.36, p=0.019). Moreover, B-CLL cells expressing P-gp were more resistant to apoptosis as compared to P-gp negative cells with the arbitrary chosen cut-off value. As far as we know, this is the first report suggesting that P-gp expression on B-CLL malignant cells may contribute to phenomenon of their longevity. PALLIS et al found negative correlation of P-gp expression and decreased spontaneous apoptosis of de novo AML samples [27]. In another study, GOLLAPUD et al reported that blocking P-gp activity by anti-P-gp antibody induced apoptosis of activated peripheral blood lymphocytes [15]. Our observation is indirectly supported by the fact that verapamil increases *in vitro* toxicity of non-MDR drugs and cause clinical response in B-CLL [3, 38]. Since this activity of verapamil is most probably due not only to P-gp inhibition, we currently study the *in vitro* effect of blocking P-gp by specific monoclonal antibody on spontaneous apoptosis in B-CLL.

The mechanism by which P-gp can protect B-CLL cells from caspase-dependent death is unclear. RUEFFLI et al have shown that although all caspases are present in P-gp expressing cells, the activation of caspase-8, but not formation of death inducing signal complex (DISC), was inhibited by P-gp [31]. PALLIS et al reported that P-gp augmented cell survival in P-gp positive AML samples by modulation of sphingomyelin-ceramide pathway [27]. Also alkalinization of intracellular environment by P-gp can lead to non-efficient caspase activation. Additionally, some cytokines including IL-2 or IL-4 that can act in autocrine/paracrine manner in B-CLL tumor were suggested to be transported by P-gp [12]. In this study we observed that apoptosis rates correlated negatively with P-gp expression, but not with P-gp activity. These data suggest that efflux-independent mechanism may promote B-CLL cell survival by P-gp. One of possible mechanisms would be a co-expression of P-gp with some anti-apoptotic molecules. Association of P-gp with bcl-2 was previously described in B-CLL patients [4].

Another finding of this study is the gender difference in the rate of spontaneous apoptosis among the B-CLL patients. The PBMC samples from female B-CLL patients showed decreased resistance to spontaneous *in vitro* apoptosis when compared with male patients (median AI 29.7 vs. 19.2, p=0.048). It was reported that women with B-CLL have more benign course of the disease than males [7]. OLIVIEIRA and co-workers have shown that resistance to spontaneous apoptosis increases with the progression of the disease [25]. STEINER et al found lower P-gp activity in female B-CLL patients and proposed this as explanation for better prognosis in women [35]. Here, we found that low P-gp protein expression is linked with higher rates of B-CLL cells undergoing apoptosis *in vitro*. Taking these data together, lower P-gp activity and higher susceptibility to spontaneous apoptosis can be related with better clinical outcome in female B-CLL patients. However, further research to verify this hypothesis is necessary.

In conclusion, we found negative correlation between P-gp expression and the rate of spontaneous apoptosis in B-CLL. We suggest that relatively high intrinsic expression of P-gp in B-CLL can be additional mechanism, in which B-CLL become resistant to apoptosis. Thus, the therapeutical strategies targeting P-gp may be justified, even though drugs typically used in B-CLL i.e. chlorambucil or purine analogs are not transported by P-gp. New types of anticancer agents combining cytotoxicity with internal activity of P-gp inhibitors (e.g. selective protein kinase C inhibitor PKC 412), or overcoming P-gp-mediated resistance by acting in caspase-independent manner, may provide an interesting alternative for treatment of B-CLL [14, 30]. The difference in the rates of spontaneous apoptosis among male and female patients, which was also detected in this study, may be related to gender-dependent variations in the prognosis of B-CLL.

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