Molecular specific and cell selective cytotoxicity induced by a novel synthetic HLA-DR antibody mimic for lymphoma and leukemia

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Abstract. Like rituximab, monoclonal antibodies reactive with human leukocyte antigen have potent antilymphoma activity. However, size limits their vascular and tissue penetration. To mimic monoclonal antibody binding, nanomolecules have been synthesized, shown specific for the ß subunit of HLA-DR10, and selective for cells expressing this protein. Selective high affinity ligands (SHALs) containing the 3-(2-([3-chloro-5-trifluoromethyl)-2-pyridinyl]oxy)-anilino)-3-oxopropanionic acid (Ct) ligand residualized and had anti-lymphoma activity against expressing cells. Herein, we show the extraordinary potency in mice with human lymphoma xenografts of a tridentate SHAL containing this ligand. After titrating antilymphoma activity in cell culture, a randomized preclinical study of a tridentate SHAL containing the Ct ligand was conducted in mice with established and aggressive human lymphoma xenografts. Mice having HLA-DR10 expressing Raji B- or Jurkat's T-lymphoma xenografts were randomly assigned to receive either treatment with SHAL at a dose of 100 ng i.p. weekly for 3 consecutive weeks, or to be untreated. Primary end-points were cure, overall response rates and survival. Toxicity was also evaluated in these mice, and a USFDA general safety study was conducted in healthy Balb/c mice. In Raji cell culture, the threshold and IC_{50} concentrations for cytotoxic activity were 0.7 and 2.5 nmol (pm/ml media), respectively. When compared to treated Jurkat's xenografts or untreated xenografts, Raji xenografts treated with the SHAL showed an 85% reduction in hazard of death (P=0.014; 95% confidence interval 32-95% reduction). There was no evidence for toxicity even after i.p. doses 2000 times greater than the treatment dose associated with cure of a majority of the mice with Raji xenografts. When compared with control groups, treatment selectively improved response rates and survival in mice with HLA-DR10 expressing human lymphoma xenografts at doses not associated with adverse events and readily achievable in patients.

Introduction

Cell surface proteins, such as CD20, CD22 and MHCII HLA-DR, have proven to be attractive targets for therapeutics for non-Hodgkin's lymphoma (NHL). One of these proteins, human leukocyte antigen (HLA) is upregulated on the surface of malignant B lymphocytes when compared to normal B lymphocytes. Moreover, intracellular HLA-DR protein is even more abundant (1). HLA-DR proteins serve as trans-membrane and cytoplasmic signaling receptors and as peptide shuttles for the immune system (2-4). The monoclonal antibody (MAb), Lym-1, binds to a well-characterized epitope on the ß subunit of HLA-DR and reacts with lymphoma tissue from about 90 and 50% of patients with B-cell lymphoma and leukemia, respectively (5-7). Although binding is restricted to the cell surface (8), Lym-1 is highly active against malignant B cells in culture and in mice (9-11).

To mimic MAb binding to HLA-DR10, while decreasing size, a series of selective high affinity ligands (SHALs) <5 kDa in size have been synthesized to bind in the Lym-1 epitopic region of the ß subunit of HLA-DR protein based on *in silico* modeling and experimental studies. Bidentate versions of these novel nanomolecules showed many desired characteristics *in vitro* and in mice (12,13) but had no antilymphoma activity (14). Strikingly, a tridentate SHAL containing the Ct ligand (3-(2-([3-chloro-5-trifluoromethyl)-2-pyridinyl]oxy)-anilino)-3-oxopropanionic acid) residualized inside and was cytotoxic for B-lymphoma cells in culture (1). Here, we titrate the cell-selective antilymphoma activity of this SHAL, demonstrate its remarkable efficacy, selectivity and safety in mice with human lymphoma xenografts and show electron microscopic evidence for SHAL-induced autophagy.

Materials and methods

Reagents and cell lines. Murine (Peregrine Pharmaceuticals, Tustin, CA) and chimeric (A. Epstein, Los Angeles, CA)

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Lym-1, that bind to the ß subunit of HLA-DR10 expressed on malignant B-cells (8), and HLA-DR10 protein isolated from Raji Burkitt's B-cells using a Lym-1 affinity column, as described previously (12) were used as references. An HLA-DR10 expressing human B lymphoma cell line, Raji (American Type Culture Collection, Manassas, VA), grown in RPMI-1640 with 10% fetal calf serum, supplemented with sodium pyruvate, non-essential amino acids and antibiotics (Gibco/Invitrogen, Carlsbad, CA), at 37˚C in a humidified, 5% CO_2 atmosphere and a non-expressing human T lymphoma cell line, Jurkat's (American Type Culture Collection, Manassas, VA), grown as recommended by ATCC, were used for these experiments.

Drug design and chemistry. Using homology modeling, HLA-DR amino acid residues critical for Lym-1 binding were mapped on a 3-D model of the HLA-DR10 ß subunit (12). Cavities within the Lym-1 epitopic surface of the protein were identified using SPHGEN (15,16). After computational ligand docking for each of the cavities, a combination of NMR spectroscopy, surface plasmon resonance (BIAcore 3000; Biacore, Piscataway, NJ) and competitive binding experiments were used to confirm ligand binding to HLA-DR10 protein. Sets of ligands were then linked to create a SHAL, as described previously (1,12). Each SHAL was synthesized on chlorotrityl-chloride resin in a polyethylene column (Pierce Biotechnology, Inc.) using Fmoc solid phase chemistry to conjugate PEG monomer units and selected ligands through the α and ε amines of the N-terminal lysine. To synthesize the tridentate SHAL, a dabsylvaline (Dv) ligand was attached to the terminal amine of a PEG spacer conjugated to the alpha amine of the 2nd lysine residue and a 4-[4-(4-chlorobenzyl)piperazino]-3-nitrobenzenecarboxylic acid (Cb) ligand was attached to the terminal amine of a PEG conjugated to the alpha amine of the 3rd lysine. Lastly, the Ct ligand was also attached to the ε amine of the 3rd lysine (Fig. 1). Conversion to DOTA (or biotinylated) SHAL derivatives was accomplished, as described previously (1). The reaction was monitored by analytical high performance liquid chromatography (HPLC), and the DOTA (or biotinylated) derivatives were purified using reverse phase, high performance liquid chromatography (RP-HPLC). Analytic electrospray ionization-mass spectrometry (Agilent 1100 instrument, Waters Symmetry C18 column) was used to confirm the elemental and mass composition of the SHAL (molecular weight within 0.07% of the theoretical molecular weight). To examine SHAL binding to isolated or recombinant HLA-DR10 protein, surface plasmon resonance experiments were conducted, as previously described (12). SHAL binding was better than nanomolar and was blocked by Lym-1 addition.

Cytotoxicity assay. Raji cells harvested in log growth phase were centrifuged, resuspended in fresh media and counted in 10% trypan-blue dye to determine initial and subsequent viability, as previously described (1,14). When untreated, cells continued to multiply and nonviable cells, initially <5% of total cells, remained so over the course of the assays. To titrate cytotoxic activity, biotinylated tridentate SHAL at concentrations ranging between 0 and 7 nM (pm/ml media) was incubated with $0.5x10^6$ cells/ml at 37˚C in a 5% CO_2, humidified atmosphere (NAPCO, Portland, OR). After 1, 2 and

3 days, cells were resuspended in trypan blue dye and counted in a hemocytometer. The fractional viability and absolute number of viable and non-viable cells/ml were determined.

Efficacy and toxicity in mice. Seven- to nine-week old female, athymic Balb/c nu/nu mice (Harlan Sprague Dawley, Inc., Frederick, MD) were maintained according to University of California animal care guidelines under pathogen-free conditions and on a normal *ad libitum* diet. Raji or Jurkat's cells harvested in log phase and having >95% viability were implanted ($6x10^6$ cells) subcutaneously in the lower abdomen 3-4 days after the mice were irradiated (400 cGy) to suppress xenograft rejection. When xenografts reached 20-500 mm^3 by caliper measurement, the mice were sorted by xenograft volume into 4 groups: SHAL-treated mice with Raji xenografts, SHAL-treated mice with Jurkat's xenografts, untreated mice with Raji xenografts or untreated mice with Jurkat's xenografts. Each mouse was injected with 100 μl of PBS or 100 ng DOTA-chelated tridentate SHAL i.p. on days 0, 7 and 14. Xenograft volume and survival were monitored for ≥84 days. The mice were also weighed 2-3 times each week for 4 weeks then weekly. Blood counts were measured prior to each SHAL or PBS dose and weekly for 4 weeks after the final dose using a Z Series Coulter Counter (Beckman Coulter Inc., Hialeah, FL) and a phase contrast microscope (Zeiss, Jena, Germany).

Xenograft volumes were calculated by the formula for hemiellipsoids (17). Responses were characterized as a cure, xenograft completely disappeared and did not regrow by 84 days, the formal censor day (>210 days to the present) complete remission (CR), xenograft disappeared for at least 7 days, but regrew, and partial remission (PR), xenograft volume decreased by at least 75% and remained stable for at least 7 days. All cures at 84 days in SHAL-treated mice persisted over a 210-day observation time for the mice. If a xenograft became >2000 mm^3, the mouse was euthanized in accordance with University of California animal care guidelines and scored as a disease-related death.

Safety in mice. Each of 3 groups of 7 healthy Balb/c mice were given PBS, 20 or 200 μg of DOTA-chelated SHAL i.p., that is, 200 or 2,000 times the SHAL efficacy dose. Immediately prior to intervention, the mice were observed, weighed and blood counts (red, white and platelets) obtained, then the mice were observed daily, weighed 3 times each week and blood counts measured weekly for 4 weeks after intervention.

Electron microscopy (EM). Raji xenografts (53-132 mm^3) were harvested for EM from mice, untreated (control) and 2, 4 or 24 h after 100 ng of biotinylated SHAL. Xenografts were fixed, as described (18), using 4% paraformaldehyde in 0.1 M Sorenson's phosphate buffer, pH 7.35. After treating with 4% uranyl acetate in 70% ethanol for one hour and LR White acrylic resin overnight, polymerization was achieved in a microwave (Pelco 34700 BioWave, Pella Inc., Redding, CA). Ultra-thin sections (Leica Ultracut UCT, Leica, Vienna, Austria) were captured on gold grids, floated on Strepavidin-20 nm gold (BB International from Ted Pella Inc.) rinsed and stained with uranyl acetate and lead citrate before viewing using a Philips CM120 Biotwin Lens (FEI, Hillsboro, OR,
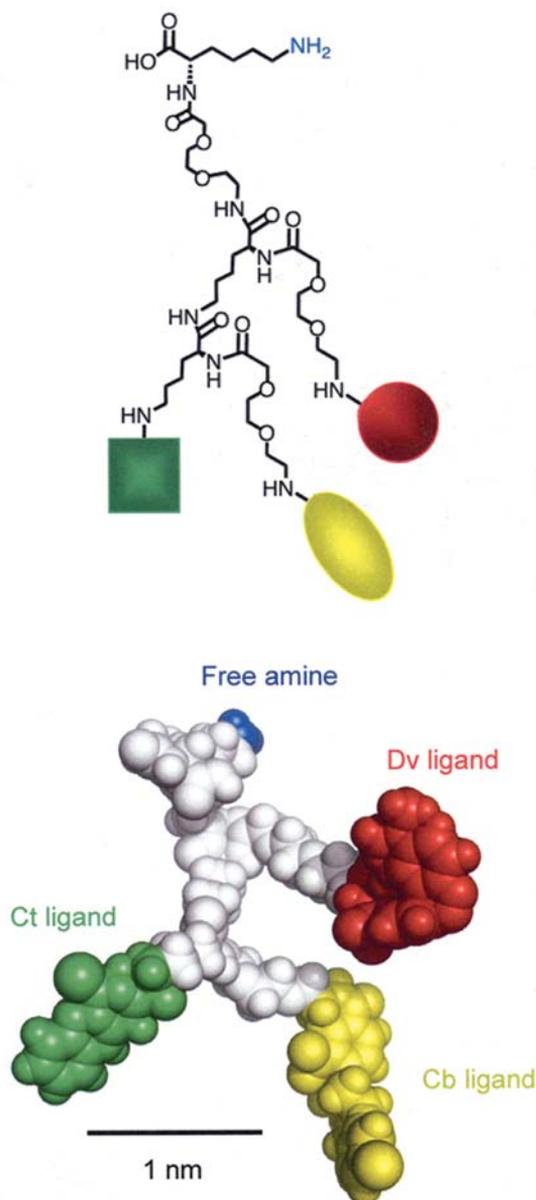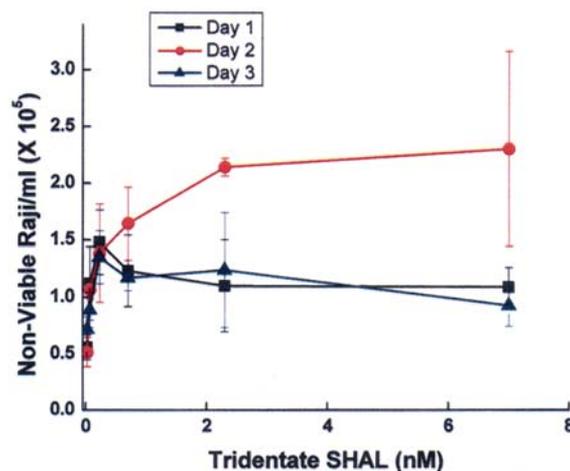
Figure 2. Titration of SHAL cytotoxic activity in Raji human HLA-DR10 expressing lymphoma cells. Absolute number of non-viable cells observed 1, 2 or 3 days after addition of SHAL at concentrations shown. SHAL threshold and IC_{50} concentrations were determined at 2 days to be 0.7 and 2.5 nM (pm/ml media), respectively (mean ± SD).

Figure 1. Structure of the tridentate SHAL containing the Ct ligand shown as a 2-D schematic (upper) and as a 3-D space-filling molecular structure (lower). The ligands used to produce this SHAL were dabsyl-L-valine (Dv), 4-[4-(4-chlorobenzyl)piperazino]-3-nitrobenzenecarboxylic acid (Cb) and (3-(2-([3-chloro-5-trifluoromethyl]-2-pyridinyl]oxy)-anilino)-3-oxopropanionic acid) (Ct). The 1st lysine residue provided a free amine for covalent binding of moieties such as metal binding macrocycles and biotin. Additional lysine residues were used to create branch points in the linker and PEG monomers were used to provide the appropriate distance between the ligands. The three ligands were identified by docking to cavities in the ß subunit of HLA-DR10, flanking the amino acid arginine 70 shown critical for Lym-1 binding and cytotoxicity. Functional molecules can be incorporated at specific positions along the linker by inserting additional lysine residues, and attaching them to the free epsilon amine of the primary lysine.

made in Eindhoven, The Netherlands and Gatan MegaScan, model 794/20, digital camera (2K X 2K), Pleasanton, CA. Gatan BioScan, model 792, Pleasanton, CA.

Biostatistical methods. Initial analysis compared response rates by exact tests and survival times by log-rank tests for untreated Raji and Jurkat's mice and found no significant differences, so these groups were pooled for subsequent analysis. Response rates for treated Raji, treated Jurkat's and untreated mice were compared by χ^2 and exact tests. Survival times were censored at 84 days for mice who had not died or been euthanized because of tumor growth before that time. Times were summarized descriptively by Kaplan-Meier curves and median survival and 95% confidence limits were obtained by the product-limit estimate. The two treated groups were compared to the untreated using a log-rank test for homogeneity across groups, followed by a proportional hazards model to estimate the effect of treatment compared to no treatment. Analyses were carried out using SAS/SAT® software (19). All tests were two-sided at level 0.05.

Results

Cytotoxicity assay. When untreated, non-viable (dead) Raji cells remained <5% of the total cells over the 3 days of observation. Raji cells treated with SHAL [over a concentration range of 0-70 nM (pm/ml media)] showed more dead cells, both fractionally and absolutely, over the 3-day period when compared to the untreated controls. The maximum number of non-viable cells was observed on day 2 at SHAL concentrations between 2.3 and 7 nM, and the threshold and IC_{50} SHAL cytotoxic concentrations were 0.7 and 2.5 nM (pm/ml media), respectively (Fig. 2).

Efficacy and toxicity. Regression in treated Raji xenografts typically began ~7 and reached complete remission ~30 days after initial SHAL treatment (Fig. 3). Although censored at 84 days, all cures in SHAL-treated Raji xenografts have persisted to the present, seemingly representing permanent cures. Because there were no significant differences in response and survival of untreated Raji and Jurkat's mice (P>0.08) they were combined for comparison to treated mice. Mice with Raji xenografts and treated with SHAL had a 69% cure rate and 77% overall response rate, significantly better than treated mice with Jurkat's xenografts (0%) and untreated mice (15% response) (P<0.001) (Table I). The median survival

Figure 3. Photographic evidence for cure in a mouse with a Raji xenograft; before SHAL treatment (upper), one week after the initial and a second 100 ng dose of SHAL i.p. By three weeks after the initial dose the xenograft had completely regressed and represented a cure that persisted over the period of observation of the mouse (>84 days; lower).

Table I. Athymic mice with lymphoma xenografts, SHAL treated or untreated.

| Treatment group | Mice | Response (%) | |
		Cure (%)	ORR (%)[a]
Raji	13	69	77
Jurkat's	9	0	0
Untreated	13	15	15

[a]Overall response rate.

times were 25 days for untreated mice and 21 days for treated mice with Jurkat's xenografts. The median survival time for treated mice with Raji xenografts was not estimable because almost all mice were alive at 84 days (Fig. 4). The proportional hazards model estimated that Raji mice had ~85% reduction
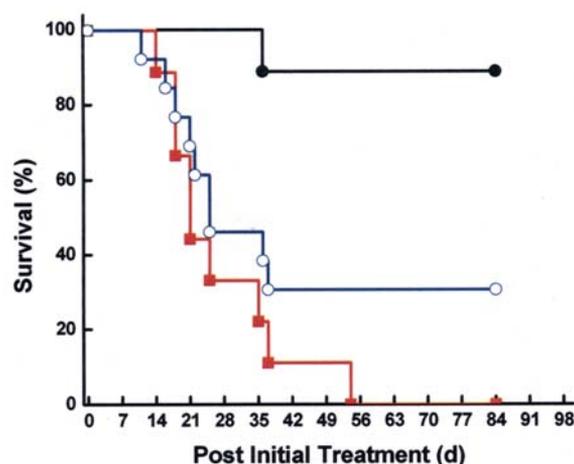

Figure 4. Overall survival effect of SHAL in mice with established Raji or Jurkat's lymphoma xenografts. Mice with Raji or Jurkat's xenografts were treated with 100 ng SHAL i.p. once weekly for each of 3 consecutive weeks, or untreated. Mice with treated Raji xenografts (dark circle) had the longest survival while mice with treated Jurkat's xenografts (square) did not survive past 56 days. Two untreated mice (empty circle) with enlarging xenografts survived 84 days.

in hazard of death compared to untreated and treated Jurkat's mice (P<0.001).

Safety in mice. All of the mice in the general safety study, including those given 2000 times the SHAL dose used in the efficacy trial, gained weight, showed stable blood counts and no adverse effects during 4 weeks of observation, thereby exceeding the requirements of the US FDA 'general safety' test guidelines.

Electron microscopy. Raji xenograft EM from untreated mice showed healthy cells having well-defined cellular and nuclear membranes, and cytoplasmic organelles, including reticular endothelium, Golgi, and liposomes (Fig. 5). In contrast, Raji EM, harvested after SHAL treatment, were characterized by highly condensed nuclear chromatin and fragmented organelles, findings consistent with autophagicy (programmed) cell death.

Discussion

Proteins on the surface of malignant cells have been used to identify them and to serve as targets for therapy and imaging. The class II major histocompatibility, human leukocyte antigens (HLA) are abundant both on the surface and inside malignant B-lymphocytes (1,8). These proteins are major signaling receptors for cell death (2,4). Whereas intact MAbs recognize malignant cells selectively, size limits their blood clearance and tissue penetration. Based on predictions from *in silico* modeling and from empiric testing, small organic ligands have been selected to bind to sites within the Lym-1 MAb epitopic region of HLA-DR10. By covalently linking sets of these ligands together, SHALs have been generated to target B-cell derived lymphomas and leukemias (20). These novel nanomolecules mimic the affinity and selectivity of MAbs because of three-dimensional interactions made between multiple ligands in the SHAL and multiple sites
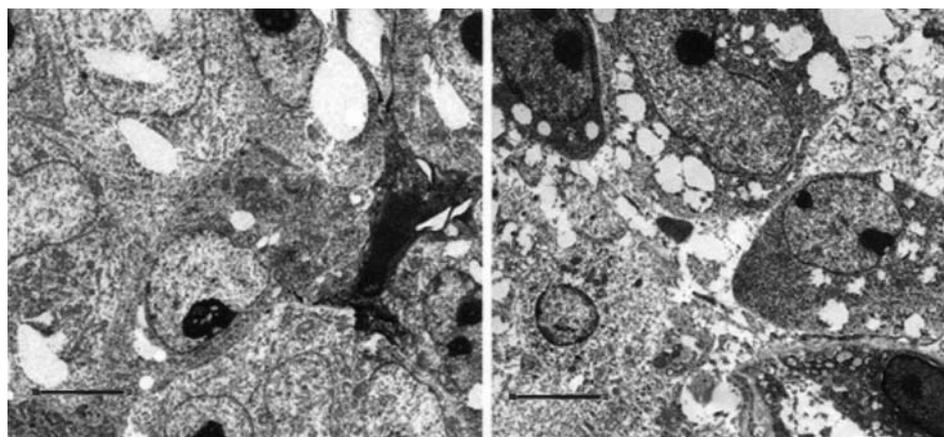
Figure 5. Electron micrographs of Raji xenografts excised from an untreated mouse and a mouse 24 h after SHAL treatment. Cellular structures were normal for this aggressive xenograft, untreated (left), whereas cells in the SHAL-treated xenograft showed condensed and fragmented chromatin and loss of cytoplasmic structures consistent with autophagic death (right) (bar, 5 μm).

located within the epitope of the target protein. SHALs are taken up rapidly by HLA-DR expressing xenografts and clear quickly from normal tissues (13) because the SHAL is ~50 times smaller than an immunoglobulin G molecule. Histochemical analyses has shown that SHALs bind to NHL tissues from patients and from mice (12). Previous studies have shown that the SHALs readily enter cells, but they do not leave cells that express HLA-DR10 and related HLA-DRs, behaving like a lobster and its trap (1).

These SHALs cross cell barriers efficiently, seemingly unaffected in HLA-DR10 expressing cells by cellular pumping mechanisms. SHALs containing a Ct (3-(2-([3-chloro-5-trifluoromethyl)-2-pyridinyl]oxy)-anilino)-3-oxopropanionic acid) ligand residualize inside and exhibit potent antilymphoma activity against live human HLA-DR10 expressing lymphoma cells (13). The present experiments demonstrate that the Ct ligand containing tridentate SHAL that has an IC_{50} of 2.5 nM (pm/ml media) for HLA-DR10 expressing cells in culture also exhibits remarkable efficacy in mice with Raji xenografts. Permanent cures were achieved in 69% of these mice at a SHAL treatment dose 2000-fold less than the maximum dose tested and confirmed to be safe in healthy mice. The anti-lymphoma effect was only observed in mice with xenografts expressing HLA-DR10; Jurkat's xenografts were not affected by SHAL treatment. Because mice treated with 2000-fold more SHAL did not exhibit signs of adverse events, the SHAL seems to have an exceptional margin of safety as a potential therapeutic. It will be important in the future to conduct a dose response study with the tridentate SHAL in mice to ascertain whether or not the cure rate can be increased with a larger SHAL dose.

Electron micrographs of xenograft tissue from mice treated with the SHAL confirmed the SHAL's cytotoxicity to the Raji lymphoma cells. Extensive vacuolization and loss of cell structure suggest the possibility that SHAL binding to HLA-DR induced cell signaling that ultimately led to apoptosis and autophagic cell death. More extensive study will be required to confirm this hypothesis and identify the mechanism of action of the SHAL.

The results provide convincing evidence that SHALs have extraordinary potential as novel nanomolecules for targeting lymphoma and leukemia for molecular therapy. SHALs represent an attractive alternative to their biological counterparts, because these chemicals are ~50 times smaller, less expensive, easier to produce with consistency, stable and expected to have a long shelf-life and be effective when given orally. Furthermore, SHAL-based therapeutics can transport and residualize other agents near critical sites inside these malignant cells. The SHAL production platform is efficient, flexible, and permits rapid synthesis and modifications.

Acknowledgements

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