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USE OF LARGE COMBINATORIAL CHEMICAL LIBRARIES FOR ANTICANCER DRUG DISCOVERY

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ABSTRACT

A new technology for drug discovery (Selectide technology) has been developed which greatly extends the capability for rapid molecular and cellular screening to large libraries containing millions of chemically synthesized compounds. Using a split synthesis approach to condensation chemistry, we recognized that each individual compound of potential interest is located on a different solid phase resin bead. Screening for binding activity on the bead surface: In order to screen for biological activity, we used several cleavable linkers which could release a portion of the compound from each bead into the solution phase in each of two sequential steps. For libraries comprised of peptides, there is sufficient residual peptide on each bead to permit sequence determination via Edman degradation. For non-peptide libraries, a system of peptide encoding is used on each bead so that the structure of the non-peptide compound can be determined by decoding the information conveyed by the peptide (which can be sequenced). We have applied this technology to various areas of drug discovery including a major interest in the development of anticancer drugs. Molecular targets under evaluation include cell surface monoclonal immunoglobulin on B-cell lymphomas, the epidermal growth factor receptor, and the HER2-neu receptor. Solution phase screening with releasable libraries is being used to evaluate anti-tumor activity using a battery of human tumor cell lines. Based on data obtained thus far, we believe that the Selectide technology has the potential for discovering novel new anticancer drugs.

Keywords: Combinatorial chemistry, peptides, drug discovery, cancer, screening.

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INTRODUCTION

The anticancer drug discovery process has proven to be difficult and has focused on two major approaches: rational drug design and random screening of natural and synthetic products. The majority of current anticancer drugs are derived from, or are semi-synthetic derivatives of, natural products (plant alkaloids, antibiotics). The alkylating agents and antimetabolites which are in use are chemical compounds discovered largely by serendipity, though there also has been some success using rational design. As a generalization, at least 50,000 agents must be screened before a promising lead compound is discovered. Both the rational and random screening approaches have yielded some success, and there are now over 40 anticancer drugs that are in clinical use. Nonetheless, there is still a major need for new anticancer drugs particularly in the treatment of common solid tumors as the existing agents have limited efficacy and substantial side effects. As in other fields of drug discovery, structural modification of lead compounds is often essential in order to move from the discovery phase through lead optimization of a new class of compounds, and then on to a final candidate drug suitable for clinical testing. This caveat applies to both natural product derived or chemically synthesized lead compounds. Within nature, peptides and proteins have been used extensively by living organisms as key molecules with a variety of functions including hormonal signaling as well as in

interactions with the internal and external environment. The diversity of peptidic compounds that can be synthesized from just the 20 eukaryotic (L) amino acids is enormous. If one wishes to synthesize the theoretical number of pentapeptides (5mers) that can be made from just these 20 natural amino acids, the approximate total is 3,200,000. If one wishes to produce 10mers from these building blocks, then the approximate number of unique sequences rises to 10,240,000,000,000. Accordingly, if such structures can be generated in random libraries and screened rapidly for biological activity, the lead discovery process for anticancer drugs might be markedly accelerated. This objective is the goal of these research efforts.

The "One-Bead, One-Compound" Concept and Related Technological Developments

Several years ago, we first introduced the concept of using large libraries of chemically synthesized peptides or peptidomimetics for drug discovery and development based on an original "one-bead, one peptide" synthesis and screening concept which we now designate as the Selectide technology or Selectide process (Lam *et al.*, 1991; Lam and Lebl, 1992; Lam *et al.*, 1993). Prior to our studies, relatively small peptide libraries had been chemically synthesized using a multi-pin technique in 96 well plates as originally described by Geysen *et al.* (1987) for epitope mapping, and the biological generation of very large peptide libraries had been accomplished by inserting a randomized oligonucleotide into the genome of the filamentous phage pIII coat protein (Scott and Smith, 1990; Cwirla *et al.*, 1990; Devlin *et al.*, 1990). Once generated, the phage library can be tested by screening the live phage, selecting those which express peptides within the pIII coat protein with the desired ligand binding activity. Identification of the active peptide is accomplished by sequencing the nucleic acid code after expanding the active phage clone through a series of plating and expansion steps. Contemporaneously with our development of the "one-bead, one peptide" concept, Houghten and his colleagues (1991) developed a modification of Geysen's multistep iterative chemical process (Geysen *et al.*, 1986) in which batches of peptides of known structure were synthesized and cleaved into solution and repetitively screened to identify peptides with various functional or binding activities. The Selectide process (which is also capable of producing free peptides in solution) is based on a technique for random compound synthesis of oligomers produced via solid phase chemical synthesis conducted in such a fashion that each individual compound in the library is represented on a separate solid phase resin bead on which it was synthesized. This "one-bead, one-peptide" situation can be achieved by synthesizing the compounds using the standard solid phase peptide synthesis method of condensation chemistry with a technique of "split synthesis" (Lam *et al.*, 1991; Furka *et al.*, 1991). We recognized that by using this approach, each resin bead would contain a single unique peptide sequence. The individual beads within the batch of resin beads used for synthesis were divided up into different reaction vessels, one for each reactant species (e.g., amino acid) to be added to a growing polymer or compound. After each cycle of deprotection and building block addition (e.g., using Boc or Fmoc protected amino acids and sufficient time to allow the reaction step to go to completion), the beads were recovered and pooled from the various vessels, washed and thoroughly mixed and then redistributed into the vessels again for the next addition step. In a model experiment using three different amino acids to produce a library of tripeptides, all 27 theoretically generated tripeptides were successfully produced and identified and found to be produced in approximately equimolar quantities. Of importance, each individual bead contained a sufficient quantity of its unique peptide to permit its sequence to be determined via Edman degradation using an automated protein sequencer. This approach proved to be readily adaptable to a generation of libraries of various sized peptides (e.g., pentapeptides to dodecapeptides), using as many as 40 amino acids.

A wide variety of peptide library formats have been synthesized using natural or unnatural amino acids, varying peptide length, and with introduction of conformational constraints to produce libraries of cyclic or alpha-helical peptidic structures. Inasmuch as each individual peptide in the library was represented separately on an individual bead, binding assays using enzyme- or fluorescent-marker coupled molecular acceptors were developed to identify unique peptides that bound tightly to a given molecular acceptor (e.g., antibody, enzyme, receptor, etc.). The technique proved to be quite effective for high volume testing as a library could be synthesized within a few days, and screening of a library of up to ten

million peptides could be accomplished within a day.

Using several model macromolecular acceptors, we then established that we could identify structurally unique high affinity ligands from within a series of pentapeptide libraries containing approximately three million different peptide structures (Lam *et al.*, 1993). The specific staining of just those few beads that bound the tagged acceptor at high dilution therefore provided a rapid means for screening for ligand or "mimotope" activity of peptides in these large libraries. Specific high affinity peptide ligands for an anti-β-endorphin monoclonal antibody (clone E-7) and for the biotin-binding site of streptavidin provided the specific demonstrations of specificity of this screening process and established that it was based on molecular-recognition principles (Lam *et al.*, 1991; Lam and Lebl, 1992; Lam *et al.*, 1993). As mentioned above, the technology based on developing unique libraries of compounds with each compound on a different solid phase bead as well as the related techniques for rapidly identifying compounds of interest within such large libraries represents the core of the Selectide technology. Development of this technology required development of suitable chemistries, materials, and assay techniques to bring its potential into focus for drug discovery. We do not believe this concept is limited to the generation and screening of peptide libraries, but rather represents a more general process for obtaining lead compounds from a chemical library with specific molecular recognition properties which may serve as the basis for drug discovery and development.

We have recently extended the Selectide technology to the testing of free peptides in solution by incorporating two cleavable linkers, with orthogonal cleavage characteristics (diketopiperazine and ester bonds), as well as a third non-cleavable linker onto each bead. The same oligimer is synthesized on each of these linkers, producing a construct where one-third of the compound can be released and tested, subsequently the second third can be released and tested, and the final third remains on the bead, ena-

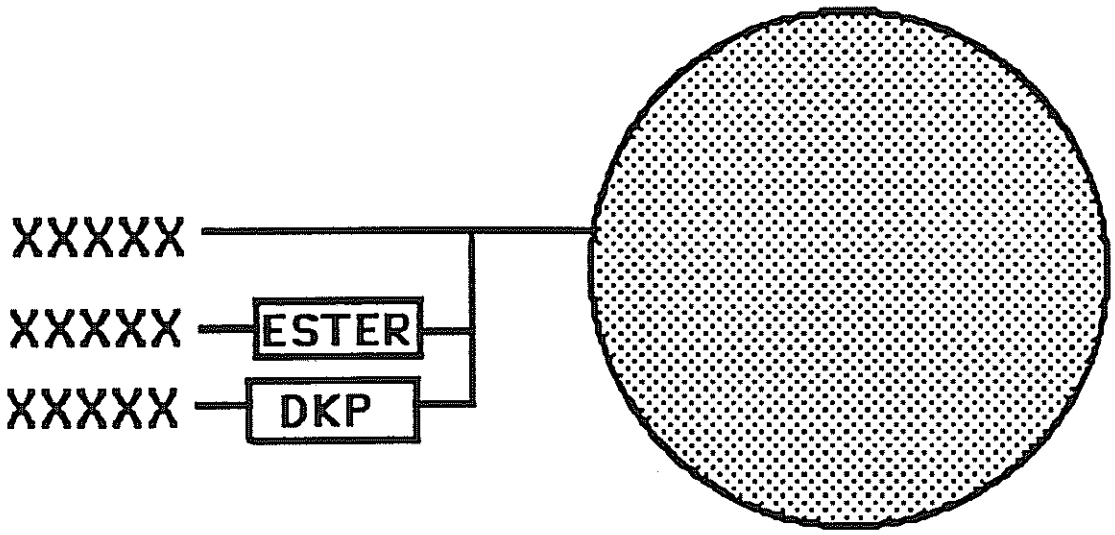

Fig. 1. Organization of the three linkers connected to a single random peptide sequence on a typical dual release-peptide random peptide bead. For simplification, only one representative linker construct is shown, whereas each actual bead contains very large numbers of identical linker constructs both within the bead and on its surface. Cleavage with reverse-diketopiperazine (DKP) formation releases the first 100 pM of peptide into solution for the first stage assay. Cleavage of an ester bond releases the second 100 pM of peptide into solution for the second stage of the assay. The remaining linker, which is non-cleavable under these conditions, retains the sequence information on the bead of origin. This remaining linker provides more than an adequate amount of the bead's peptide sequence so that microsequencing of a single bead can be accomplished readily after a positive second stage assay has been completed. More detail on assay technology has been published elsewhere (Lebl *et al.*, 1993; Salmon *et al.*, 1993). With current plating volumes, the maximum concentration of any one peptide in a single microwell is in the range of 1 μM.

bling its identity to be determined (Lebl *et al.*, 1993). Figure 1 provides a conceptual illustration of the construction of the dual release, sequenceable peptide bead. This approach provided a means to test libraries of millions of peptides comprised of either natural or unnatural amino acids with conventional microplate pharmacological assays of growth, signaling, etc. A two-stage design was developed for solution assays wherein for the first phase, 500-1,000 beads were placed in each of the 96 wells in a microassay plate and the first linker (a diketopiperazine-type linkage) was cleaved at neutral pH. Thus, each individual 96 well plate contained from about 50,000-100,000 unique peptides released into solution (depending upon the number of beads distributed in the wells). The peptide solution from each well of each "master" Millipore filtration plate was then transferred by filtration into a corresponding test well for solution phase testing. After identifying the "active" well(s) in the first stage assay, the beads from the corresponding master well(s) were then recovered and redistributed into new master plates at a concentration of about one bead per well (range 0-6) and the second stage linker (an ester bond) was cleaved using either base or gaseous ammonia. Following transfer by filtration and assay, the active well(s) were then identified and the corresponding beads from the second stage master well(s) were recovered for peptide sequencing. As demonstrations of the specificity of this release-technology for finding specific ligands, we utilized two molecular targets: the same anti-β-endorphin monoclonal antibody as used in defining the specificity of the bead binding assay, and the human platelet-derived gp IIb/IIIa receptor for fibrinogen (Salmon *et al.*, 1993). Again, we were able to find specific high affinity ligands using the release technology. This release technology is therefore useful for testing peptides for growth inhibition effects or other signaling assays that can directly evaluate functional as well as structural interactions. It is the release assay that we have used in an effort to discover new anticancer drugs.

These studies established that we could identify peptides which bound to the active sites of antibodies and of cell surface receptors. More recently, the Selectide Corporation has also established that this technology can be successfully applied to identify potent enzyme inhibitors. We recognized that it would be of value to move the Selectide technology beyond peptides to the more general world of organic compounds, as most pharmaceutical agents are not peptidic in structure. Although there are exceptions, most peptides are not active orally as pharmaceuticals, and furthermore, in most instances, peptidic agents do not gain access to the intracellular environment. The cyclic D-amino acid containing peptide antibiotic cyclosporine is a distinct exception to this generalization. In this instance, cyclosporine is internalized via a cell surface receptor for cyclophyllin. The technology for producing libraries of non-peptides (including both polymeric structures based on subunits linked as amides, ureas etc., or as derivatives of biologically interesting molecular scaffolds such as steroids, sugars, etc.) proved to be relatively straightforward and we determined that such structures could be built with the Selectide process by using a variety of precursor organic molecules that are available for chemical syntheses achieved via condensation chemistry. Screening of such libraries is of course feasible using either the bead-binding or release assay screening technologies as described above, after which the active bead(s) can be recovered. A major problem that had to be addressed with the non-peptidic libraries was that of structure determination of functionally active compounds. While some structures can be determined using techniques such as mass spectroscopy, this is not always feasible. Therefore, we developed an encoding technique using peptide encoding wherein non-sequenceable structures could be identified by incorporating an amino acid coding unit on each bead that identifies each non-sequenceable subunit. Accordingly, such beads have two separate structures represented: a non-sequenceable oligomer or other organic compound and a readily sequenceable peptide (the amino acids which serve as the code for the structure of the non-sequenceable compound) (Nikolaiev *et al.*, 1993). Related techniques for encoding other forms of combinatorial chemical libraries have recently been reported by Brenner and Lerner (1992) using a nucleoside encoding technique and with peptide encoding by Kerr *et al.* (1993).

Current Applications of the Selectide Process to Anticancer Drug Discovery

We are currently using the Selectide technology in an effort to discover new anticancer drugs. One target identified by one of us (KSL) was the cell surface immunoglobulin on the surface of malignant B-cells. The concept in this instance was to develop

"antigene-directed immunotherapy" (ADI) for B-cell non-Hodgkin's lymphoma (Lam, 1993). This approach is intended to be patient specific and designed to identify peptide ligands for the cell-surface monoclonal immunoglobulin "idiotype" (id) which is unique for every individual patient's lymphoma (Miller *et al.*, 1982). This approach appears feasible from several standpoints: a) the first proofs of principle for the Selectide process were obtained by demonstrating that the technology could be used to identify mimotopes for the specific antigen-epitope which binds to the combining site of individual murine monoclonal antibodies, and b) anti-id antibodies have shown therapeutic benefit in some patients with B-cell lymphoma (Miller *et al.*, 1982). The overall concept of ADI as envisioned involves recovery of cell surface id, identification of a highly specific peptide (with the Selectide process) which binds to the id, followed by resynthesis of and radioiodination of the patient-specific peptide for use in radioimmunotherapy (Lam 1993). Use of a peptide to deliver tumor-specific radiotherapy would have more favorable pharmacokinetic characteristics than a monoclonal antibody for tumor imaging, dosimetry and therapy. This concept is currently being tested in preclinical models at the Arizona Cancer Center. If this patient specific technology can be perfected, we envision that the whole program of discovery and synthesis of an individualized radioactive peptide for patient therapy might be accomplished within one month from the time of biopsy of the patient's lymphoma. We recognize that there are other obstacles to be overcome, including the potential of *in vivo* evolution of tumor clones expressing variants of the idiotype or which lack cell surface immunoglobulin (Renschler and Levy 1983).

Our efforts at drug discovery using the Selectide technology were substantially expanded in 1992 with establishment of collaborative interactions between a series of laboratories at The University of Arizona, New York University, The Max Planck Institute, The Selectide Corporation and The National Cancer Institute (NCI). This program is intended to discover new anticancer drugs which may be of more general use than the patient specific approach as described above. It is therefore amenable to more conventional drug development involving the pharmaceutical industry. The initiative is supported in part with "National Cancer Drug Discovery Group" funding from the NCI (which involves ongoing close cooperation with the NCI). The discovery program utilizes both bead binding technology (for specific receptors produced with recombinant DNA technology), and the release assay technology for identifying peptides with antiproliferative activity. While this program was only initiated recently, we have already obtained preliminary evidence that the Selectide technology can be used for lead discovery for anticancer agents.

Molecular Recognition of Cancer-related Receptors

We have initiated a collaborative program to identify peptidic ligands which bind to the external domains of tyrosine kinase receptors known to be overexpressed on certain forms of cancer (Ullrich and Schlessinger 1990). After binding with their growth factor ligands, these receptors transduce signals thought to stimulate cancer growth. The specific receptors being studied are the epidermal growth factor receptor (EGFR) and the HER2-neu oncogene expression product, which also appears to be a receptor (HER2R). The external domains for both of these receptors have been synthesized via recombinant DNA technology in forms that are of particular use for our screening efforts. It is our hypothesis that a binding peptide that occupies the growth factor binding site on these receptors may function as a competitive inhibitor of growth factor action, and thereby inhibit cancer growth. A second alternative approach to antagonism of signaling by such growth factor receptors would be to identify a peptide ligand which blocks receptor dimerization (dimerization resulting from ligand interaction is thought to be necessary to initiate the tyrosine-kinase-linked signaling process). For both EGFR and HER2R, the bead binding technology was employed after coupling the relevant receptor to either alkaline phosphatase or fluorescent markers. Each of these markers has certain advantages, and they have been used differentially for the two receptor targets. An advantage of the EGFR target is that the natural ligand (EGF) is available for competitive binding studies which increase the specificity of the screening assays and provide experimental evidence that the discovered peptides bind at the ligand binding site. To date we have identified several 7mer cyclic peptides which bind to EGFR and can block the binding of EGF. We are not yet certain of the affinity of these peptides for EGFR and they have yet to be tested in functional assays. Further screening as well as lead

optimization based on the initial cyclic peptide leads is now underway.

Molecular screening for binding ligands for HER2R is more difficult, because the natural ligand for this tyrosine-kinase receptor-like molecule has not been clearly identified, and has not been available for our studies. Accordingly, we cannot carry out competitive binding studies. Thus, peptide ligands which we determine to bind to HER2R may well bind at alternative sites on the receptor than at the ligand binding site. These peptides may prove of interest (e.g., if they block receptor dimerization), but functional assays are required to determine their relevance to signaling. To date, we have identified some 13-mers which bind with specificity to HER2R. Several of these appear to inhibit functional kinase activity. Characterization of affinity and effect on cell proliferation is in progress.

Cancer Cell Line Screening

Using the release assay, we have initiated an active screening program using six human tumor cell lines (breast, colon, lung, melanoma, prostate, leukemia) using a replicate plating technique. The cell lines tested were obtained from the American Type Tissue Culture Collection (Rockville, MD). For first stage screening for antitumor activity, the released peptide from each well of the 96 well plates (500-1,000 beads per well) is divided and simultaneously tested against 2-3 cell lines in a 96-hour cell growth inhibition assay. Based on bead content and peptide release characteristics as well as plating volumes of releasate, the maximal concentration of each test peptide in the assay wells is approximately 1 μM. Measurement of drug effect is determined after four days of culture with the sulforhodamine B (SRB) assay (Monks *et al.*, 1991). The growth-inhibition endpoint for the SRB assay is determined spectrophotometrically, with a positive well defined as exhibiting greater than 30% reduction in OD compared to controls. With releasate from 500 beads per well, each 96 well test plate permits the simultaneous evaluation of close to 50,000 peptides. A single investigator can set up 20 plates per day with each cell line, permitting the evaluation of almost 1,000,000 peptides in each four-day assay with three assays per week. Once a positive well is identified in the first stage assay, the beads from the master plate for that well are recovered and redistributed with 1-2 beads per well for second stage testing. The second stage test is performed with the specific cell line that yielded the positive screening result in the first stage assay. With a positive second stage assay, the 1-2 beads from the master well are then recovered for sequencing. The identified peptide is resynthesized and retested against all six cell lines in dose-response fashion. Over the past nine months we have conducted first stage screening against ten different library formats which were prepared with the dual cleavable linkers, and tested more than 30 million individual peptides. At this stage of technology development, library format is exploratory since specific molecular targets have not been identified from the cell line testing. Structural modifications tested to date have included libraries of differing peptide chain length (up to 15mers), cyclic structure, or incorporating various end-groups added to the amino terminus of the peptides. Complete screening of a library was feasible for peptide lengths up to 5mers, but only a statistically representative fraction of longer chain length libraries. At least three million individual peptides were tested from each of the libraries tested. In first stage screening of the libraries, a total of approximately 74,000 microwells were assayed against the various cell lines, out of which eight were positive. Of the beads recovered from these eight wells and retested in second stage testing, one positive

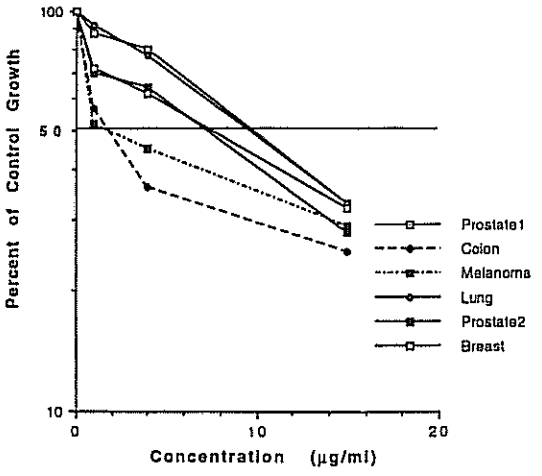

Fig. 2. Effects of SEL1202 on the *in vitro* growth of human tumor cells. The cancer cell lines used to obtain the results depicted in this figure include: breast (MCF7), colon (SW480), lung (A549), melanoma (A375), prostate 1 (LNCaP) and prostate 2 (PC3).

well was obtained (from a pentapeptide library). That specific well contained four beads, which were sequenced and shown to contain four different pentapeptide sequences. These four peptides were then resynthesized and tested and one of the peptides proved to be active on retesting with a preliminary estimate of its IC_{50} in the range of 1 μM. The lead peptide discovered (SEL1202) is a lipophillic pentapeptide analog with differential antitumor activity against the cell lines tested in the primary screen as well as in the testing against six solid tumor cell lines. The activity profile of this peptide against the various cell lines was quite different than that of an index antitumor agent (mitomycin C) used as a positive control. The greatest degree of growth inhibition with SEL1202 was observed with the melanoma and colon cell lines, and which had IC_{50}s almost a log lower than observed with some of the other lines tested (Figure 2). However, SEL1202 must be considered, at best, just a preliminary lead, and detailed study will be required to better characterize this compound and determine whether it is a true "hit" or not. We are continuing prospective screening with the release assay using novel library formats including libraries with conformational constraints, unnatural amino acids, and non-amino acid building blocks for the synthesis of large libraries of small organic compounds which may have far better capabilities for cellular uptake (Nikolaiev *et al.*, 1993).

DISCUSSION

These studies provide an initial indication that the Selectide process can be applied to the anticancer drug discovery process. We have focused on cell surface receptors on cancer cells for our initial molecular targets. However, it appears that enzymes associated with invasion and metastasis represent potential targets of interest, as the Selectide process appears to be applicable to the identification of protease inhibitors. Leads discovered from peptide libraries may serve as templates for subsequent development of peptidomimetics, or in some instances may themselves serve as therapeutic agents. We are clearly interested in pursuing non-peptide libraries of organic molecules, as these may be be easier to optimize to produce useful drugs. We plan to evaluate non-peptide libraries using our multiple cell-line screening assay with the knowledge that the potential chemical diversity of the libraries will be much greater than that afforded by amino acids. We also assume that compounds within such non-peptide libraries will have a greater likelihood of crossing cell membranes and acting on intracellular targets within cancer cells. While the initial stage of our approach to drug discovery appears more empirical than rational, its "brute force" nature provides the potential of providing a substantial number of lead compounds against various targets which can then be optimized through the use of optimization via further combinatorial library approaches as well as via more traditional medicinal chemical approaches as well as with the application of computational methods and molecular modeling on the lead structures and analogs.

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