Target validation: A door to drug discovery

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ABSTRACT: From ancient times to today, drug discovery transitioned from serendipity to rationality over its long history. Proper drug target selection and validation are crucial to the discovery of new drugs. This review discusses the definition of drug targets and proposes several characteristics for drug targets. The limitations of the term 'target' itself are summarized. The drug target validation process is also discussed in detail and pitfalls during this process are outlined. Small active chemical compounds obtained from the target validation process are useful tools in target validation and target function research. The validation of lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1) as a new potential anti-atherosclerotic drug target is cited as an example in order to elucidate the target validation process.

Key Words: Drug discovery, drug target, drug target validation, high-throughput screening, lectin-like oxidized low-density lipoprotein receptor-1

Introduction

In medicine, biotechnology, and pharmacology, drug discovery is generally thought of as the discovery, creation, or design of a compound or a complex that possesses the potential to become a useful therapeutic. It is really an expensive, time-consuming, and difficult process that involves the identification of candidates and synthesis, characterization, screening, and assays of their therapeutic efficacy. The word 'target' has been widely used in both medical and pharmaceutical research. However, the definition of "target" itself is vague and is debated within the pharmaceutical industry. The number of drug targets is also controversial, due in large degree to disputes over the definition of what a target is. The exact connotation of the term "drug target" needs to be elucidated. Target validation is the first step in completely new drug discovery. Validation of new drug targets is the process of physiologically, pathologically, and pharmacologically evaluating a biomolecule and might be performed at the molecular, cellular, or whole animal level.

Lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1) was identified as a special receptor for oxidized low-density lipoprotein (ox-LDL) in endothelial cells (*1*). Accumulated studies revealed that LOX-1 might play an important role in the pathogenesis of atherosclerosis (*2-7*). Review of ten years of studies on LOX-1 suggested that LOX-1 might be a specific new drug target, and validation results here revealed that LOX-1 is a new promising anti-atherosclerotic drug target.

Drug discovery: From serendipity to rationality

Drug discovery is one of the most crucial components of the pharmaceutical industry's Research and Development (R&D) process and is the essential step in the generation of any robust, innovative drug pipeline. However, new drug discovery is an expensive, time-consuming, and difficult process. Moreover, the end result is never guaranteed. A single new drug can cost 1.2 billion euros and take 10 years to develop (*8*).

The early history of drug discovery is all about natural products and herbal remedies, the use of which dates back thousands of years. In ancient times, drug discovery was mainly based on the accumulation of experience from generation to generation. It is a rather long process that involves huge costs in both money and even lives. Just like the discovery of penicillin, serendipity has been the key to the pharmaceutical industry's success over many decades. Now with the development of modern chemistry, hundreds and sometimes thousands of chemical compounds have to be made and tested in an effort to find one that can achieve a desirable result. Thus, traditional drug discovery strategy based on experience and serendipity is no longer able to meet the needs of pharmaceutical companies. A shift from serendipity to rationality in drug discovery is underway (Figure 1). Rational drug discovery based on knowledge of the biological system being investigated allows highly specific selective

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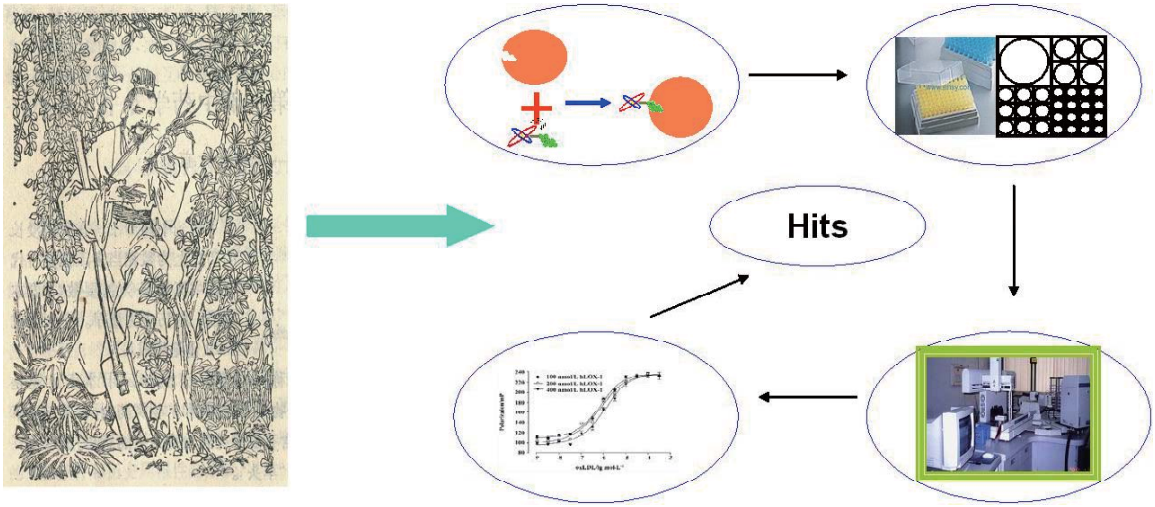

Figure 1. Drug discovery: From 'Tasting to Testing.' The emergence of an "accidental" approach to drug discovery has its origins in early history with traditional natural herbal remedies that were passed from generation to generation in local communities or tribes. The ancient Chinese legend that "Shen Nong tastes a hundred herbs in a single day and meets seventy toxins" demonstrates the great sufferings of our ancestors encountered during the discovery of new drugs. This experience-based drug discovery mode lasted for several thousands years. It was not until the late 18th and early 19th centuries that the active components of medicinal plants and herbal remedies were analyzed, resulting in the discovery of alkaloids. HTS and ultra-HTS developed during the later 1980s represent a new mode of drug discovery. These methods represent a multifunctional, multiskilled environment that connects a range of discovery functions in a high-capacity, integrated process producing a product that consists of a cohort of tractable chemical leads with respect to targets of interest.

antagonists and agonists to be developed. These molecules can then be developed as lead compounds, drug candidates, and even drugs.

What are drug targets?

Target identification and validation are the first key stages in the drug discovery pipeline (*9*). But what is a drug target? Generally speaking, a drug target is the specific binding site of a drug *in vivo* through which the drug exerts its action. A specific drug target might have the following characteristics: 1) The drug target is a biomolecule(s), normally a protein that could exist in isolated or complex modality. 2) The biomolecules have special sites that match other molecules (commonly small molecules with special structures). These molecules could be endogenous or extraneous substances such as chemical molecules (drugs). 3) The biomolecular structure might change when the biomolecule binds to small molecules and the changes in structure normally are reversible. 4) Following the change in the biomolecule's structure various physiological responses occur and induce regulation of the cell, organ, tissue, or body status. 5) The physiological responses triggered by the changes in biomolecule structure play a major role in complex regulation and have a therapeutic effect on pathological conditions. 6) The expression, activity, and structure of the biomolecule might change over the duration of the pathological process. 7) Small molecules binding to the biomolecules are drugs (*10*).

As is apparent from the above discussion, a drug target is a key molecule involved in a particular metabolic or signal transduction pathway that is specific to a disease condition or a specific disease. However, the term 'drug target' itself has several limitations and is debated within the pharmaceutical industry. In this respect, several points should be kept in mind.

First, a drug target is a relative concept. For starters, a drug target is, just like other biomolecules, also a biomolecule involved in a transduction pathway. The difference between the two is only in their location and role in the transduction pathway. Another aspect is that a drug target is disease-dependent, that is, every target is involved in a special spectrum of diseases.

Second, most human diseases are rather complicated and involve many risk factors, so there are clearly many different drug targets with respect to a specific disease. Targeting a specific target could not conceivably cure a kind of disease. However, the involvement of many targets in a disease does not mean that each target shares equally in the pathogenesis of the disease and thus drugs targeting these targets would not be equally effective in the therapy of the disease.

Third, drug targets can change, which means that with the development of insights into biomolecules and their role in the pathogenesis of a certain disease, drug targets might be not as important as or may be much more important than currently believed. In fact, the establishment of drug targets is based on understanding of the pathogenesis of the disease.

Fourth, there are many drugs targeting the same target and one drug may have more than one target. The relationship between a drug and its target is not one-to-

one but one-to-many or many-to-one.

Fifth, when a new drug target is discovered and validated, researchers usually hope to obtain more specific drugs targeting the target. However, a key understanding to keep in mind is that the body is a subtle organism and a more specific drug might disrupt the homeostasis of the body. Compared to aspirin, rofecoxib is a specific COX-2 inhibitor. However, studies had shown that rofecoxib increases cardiovascular risks, resulting in rofecoxib's withdrawal from the drug market.

Sixth, a drug target usually refers to a single biomolecule. This connotation should be revised. Recent research has noted that a complex, like HDL, for example, or even a kind of cell, like an endothelial cell, could be a potential drug target. However, drug target validation based on this concept is very difficult since reliable, accurate, and robust indexes to evaluate the effect of drugs targeting these targets are rare.

According to whether there are drugs available, a drug target can be classified into two classes: established drug targets and potential drug targets. The former are those for which there is a good scientific understanding, supported by a lengthy publication history regarding both how the target functions in normal physiology and how it is involved in human pathology. Furthermore, there are many drugs targeting this target. The latter are those biomolecules whose functions are not fully understood and which lack drugs targeting them. Potential targets suggest directions for completely new drug research.

How many drug targets are there?

With the development of modern science and technology, humans became more informed about themselves than at any time in history. Thousands of drugs had been discovered and created. However, the mechanisms of their action and the targets of their action were poorly understood. Furthermore, the number of drug targets in the body is less consistent than the definition of a drug target. How many drug targets are there in the body? Drews and Reiser were the first to systematically pose and answer this question, identifying 483 drug targets. Later, Hopkins and Groom revised this figure downward to only 120 underlying molecular targets. Subsequently, Golden proposed that all then-approved drugs acted through 273 proteins. By contrast, Wishart *et al*. reported 14,000 targets for all approved and experimental drugs, although they revised this number to 6,000 targets on the Drug Bank database website (*11*). Imming *et al*. catalogued 218 molecular targets for approved drug substances (*12*), whereas Zheng *et al*. cited 268 'successful' targets in the current version of the Therapeutic Targets Database. John *et al*. proposed a consensus number of 324 drug targets for all classes of approved therapeutic drugs (*11*). With the publication of draft maps of the human genome and an interim agreement that the human genome consists of approximately 21,000 genes, there has been considerable anticipation that many novel disease-specific molecular targets will be rapidly identified and that these will form the basis of many new drug discovery programs (*13*). According to the current definition, one could rationally predict that there are 5,000 to 10,000 established and potential drug targets in humans (*10*).

Target validation

New target validation is the basis of completely new drug exploration and the initial step of drug discovery. New drug target validation might be of great help not only to new drug research and development but also provide more insight into the pathogenesis of target-related diseases. Basically, the target validation process might include six steps:

1. Discovering a biomolecule of interest.
2. Evaluating its potential as a target.
3. Designing a bioassay to measure biological activity.
4. Constructing a high-throughput screen.
5. Performing screening to find hits.
6. Evaluating the hits.

The drug discovery process starts with the identification, or growing evidence of, biological targets that are believed to be connected to a particular condition or pathology. Information supporting the role of these targets in disease modulation can come from a variety of sources. Traditionally, the targets have been researched and largely discovered in academic laboratories, and to a lesser extent in the laboratories of pharmaceutical and biotechnology companies. Basic research into understanding the fundamental, essential processes for signaling within and between cells and their perturbation in conditions has been the basic approach for establishing potential targets suitable for drug intervention (*14*).

After the identification of a biological target of interest, the next challenge begins with the conversion of the target into a bioassay that can give a readout of biological activity. The range of potential targets is large, from enzymes and receptors to cellular systems that represent an entire biochemical pathway or a disease process. Consequently, the range of assay design techniques and types of assay available have to be correspondingly comprehensive. Once an assay that measures the biological activity of the target, by some direct or indirect means, has been developed, then compounds can be tested in the bioassay to see if they inhibit, enhance, or do nothing to this activity (*14*).

After a bioassay to measure biological activity is designed, the next key step is the establishment of a high-throughput screening (HTS) method. The basic requirements for HTS assay are that it be sensitive,

stable, highly reproducible, and robust and suitable for screening thousands or even millions of samples. With sufficient luck, several 'hit' compounds will be discovered by primary screening.

The 'hit' compounds must be rescreened to exclude false positive results. Then, the next step is 'hit' identification, which may include its chemical characteristics, *i.e.* mainly its stability, its toxicity *in vivo* and *in vitro*, and its pharmacological evaluation, and particularly its effects in cells and animal models.

In summary, target validation should be performed at three levels: the molecular level, the cellular level, and the whole animal model level (Figure 2). Small chemicals obtained from HTS provide useful tools for the validation of new drug targets. Most HTS models are at the molecular level, that is, cell-free systems. For example, screening of a specific enzyme inhibitor usually involves mixing the enzyme and samples together to detect a decrease in the substrate or to determine an increase in the product in this enzyme catalytic process. The results obtained from this level are not absolutely reliable since there are many predictable and unpredictable factors. However, true results from this level convey the point that hits truly act with the target. There is a significant difference between a cell and cell-free system. Validation at the cell level provides confirmation of cell-free results. At this level, the pathological significance of the target might be rendered more apparent using small chemicals. The effect of the small chemicals on a cell system will provide a tentative outline of these chemicals. Animal models are used to validate the target at the whole level. At this level, the primary concern is the effect of the 'hit'. If the hit obtained from HTS displays a therapeutic effect in animal models, then it may be promising. However, more often than not a 'hit' displays no effect in an animal model and the result should be interpreted with caution. Common shortfalls and/or pitfalls that need to be considered include (*15*):

1. Using the wrong animal model.
2. Using the wrong route or dosing regimen.
3. Using the wrong vehicle or formulation of test material.
4. Using the wrong dose level. In studies where several dose levels are studied, the worst outcome is to have an effect at the lowest dose level tested (*i.e.*, the safe dosage in animals remains unknown). The next worst outcome is to have no effect at the highest dose tested (generally meaning that the signs of toxicity remain unknown, invalidating the study in the eyes of many regulatory agencies).
5. Making leaps of faith. An example is to set dosage levels based on others' data and to then dose all test animals. Ultimately, all animals at all dose levels die, the study ends, and the problem remains.
6. Using the wrong concentration of test materials in screening. Many effects are very concentration-dependent.

Validation of LOX-1 as a new potential anti-atherosclerotic drug target

LOX-1 is a new kind of ox-LDL receptor discovered from bovine aortic endothelial cells (BAEC) by Sawamura *et al.* in 1997 (*1*). Studies have shown that LOX-1 is the major receptor for ox-LDL in endothelial cells of large arteries although other ox-LDL receptors have also been reported (*1*). Accumulated studies revealed several ligands for LOX-1 that are expressed in different types of cells and that could be regulated at the level of transcription. Changed expression of LOX-1 at the mRNA and protein level has been reported in several cardiovascular processes such as atherosclerosis, hypertension, myocardial ischemia, ischemia-reperfusion injury, and diabetes (*2-6*). Identification, regulation, and understanding of LOX-1 signal transduction pathways might improve current insights on the pathogenesis of some cardiovascular diseases and provide a selective treatment tool for physicians. LOX-1 might be a potential and promising target for cardiovascular drug research.

The relationship between LOX-1 and atherosclerosis could be summarized as six e's: endocytosis of ox-LDL, expression co-location with atherosclerosis, enhanced by atherosclerosis-related risk factors, elevated LOX-1 protein in cardiovascular diseases, effects involved in pathogenesis of atherosclerosis, and eliminated by anti-atherosclerotic drugs. Furthermore, LOX-1 is related to the stability of atherosclerotic plaque (*6*).

A review of ten years of studies on LOX-1 reveals that in principle LOX-1 is consistent with these characteristics for a specific drug target. LOX-1 is a 50 kDa type II membrane protein that structurally belongs to the C-type lectin family with a short intracellular N-terminal hydrophilic and a long extracelluar C-terminal hydrophilic domain separated by a hydrophobic domain of 26 amino acids (*1*). The lectin domain is the ligand recognition site and the binding activity of LOX-1 to its ligands depends on the interaction of positively charged residues with negatively charged ligands (*16,17*). Based on the arrangement of critical binding residues on the LOX-1 structure and comparing the size of the dimmer surface of LOX-1 with the diameter of ox-LDL, the binding mode for the recognition of ox-LDL was proposed, indicating that three LOX-1 molecules are needed to bind to one ox-LDL molecule (*18,19*). Furthermore, two different fragments of the ligand-binding domain of human LOX-1 have been crystallized and analyzed by X-ray (*20*). The binding of LOX-1 to ox-LDL induces intracellular reactive oxygen species (ROS) production, p38 mitogen-activated protein kinase (p38 MAPK) and nuclear factor kappa B (NF-κB) activation, and expression of intercellular adhesion molecule-1

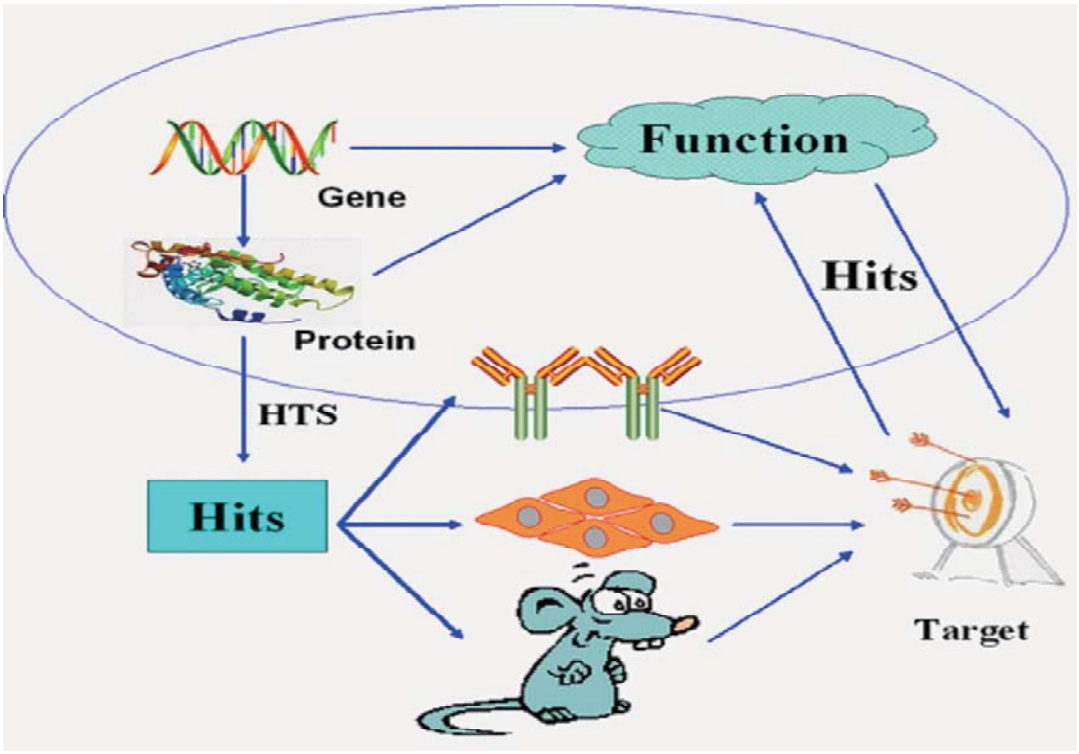

Figure 2. Drug target validation: hit discovery and target function research. New drug target validation is a difficult process. However, hit compounds obtained from HTS could be a useful tool for target validation and target function research. A HTS model is established to obtain hits. The screened hits will be evaluated at the molecular, cellular, and whole animal level and their effects will be of great use in validating the target. At the same time, a drug target validation process based on this strategy also serves as the process of target function research.

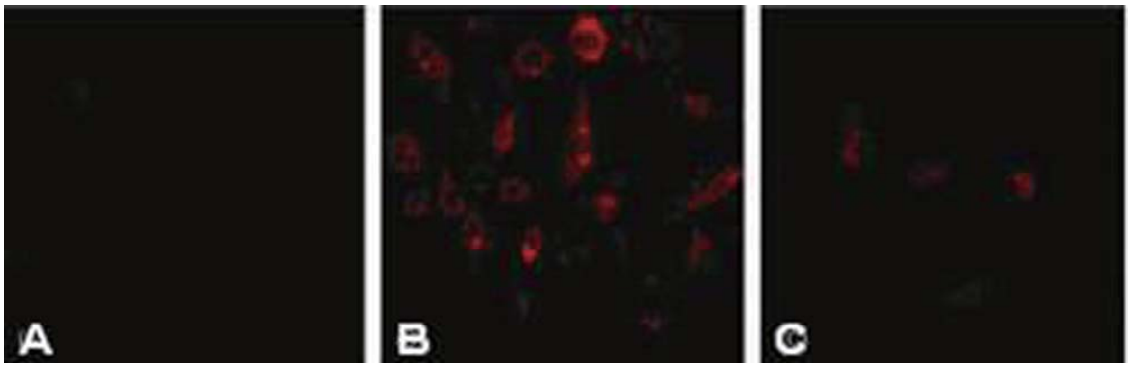

Figure 3. LOX-1 ligand 6306 obtained from HTS inhibits LOX-1 mediated DiI-ox-LDL uptake. hLOX-1-CHO-K1 cells were incubated with DiI-ox-LDL (10 μg/mL) with and without 6306. The uptake of DiI-ox-LDL was measured with a fluorescence microscope. A, blank; B, without 6306; C, 20 μM 6306.

(ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), and monocyte chemoattractant protein-1 (MCP-1). Changed expression of LOX-1 had been reported in several cardiovascular diseases such as atherosclerosis, hypertension, myocardial ischemia, ischemia-reperfusion injury, and cardiovascular complications of diabetes (*2-7*). However, further study is needed to provide more evidence that LOX-1 is a promising drug target.

To validate whether LOX-1 could serve as a new potential target, a competitive fluorescence polarization-based (FP) HTS method was first established to screen LOX-1 ligands in a 384-well microplate using recombinant human LOX-1 protein. The human LOX-1 gene was obtained by RT-PCR from THP-1 cells stimulated with histamine. The purified hLOX-1 gene was cloned into a pMD 18-T vector, which was amplified in Escherichia coli strain DH5α. hLOX-1 cDNA was subcloned into pPIC9K to provide the recombinant plasmid pPIC9K-His-hLOX-1. The plasmid pPIC9K-His-hLOX-1 was transformed into Pichia pastoris GS115. The hLOX-1 protein was

purified with HiTrap Chelating HP and labeled with fluorescein isothiocyanate (FITC).

Assay was based on receptor-ligand interaction: human LOX-1 was labeled with FITC and bound to its special ligand, ox-LDL and different chemicals from the sample bank were used to compete with ox-LDL. A total of 12,700 compounds were screened with an excitation filter of 485 nm and emission filter of 530 nm. This yielded a Z' factor of 0.75, and three chemicals of LOX-1 mimic ligands with an EC_{50} below 40 μM were identified (*21*,*22*).

To further evaluate the binding activity of these chemicals, one of the three compounds, 6306, was studied further using cell models. Ox-LDL was labeled by DiI and the uptake of DiI-oxLDL was studied with hLOX-1-CHO-K1 cells (CHO-K1 cells transfected with the human LOX-1 gene). Fluorescence microscopy of hLOX-1-CHO-K1 cells incubated with DiI-ox-LDL showed that hLOX-1-CCHO-K1 cells internalized significant amounts of DiI-ox-LDL (Figure 3B) although the control did not (Figure 3A); this internalization was also blocked by excess amounts of unlabeled ox-LDL (200 μg/mL). Pre-cultured 6306 (20 μM) significantly decreased LOX-1 mediated DiI-ox-LDL endocytosis (Figure 3C). This suggests that 6306 has a high affinity to hLOX-1 protein under physiological conditions.

Previous studies revealed that the binding of ox-LDL to LOX-1 induced intracellular reactive oxygen species (ROS) production (*23-25*). Ox-LDL may decrease intracellular nitric oxide (NO) concentration due to the increase in intracellular O_2^-. Using hLOX-1-CHO-K1 cells, the effects of the screened LOX-1 ligand 6306 on ROS and O_2^- production were determined.

Results showed that 6306 had similar effects to ox-LDL on the ROS and O_2^- production in LOX-1-CHO-K1 cells. 6306 significantly reduced the NO_2^- level in the supernatant of cultured cells (data not shown). These results suggest that 6306 might activate LOX-1 and result in effects similar to those of ox-LDL. Due to the important role ox-LDL plays in atherosclerosis, 6306 was not used in animal studies.

Another ligand, 6302, may inhibit ox-LDL induced hLOX-1-CHO-K1 cell intracellular ROS and O_2^- formation and NO_2^- decrease. A rat model of atherosclerosis induced by a high-fat diet was established to explore the role of LOX-1 in the pathogenesis of atherosclerosis and to test if LOX-1 ligand had potential to serve as a leading anti-atherosclerotic compound, and the effects of LOX-1 ligand 6302 were studied in this model.

The results revealed that LOX-1 ligand 6302 attenuated aortic intima injury induced by a high-fat diet in rats and inhibited atherosclerotic plaque formation. The serum levels of total cholesterol (TC), triglyceride (TG), and low-density lipoprotein-cholesterol (LDL-C) decreased while the high-density lipoprotein-cholesterol (HDL-C) level increased in a model of atherosclerosis where 6302 was administered. The serum malondialdehyde (MDA) level also decreased (data not shown). These results suggest that LOX-1 inhibition might have a beneficial effect on atherosclerosis.

Conclusion

Drugs are a physician's most powerful weapon to combat disease. The discovery of new drug targets is the basis of new drug development and examination of new drug mechanisms. Though the advent of a pharmacogenomics era opens the door for new drug target research, there are still numerous obstacles to the identification and validation of new drug targets and a great deal of work must be done.

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