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From Epoxomicin to Carfilzomib: Chemistry, Biology, and Medical Outcomes

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Abstract

The initial enthusiasm following the discovery of a pharmacologically active natural product is often fleeting due to the poor prospects for its ultimate clinical application. Despite this, the ever-changing landscape of modern biology has a constant need for molecular probes that can aid in our understanding of biological processes. After its initial discovery by Bristol-Myers Squibb as a microbial anti-tumor natural product, epoxomicin was deemed unfit for development due to its peptide structure and potentially labile epoxyketone pharmacophore. Despite its drawbacks, epoxomicin's pharmacophore was found to provide unprecedented selectivity for the proteasome. Epoxomicin also served as a scaffold for the generation of a synthetic tetrapeptide epoxyketone with improved activity, YU-101, which became the parent lead compound of carfilzomib (KyprolisTM), the recently approved therapeutic agent for multiple myeloma. In this era of rational drug design and high-throughput screening, the prospects for turning an active natural product into an approved therapy are often slim. However, by understanding the journey that began with the discovery of epoxomicin and ended with the successful use of carfilzomib in the clinic, we may find new insights into the keys for success in natural product-based drug discovery.

1 Introduction

Mankind has long been plagued by a myriad of diseases as a result of invading organisms or the dysfunction of our cells. To fight these diseases and the hardships they cause, natural resources such as plant extracts have provided powerful weapons for thousands of years.¹ However, only in the past few hundred years have pharmacologically active compounds been identified, isolated in their pure forms, and administered in exact dosages to relieve disease symptoms.² In early years, medicinal plants were a major source of many clinically used drugs, such as morphine, quinine and artemisinin. This is not surprising since plant extracts have been used for centuries for medicinal purposes, even before their active ingredients were isolated and used in formal medical practice. In addition, drugs originating from microbial species including penicillin, camptothecin, lovastatin, and cyclosporin, to name a few, have made substantial contributions to the health of humankind.¹ More recently, researchers have also made significant inroads into marine-based natural products for drug discovery leading to novel therapeutics and natural product-inspired drugs such as gemcitabine, eribulin, ziconotide, and trabectedin.³

Viewing current drug discovery efforts from a historical perspective, it is clear that natural products have served as rich sources for a significant fraction of our pharmacopeia today. Quite expectedly, the pharmaceutical industry has invested heavily in efforts to discover new pharmacologically-active natural via extensive and systematic screening of natural sources, ranging from medicinal plants to microbes found in soil as well as marine environments.^{4, 5} These screening efforts have resulted in large collections of active natural products over the years. However, initial excitement generated by these compounds quickly faded due to their unfavorable physicochemical properties and poor *in vivo* efficacy. In the 1990s, with the advent of the modern chemical biology era, these natural products once deemed as nonstarters have found a new purpose in the exploration of novel and potentially critical signaling pathways.⁶⁻⁹ With this renewed interest in these natural products as chemical probes, researchers have made conscious efforts to identify the target proteins of natural products. These efforts often provide a starting point for unraveling complex biological phenomena and may open the door to the discovery of new drug targets in the treatment of human disease.

2 Epoxomicin: Total Synthesis and Mode of Action Studies

Epoxomicin, which is a member of linear peptide epoxyketone natural product family, was first discovered by Bristol-Myers Squibb (BMS, Tokyo, Japan) from an unidentified *Actinomycetes* strain, No. Q996-17, based on its *in vivo* antitumor activity against murine B16 melanoma tumors.¹⁰ Despite its promising antitumor activity, epoxomicin did not draw much attention as a potential drug lead from the pharmaceutical industry, presumably because epoxomicin has seemingly poor drug-like properties, namely the presence of a peptide backbone and the labile epoxy ketone pharmacophore (Fig 1).¹¹ More importantly, at that time its mode of antitumor action was unknown.

Our initial interest in epoxomicin stemmed from its potent antitumor activity. We hoped to use the compound as a molecular probe to uncover novel signaling pathways that are crucial for tumor growth. Our first step towards this goal was to develop a strategy for identifying its intracellular target. Looking at the chemical structure of epoxomicin, we speculated that the epoxide ring, which is prone to nucleophilic attack, would aid in expediting the identification of epoxomicin-binding proteins via their covalent modification by the compound. The idea of covalently labeling target proteins by epoxomicin led us to design a biotin-tagged epoxomicin analog for use as an affinity reagent (Fig 2A). As had been done with natural products,^{12, 13} our goal was to use biotin-tagged epoxomicin to label target proteins with biotin through an irreversible linkage, enabling target proteins to be efficiently pulled down via an avidin-coated resin. Finally, isolated target proteins could be eluted from the resin and visualized by SDS-PAGE in conjunction with staining and identified by western blotting as well as mass spectrometry (Fig 2B).

At that time, however, there was no epoxomicin available for its mechanistic studies or for testing its pharmacological activity since the BMS screening project in Japan had been discontinued. Worse still, a synthetic strategy for epoxomicin had not yet been developed and thus, securing epoxomicin became our first priority before any studies could be conducted. To this end, a convergent scheme for the synthesis of epoxomicin was designed.

We successfully accomplished the first total synthesis of epoxomicin, coupling the acetyl-capped and N-methylated tripeptide left-hand fragment with the right-hand leucine residue bearing the C-terminal epoxy ketone pharmacophore (Fig 1).¹⁴ This also allowed the determination of epoxomicin's otherwise ambiguous stereochemistry for the first time and facilitated the preparation of biotin-tagged epoxomicin.¹⁴ Using this biotin-tagged epoxomicin, we were then able to determine that the target protein of epoxomicin is the proteasome (Fig 2).¹⁵ The proteasome is a large multisubunit protease complex found in species ranging from archaeobacteria to yeast to humans.¹⁶ Their ubiquitous presence across many species and high abundance in cells reflect their central role in cellular protein turnover, a fundamental biological process regulating protein homeostasis and many important signaling pathways for cell proliferation, survival and carcinogenesis.^{17, 18} Using freshly prepared epoxomicin, we also found that it possesses not just potent antitumor activity but also anti-inflammatory activity,¹⁵ making the proteasome a more attractive therapeutic target.

3 Epoxyketones: Proteasome-specific Pharmacophores

While investigating the activity of epoxomicin against the common proteases, we quickly realized that epoxomicin possesses unprecedented selectivity for the proteasome over previously known proteasome inhibitors, which commonly demonstrate cross-inhibition with non-proteasomal proteases.¹⁴ This unusually high specificity was rather surprising because we originally speculated that the inhibition of the proteasome by epoxomicin was due to a nucleophilic attack on the epoxide ring by the hydroxyl group of *N*-terminal threonine of the proteasome, resulting in the formation of an ether linkage (Fig 3A), as do proteasome inhibitors containing reactive electrophilic moieties. To our surprise, however, X-ray crystallographic studies performed in collaboration with Michael Groll and Robert Huber revealed that the unique specificity of epoxomicin is a result of the irreversible formation of a six-membered morpholino ring between the amino terminal catalytic Thr-1 of the 20S proteasome and the α' , β' -epoxy ketone pharmacophore of epoxomicin (Fig 3B).¹⁹ Mechanistically, this unusual ring formation is explained by a sequential double nucleophilic attack on the epoxy ketone residue of epoxomicin by two nucleophiles of the *N*-terminal Thr, the hydroxyl side chain and the free amine. Therefore, it is expected that only Ntn (*N*-terminal nucleophile) proteases such as the proteasome can form the morpholino ring by reaction with epoxy ketones, thereby rendering epoxomicin exceptionally specific for the proteasome over alternative mechanism-based proteasome inhibitors.

4 YU-101: Improving on Mother Nature

This unusually high specificity and potency led us to believe that epoxomicin may have promising potential for cancer treatment. Initially, there was some skepticism about whether the proteasome can be a viable anticancer target considering its essential functions in all eukaryotic cells. Contrary to this assumption, some studies began to suggest that a reasonable therapeutic window in which one can selectively kill cancer cells might exist.^{20, 21} It is now known that rapidly proliferating cancer cells generally display elevated levels of proteasome activity and increased cell death upon proteasomal inhibition compared

to normal cells. In order to understand and improve epoxomicin's anticancer activity, we put forth an effort to optimize the compound via a classical medicinal chemistry approach.²² This effort eventually led us to YU-101 (Fig 4), which showed more potent inhibitory activity against the proteasome than both epoxomicin and bortezomib, a boronic acid-based synthetic proteasome inhibitor developed initially by the biotech company Proscript and later acquired and developed further by Millennium to the yield the FDA-approved drug Velcade®.²¹ While YU-101 was being developed, bortezomib rapidly progressed into clinical trials for the treatment of multiple myeloma. At that time, it was already clear that YU-101 had better proteasome inhibitory and antitumor activities than bortezomib. Importantly, it is now shown to be substantially more selective for the proteasome than bortezomib. Indeed, it was later found that severe and dose-limiting peripheral neuropathy associated with bortezomib is most likely due to cross-inhibition with HtrA2/Omi, a neuronal serine protease implicated in neuron survival.²³ Carfilzomib, a YU-101 derivative developed by the South San Francisco-based biotech company Proteolix, does not inhibit serine proteases including HtrA2/Omi, most likely due to the properties of its epoxomicin-derived pharmacophore.²³

5 From Improved Natural Product to Drug Lead

The development of carfilzomib for the treatment of multiple myeloma began with the establishment of the South San Francisco-based company Proteolix in 2003. The birth of the company was primarily based on the promise of YU-101's superior antitumor activity and its specificity for the proteasome over other proteasome inhibitors known at that time. The initial development effort using YU-101 as a lead drug was bolstered by the FDA approval of bortezomib (Velcade®) for the treatment of multiple myeloma in 2003,²¹ which removed any doubt regarding the proteasome as a potential anticancer target. Initially, the company generated several hundred of YU-101 derivatives with the aim of finding better improved inhibitors but ultimately concluded that YU-101's impressive potency against the proteasome could not be further increased. Despite YU-101's impressive antitumor activity, it was soon realized that YU-101's poor water solubility would be a liability moving forward. By adding a morpholine ring to the *N*-terminus of YU-101, solubility was increased by several orders of magnitude, resulting in carfilzomib (Fig 4). Clinically, carfilzomib was found to be effective, even in those patients with acquired resistance to bortezomib.²⁴ Through arduous pre-clinical and clinical work,^{24–26} carfilzomib (Kyprolis™) finally joined the modern pharmacopeia in July 2012, thus concluding the long journey from natural product pariah to FDA-approved cancer drug.²⁷

While the initial journey of epoxomicin has ended with the FDA approval of carfilzomib, a new one is just beginning. Currently, carfilzomib is being evaluated in a Phase III trial (ASPIRE) to test its efficacy in combination with the critical myeloma therapy lenalidomide (Revlimid®) and low dose dexamethasone in patients with relapsed multiple myeloma.^{28, 29} Separately, a new Phase III trial (ENDEAVOR) has been launched to evaluate the efficacy of carfilzomib in combination with dexamethasone in comparison with bortezomib and dexamethasone in patients with relapsed multiple myeloma. Additionally, Phase II clinical trials are underway to evaluate the efficacy of carfilzomib in patients with relapsed solid tumors (NCI clinical trial identifier: NCT00531284). There is no doubt that the journey

taken by epoxomicin will continue to make real-life improvements to the lives of patients and their families.

6 Conclusions

The journey from epoxomicin to a FDA-approved cancer drug had its origins in pure scientific curiosity, thus serving as an a testament to drug discovery efforts emanating from natural product mode of action studies, rather than brute force small molecule screening for activity. As we see in virtually every issue of various natural product-related journals, the discovery of new biologically active compounds from natural sources will continue. It is also likely that the majority of these molecules will ultimately be abandoned for various reasons such as systemic toxicity, unknown mechanisms of action, and potentially unfavorable drug-like properties. In this era of modern chemical biology, new opportunities will, however, inevitably arise to revitalize these abandoned bioactive compounds as molecular probes for the study of complex biological processes. In the era of rational drug design and high-throughput screening, it has become an increasingly daunting challenge to find suitable drug leads from natural products. Now, the door has been opened for future drug discovery endeavors using once abandoned natural products. The model of carfilzomib's development from epoxomicin highlights new insights into the keys for future success in natural product-based drug discovery.

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Epoxomicin

Yale University

YU-101

Proteolix/Onyx

Kyprolis[™]
(carfilzomib)

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