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Discovery and development of the anticancer agent salinosporamide A (NPI-0052)

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ABSTRACT

The discovery of the anticancer agent salinosporamide A (NPI-0052) resulted from the exploration of new marine environments and a commitment to the potential of the ocean to yield new natural products for drug discovery and development. Driving the success of this process was the linkage of academic research together with the ability and commitment of industry to undertake drug development and provide the resources and expertise to advance the entry of salinosporamide A (NPI-0052) into human clinical trials. This paper offers a chronicle of the important events that facilitated the rapid clinical development of this exciting molecule.

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1. Introduction

Beginning in the late 1980s, Fenical and Jensen at the Scripps Institution of Oceanography (SIO; University of California, San Diego) began to explore the microbial diversity of marine habitats and the relationship of this diversity to natural product discovery. It seemed a natural extension of the productive drug discovery activities that had taken place with soil bacteria to apply the same concepts to microbiologically uncharacterized habitats in the ocean. In the beginning, the Scripps group focused its efforts on the isolation of actinomycete bacteria, the same bacterial group that is highly represented in soil microbial communities. This group is historically recognized as the most prolific source of microbially derived bioactive molecules discovered to date,[1] yet it was unclear at the time if strains could be readily cultured from marine samples and if, or how, these strains differed genetically, chemically and biologically from those that occur on land.

During the initial studies, it was apparent that actinomycetes could be readily cultured from marine samples, however, it took a considerable investment before the biological and chemical novelty of these strains became apparent. Although the SIO group was not the first to explore the ocean for the isolation of actinomycete bacteria,[2,3] the potential applications of these bacteria for research purposes remained poorly developed and were not widely recognized for many decades. Indeed, early studies in Europe reported the isolation of actinomycetes from deep-ocean sediments,[2] however, there was no evidence that these bacteria were 'marine', and a long-standing debate developed questioning if actinomycetes were natural inhabitants of the ocean or merely terrestrial strains that had been washed into the sea, most likely as dormant spores.[4]

Studies at SIO both confirmed the presence of strains that occur both on land and in near-shore sediments and illustrated that the taxonomic profiles of actinomycete communities were a function of the depth from which the sediments were collected. As part of a program to explore the diversity and biosynthetic potential of marine-derived strains, a survey of actinomycete distributions in near-shore marine sediments was conducted in the Bahamas in 1989 (Fig. 1). One unusual result from this survey was the cultivation of strains that required seawater for growth, a physiological trait not previously reported for actinomycetes.[5] This was the first indication that unique actinomycete bacteria reside in marine environments. To follow-up on these observations, devices were designed to sample the sea bottom at depths up to 2000 m. Consistent with Weyland's findings,[2] the presence of culturable actinomycete bacteria was evident in these remote environments. This observation supported the proposal that obligate marine actinomycetes did indeed exist.

1.1. Discovery of the genus *Salinispora* at SIO

The seawater-requiring actinomycetes cultured as part of the Bahamas survey were identified as members of the Family *Micromonosporaceae* and most closely related to the genus *Micromonospora* using the traditional taxonomic tools available at the time.

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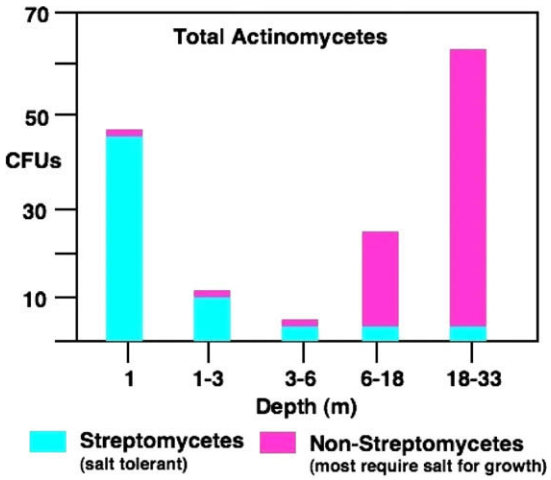

Figure 1. Distribution of culturable actinomycetes in marine sediments, collected in the Bahamas Islands, as a function of depth. Adapted from Jensen et al.[5]

Based largely on the unprecedented requirement of seawater for growth, it was proposed that these bacteria represented a new species in the genus.[5] Cursory chemical analyses of organic extracts of a subset of these strains did not reveal any compounds of interest and no further studies were performed at that time.

In 1999, a second year graduate student at SIO, Tracy Mincer, became interested in molecular phylogenetics and initiated a study of the taxonomic affiliations of these seawater-requiring actinomycetes using 16S rRNA gene sequence data. These analyses revealed that the marine strains were in fact distinct from all previously described *Micromonospora* species. This distinction, however, did not appear to reside at the species level, as the phylogenetic tree placed these bacteria in a distinct clade that was independent of all other genera within the Family. Based on these observations, it was suggested that the seawater-requiring actinomycetes more appropriately constituted a new genus for which the name '*Salinospora*' was originally proposed.[6] In 2003, a collaborative program was established between the SIO group and Alan Ward and Michael Goodfellow, at the University of New Castle Upon Tyne, to prepare a formal taxonomic description of the genus, which was published in 2005 along with the description of two species, *Salinispora arenicola* and *Salinispora tropica* and a change in the spelling of the genus name to *Salinispora* to meet nomenclatural standards.[7]

Once the phylogenetic novelty of the *Salinispora* strains was recognized, a new round of highly analytical chemical studies was initiated at SIO. This quickly focused on one *Salinispora* strain, CNB-440, which showed very potent activity in an anticancer bioassay using the HCT-116 human colon carcinoma cell line. Cultivation in scale (20 L), followed by bioactivity-guided (HCT-116 cytotoxicity) fractionation of the crude culture extract, led to the isolation of a structure designated by the SIO group as salinosporamide A (**1**; Fig. 2).[8] These findings mark some of the important events in the discovery and development of this important anticancer agent, which are highlighted in Figure 3.

1.2. Isolation and structure elucidation salinosporamide A

The assignment of the full structure of **1** required considerable effort. Spectral data illustrated the presence of the β-lactone functionality, but NMR data alone could not differentiate between several competing structures. Consequently, the full structure of **1**, including its absolute configuration, was defined by X-ray crystallographic methods. Compound **1** showed IC_{50} values of less than 2 ng/mL against HCT-116 cells, and was found to be a highly selective tumor cell growth inhibitor, as measured using the 60 cell line panel at the National Cancer Institute (GI_{50} < 10 nm). Based on the structural relationship of salinisporamide A (**1**) to the previously described β-lactone omuralide (**2**; Fig. 2), we had reason to believe that this new β-lactone could be similar to omuralide in its pharmacological mode of action. Omuralide had been earlier shown to be a potent inhibitor of the 20S proteasome and was considered the 'gold standard' in that field of cell biology.[9] At this time, Nereus Pharmaceuticals (San Diego, CA) voiced a strong interest in the compound, and it was licensed to Nereus by UC-San Diego in 2001.

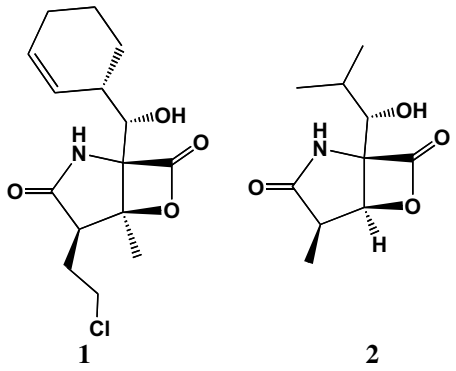

Figure 2. Structures of salinosporamide A (**1**) and omuralide (**2**).

2. Preclinical biology and mechanism of action studies at Nereus

Nereus researchers initiated studies to develop a better understanding of the mechanisms of action of salinosporamide A (designated as NPI-0052 for Nereus studies and developmental purposes) and to evaluate its potential for clinical development. In parallel, the ubiquitin-proteasome system was receiving considerable attention for its role in the degradation of intracellular proteins[10,11] and in 2003, the proteasome was effectively validated as a target in cancer chemotherapy based on the regulatory approval of bortezomib (PS 341, Velcade®) for the treatment of relapsed and relapsed/refractory multiple myeloma (Fig. 3).[12,13] The 26S proteasome is a multicatalytic enzyme complex comprising one or two 19S regulatory caps and a proteolytic 20S core particle in which protein degradation occurs.[14–16] The 20S proteasome contains three pairs of proteolytic subunits with chymotrypsin-like (CT-L), trypsin-like (T-L) and caspase-like (C-L) activities.[17] In addition to bortezomib, several small molecule 20S proteasome inhibitors were already well established as research tools,[16] including the β-lactone-γ-lactam inhibitor omuralide. Based on the structural relationship of salinosporamide A (NPI-0052; **1**) to omuralide, we had reason to believe that the two molecules may share a common molecular target. In order to confirm this hypothesis, the two compounds were screened against purified rabbit 20S proteasomes and **1** was found to inhibit all three catalytic functions, that is, the CT-L, T-L and C-L activities, with IC_{50} values in the low to mid nM range, activities significantly more potent than omuralide.[8] Compound **1** was subsequently screened against human 20S proteasomes, with similar results. The most potent inhibition is observed with respect to the CT-L and T-L activities of the 20S proteasome.[18] Other proteasome inhibitors such as bortezomib inhibit the CT-L activity to a similar degree compared to **1**, but exhibit a different inhibition profile for the T-L and C-L activities. In mice, treatment with **1** induced a prolonged duration of inhibition compared to bortezomib of all three catalytic functions of the 20S proteasome in packed whole blood (Fig. 4),[18] thereby confirming the molecular target in vivo. Compound **1** exhibits at least a 3-log specificity for inhibition of protea-

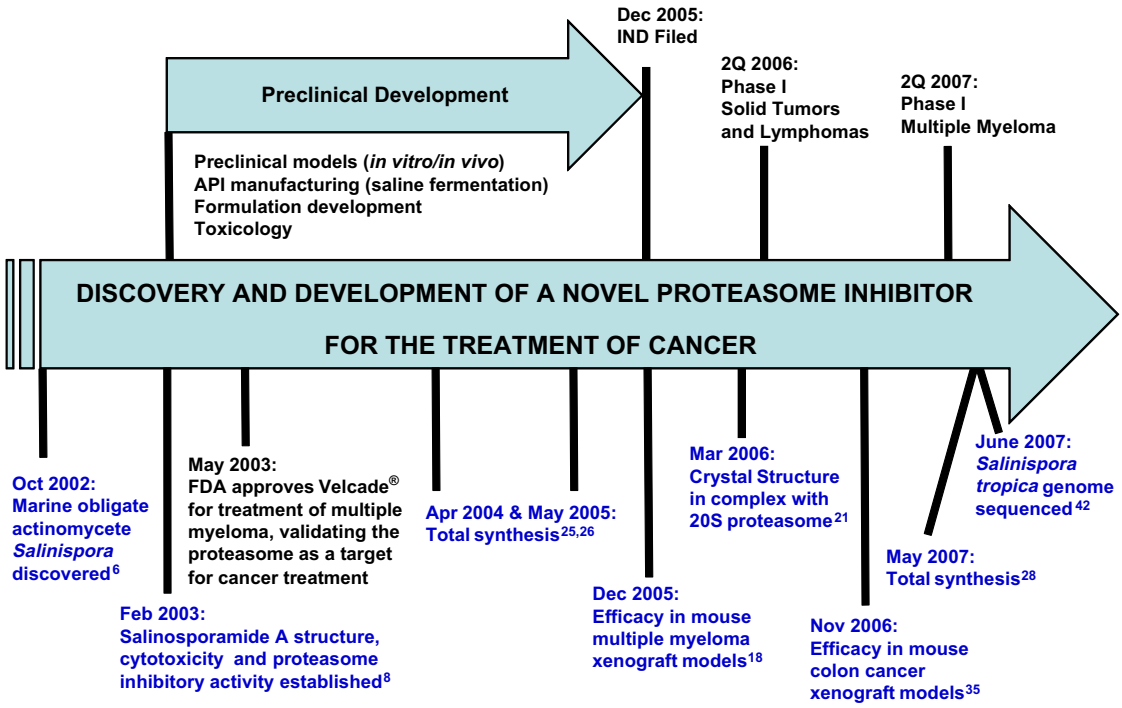

Figure 3. Timeline of events from the discovery of **1** to its entry into Phase I clinical trials, with select publications in blue.[42] (see above-mentioned references for further information).

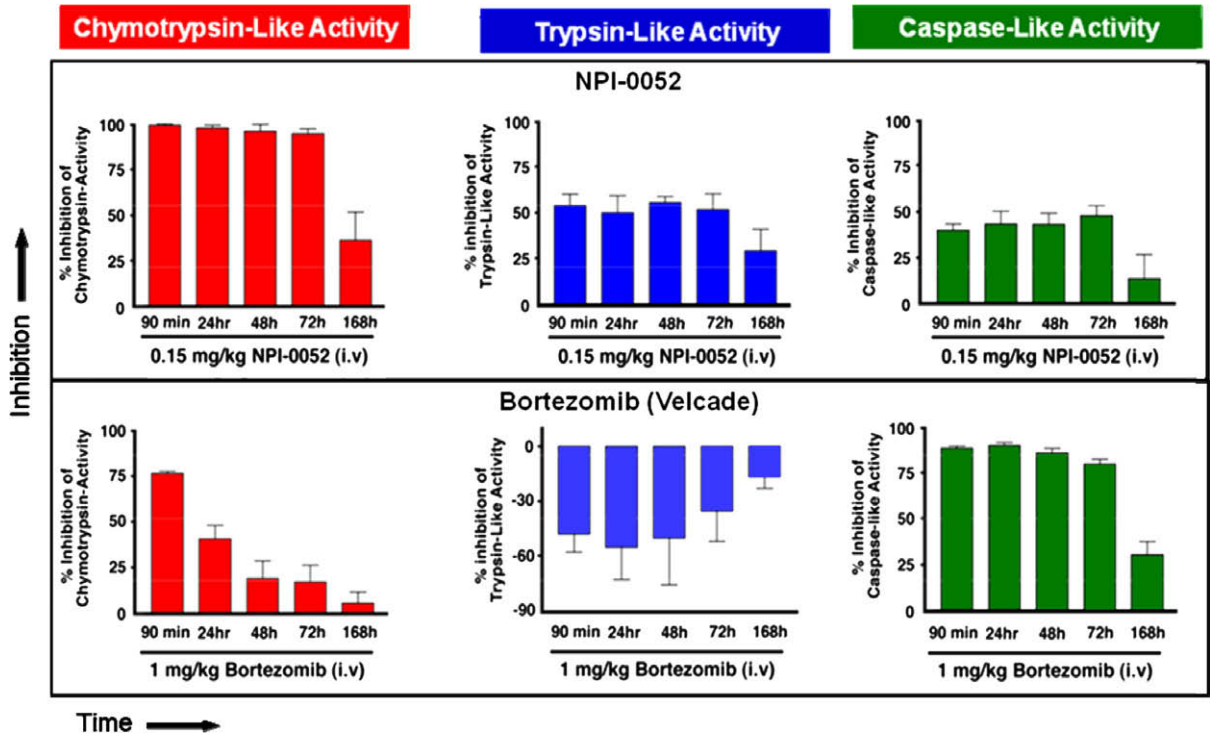

Figure 4. Inhibition of CT-L, T-L and C-L proteasomal activities in packed whole blood after IV administration of NPI-0052 (**1**) or bortezomib. Compound **1** exhibits a broader and longer 20S proteasome inhibition profile than bortezomib.[18]

some proteolytic activities as compared to other proteases such as chymotrypsin, trypsin, cathepsin A and cathepsin B.[18]

Salinosporamide A (NPI-0052; **1**) inhibits all three active proteolytic sites; this property is to be considered a potential advantage for **1**. Recent elegant studies by Kisselev et al.[19] have demonstrated that the C-L and T-L sites also play a significant role in protein breakdown and their relative importance varies markedly with the target protein. Moreover, measuring only the CT-L activity may not accurately reflect the degree of proteasome inhibition in blood, tissues and tumors and subsequently the effect on the level of protein degradation.[19] Therefore, inhibiting only the CT-L site may not be sufficient to markedly block protein degradation. Fur-

ther evaluation of the mechanism of action indicated that **1** inhibits the activation of NF-κB and 13 genes regulated by NF-κB, a hallmark downstream event of proteasome inhibition that is involved in inducing apoptosis in multiple myeloma (and other) cell lines.[20]

Structural biology provided further confirmation of the molecular target. In collaboration with Groll and Huber at the Max Planck Institute, the crystal structure of **1** in complex with the yeast 20S proteasome showed that the molecule occupies the active sites of all three pairs of catalytic subunits. The inhibitor is covalently bound at each subunit active site via an ester linkage between the catalytic *N*-terminal Thr1 O^{γ} of the proteasome and the carbonyl derived from the β-lactone ring. β-lactone ring opening is followed by chlorine elimination, giving rise to a 5-membered cyclic ether (Fig. 5); it was proposed that this unique reaction sequence renders the inhibitor irreversibly bound.[21] These findings provided a detailed understanding of the proteasome-ligand interactions at the molecular level that reveal **1** to be a remarkably well designed proteasome inhibitor. While many proteasome inhibitors comprise peptides with reactive head groups,[11,16] salinosporamide A may be described as the 'minimalist' among them, with dense but entirely purposeful functionality. In fact, every atom seems to be ideally situated for optimal binding to the proteasome active site and/or triggering the two-step reaction sequence that renders the molecule irreversibly bound. The exquisite design of this unique compound reflects the beauty and power of a selection process offered only by nature.

The identification of salinosporamide A (NPI-0052; **1**) as a novel chemical entity with known mechanism of action against a validated molecular target set the stage for an accelerated preclinical development program at Nereus Pharmaceuticals. In view of the established efficacy of the proteasome inhibitor bortezomib in multiple myeloma,[12,13] **1** was initially evaluated in a multiple myeloma xenograft model in mice, where it was shown to be efficacious when administered twice weekly at very low doses after either IV (0.15 mg/kg) or oral (0.25 and 0.5 mg/kg) administration.[18] Additional preclinical studies were also performed to define the activity of **1** in models of both hematologic and solid tumor malignancies. To date, compound **1** has demonstrated efficacy either as a single agent, or in combination potentiating the effect of Standard of Care (SOC) therapies in models for lung, prostate, pancreas, colorectal and many B-cell malignancies (see below). Of potentially significant clinical importance, NPI-0052 maintains efficacy against multiple myeloma cells isolated from patients refractory/resistant to bortezomib, lenalidomide and/or thalidomide.

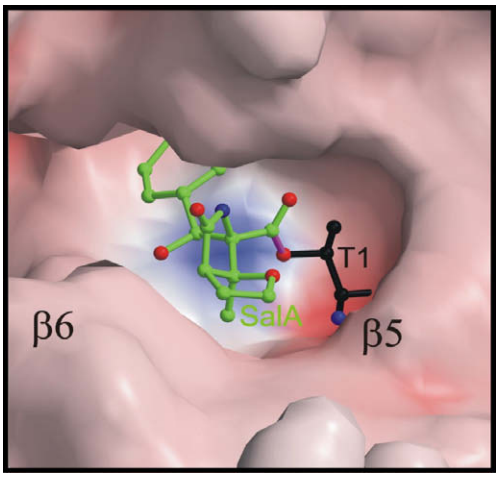

Figure 5. Surface model of **1** in complex with the β5 subunit of the yeast 20S proteasome.[21]

3. IND-enabling studies and clinical development of salinosporamide A (NPI-0052)

With the established mechanism of action and early indications of efficacy in MM preclinical models, an intensive program was undertaken to support the filing of an Investigational New Drug Application (IND) with the FDA for NPI-0052. This encompassed production of the active pharmaceutical ingredient (API), a formulated drug product for both clinical studies and IND-enabling safety/toxicology studies in two animal species, the establishment of drug pharmacodynamics (i.e., inhibition of CT-L, T-L and C-L activities in packed whole blood) and pharmacokinetics. The IND was filed in December 2005 (Fig. 3).

3.1. API manufacturing

Salinosporamide A (NPI-0052; **1**) API is currently manufactured under cGMP through a robust saline fermentation process, which employs a wild type strain that was obtained as a single colony isolate from *S. tropica* strain CNB-476. Like strain CNB-440, strain CNB-476 was isolated from a marine sediment collected in the Bahamas.[5] The original fermentation conditions for laboratory scale production of **1** yielded a few milligrams per liter. In order to industrialize the process and manufacture the compound at suitable scale and quality for administration to humans, key fermentation ingredients, such as natural seawater and animal-derived nutrients, had to be replaced, and foaming and agitation issues during scale-up production needed to be overcome. Published literature suggested that the 316 type stainless steel fermentors commonly found in manufacturing facilities are not suitable for saline fermentation and that special fermentors are required.[22] Consequently, Nereus developed a process to overcome the corrosive effect of saline culture media on common stainless steel fermentors. In addition, the inherent instability of the β-lactone ring in aqueous solution[23] was overcome by addition of a solid resin to the fermentation in order to bind and capture the API at an optimized time during the production cycle and to avoid the detrimental foaming problem associated with the production process.[24] The final process development standardized parameters such as temperature exposure, operating parameters, cleaning and passivation to overcome the corrosive effect of saline fermentation to the 316 stainless steel fermentors. This, together with careful design and optimization of the seed and production media, resulted in production titers of 450, 350 and 260 mg/L in shake flasks, lab fermentors, and 500–1500 L industrial fermentors, respectively. The final pharmaceutical grade cGMP drug substance (>98% purity) is obtained in overall ~50% recovery from the crude extract. Based on the potency of **1**, the production titer is adequate for both clinical development and commercial production. To our knowledge, this represents the first manufacture of clinical trial materials by saline fermentation.

The unique structure and dense functionality of **1** includes five contiguous stereocenters within a fused β-lactone-γ-lactam ring system decorated with chloroethyl, methyl, and cyclohexenylcarbinol substituents, a structure elegantly assembled by nature into a low (313 amu) molecular weight compound.[8] The molecule has attracted considerable attention from the synthetic organic chemistry community, and a number of strategically important total synthetic routes have been reported (Fig. 3), which have also provided opportunities to synthesize previously inaccessible analogs.[25–30] However, while a variety of synthetic and semi-synthetic analogs have been evaluated,[31–33] *it is the natural product itself that has entered clinical trials*, and at this time and for the foreseeable future, fermentation remains the most cost-efficient and robust API production method. This clearly exemplifies marine

actinomycete bacteria as a renewable resource and that the commercial development of their secondary metabolites are not universally subject to problems associated with the natural products 'supply' issue.

3.2. Formulation development and drug product manufacturing

Several factors were considered in the development of a suitable formulation for IV administration of the drug, including its solubility, high potency, and the lability of the β-lactone ring, which required that the drug product be formulated and stored in an aqueous-free environment until the time of dosing. The Phase I parenteral formulation comprises a co-solvent system of propylene glycol–ethanol, which is further diluted with citrate buffer pH 5 just prior to administration, thereby limiting aqueous hydrolysis. To take advantage of the excellent stability of the API, a lyophilized drug product was recently developed. The manufacturing process involves lyophilization from *t*-butanol in the presence of a suitable bulking agent to minimize exposure to an aqueous environment. The resulting lyophilized powder is reconstituted in a co-solvent system for administration by IV injection.

3.3. Clinical and translational biology studies of NPI-0052

Salinosporamide A (NPI-0052; **1**) has been evaluated in a wide range of non-clinical studies (vide supra), including in vivo models for multiple myeloma[18,34] colon,[35] pancreatic,[36] non-Hodgkin's Lymphoma (NHL),[37] Waldenstrom's macroglobulinemia (WM),[38] acute lymphocytic leukemia (ALL) and acute myeloid leukemia (AML),[39] as well as in vitro studies in chronic lymphoid leukemia (CLL).[40] The translational biology studies clearly demonstrate single agent activity of **1** against solid tumor and hematologic malignancies. In addition, more complex studies in animal models using SOC drug combinations confirmed the broadening potential of using **1** in combination with biologics and/or chemotherapeutics.[35,36,39] Of significant interest are recent data by Roccaro et al.[38] and Chauhan et al.[34] that indicate the enhanced antitumor activity of low dose **1** used in combination with low dose bortezomib, a proteasome inhibitor with a markedly different pharmacodynamic profile. These findings are consistent with the recent suggestions of Kim and Kaelin that the optimal combination of molecular anticancer therapies may be achieved either by 'horizontal combinations' where numerous differing signaling pathways downstream of a known oncogenic molecule are inhibited, or 'vertical combinations' where only one key oncogenic pathway is targeted but at multiple levels to obtain a greater level of inhibition such as obtained with NPI-0052 and bortezomib combinations.[41] NPI-0052 is currently proceeding through dose escalation in several concurrent Phase I clinical trials for evaluation as a single agent in patients with multiple myeloma, solid tumors or lymphomas (Fig. 3). Phase Ib studies are also underway in combination with a specific targeted therapy. The differences between NPI-0052 and bortezomib with regards to speed and duration of action and the inhibition profile of the 20S proteasome and the possible off-target activities of bortezomib indicate that NPI-0052 may provide for a greater therapeutic index and greater activity in diseases where bortezomib showed minimal activity.

4. Summary

From the above recital, it should be clear that the successful discovery and development of salinosporamide A (NPI-0052; **1**) could only have occurred as a result of a close collaboration between a highly specialized academic research institution and a committed, focused pharmaceutical company. During this process, several scientific advances and discoveries were achieved, including: (1) the proof that marine microbiology is a fertile discovery resource for new chemistry with clinical pharmaceutical application; (2) the discovery of a new actinomycete genus, *Salinispora*; (3) the discovery and subsequent development of **1** as an exciting new anticancer agent; (4) the demonstration that saline fermentation is a viable pharmaceutical manufacturing process; (5) the in-depth description of the biological characteristics of **1** as a novel proteasome inhibitor differentiated from others of this class; and (6) the entry of a marine microbiology natural product into clinical trials in oncology. These advances, together with the continued development of salinosporamide A (NPI-0052), should stimulate both academia and industry to further exploitation of marine microbiology for drug discovery and development.

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