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This article reviews the advantage of multi-target drugs and introduces our proof-of-principle studies to find a practical methodology for identifying antinociceptive multi-target drugs.

Foundation review: A series of case studies: practical methodology for identifying antinociceptive multi-target drugs

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Since the introduction of drug discovery based on single targets, the number of newly developed drugs has steadily declined, and the reliablility of the current drug-discovery paradigm has been unceasingly questioned. As an alternative, an emerging approach pursuing multi-targeting drugs has arisen to reflect multifactorial diseases caused by the complex networks of various mechanisms. The purpose of this paper is to review multi-target drugs and introduce our progress in establishing a practical methodology for identifying antinociceptive multi-target drugs. We have adopted a system of *ex vivo* efficacy screening using long-term potentiation in rat spinal cord as a surrogate biomarker for neuropathic pain. A bait-target approach is also adopted to lure an unknown target combination that induces synergistic mechanisms.

Introduction

Emerging concept of multi-target therapeutics

Over the past few decades, strategies of drug discovery have been generally focused on an approach based on single targets along with the rapid growth in genetics and molecular biology. During this time, the number of newly developed drugs that succeed in the market as safe and effective therapeutics has steadily declined. This decline coincided with the introduction of target-based drug discovery [1]. The basic assumption of the single-target approach is that affecting a single receptor or mechanism is sufficient to obtain therapeutic effects on a disease, so applications of this approach on multifactorial diseases caused by the complex networks of various mechanisms should have limitations on clinical effects [2]. In fact, agents developed through this approach often show poor efficacy because changing the functions of a single target can have insignificant effects on entire networks, or the agents might be rendered ineffective by backup systems that prevent detrimental changes and maintain the balance of biological networks and systems [3].

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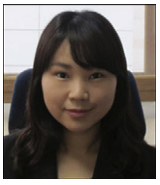

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Yuntae Kim, Ph.D. received BS and MS in chemistry from Seoul National University in 1985 and 1987, respectively. He obtained PhD in synthetic organic chemistry from the University of Pittsburgh followed by post-doctorial training at the California Institute of Technology. In 1997, he joined Merck Research Laboratories (West Point, PA, USA) to identify two clinical compounds for cancer and osteoporosis. Currently, he is working at CKD Pharm (Seoul, Korea) as a director of a medicinal chemistry group managing a number of drug discovery programs. His works resulted in 13 journals, 14 patents, and 1 oral presentation, and 3 poster presentations.

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The current paradigm of drug discovery mostly depends on the *in vitro* activities related to a selected target to identify the most potent and selective ligand to a given target. A high level of *in vitro* potency to a single target, however, is difficult to replicate at high efficacy in many cases of *in vivo* studies. As an example, antagonists of the neurokinin 1 (NK1) receptor, which have high affinity and selectivity, were developed by leading pharmaceutical companies – including Merck, Pfizer and Eli Lilly – as promising candidates for analgesics. However, numerous compounds were abandoned in the early stages of preclinical studies because of poor efficacy in antinociceptive tests [4,5]. Also, even after the successful completion of the preclinical stage, a large number of highly potent inhibitors failed at the Phase II clinical stage because of low bioavailability or lack of a significant analgesic effect [6,7]. Such a large discrepancy between *in vitro* potency and *in vivo* efficacy is one of the most serious problems facing the current paradigm of drug discovery.

From the turn of the century, the clear limitations of the single-target approach led researchers to reach a consensus on the directions needed to develop multi-target drugs. The conceptualization and rationale of multi-target therapeutics have both been proposed based considerably on network pharmacology, system biology or polypharmacology [1–3,8]. Drugs targeting multiple receptors or mechanisms are often less potent to each of their targets than a single-target drug but are not necessarily less efficacious [8]. Numerous endogenous ligands in our body's systems, such as antibodies, neurotransmitters and signaling molecules, bind weakly to target receptors or binding sites of cellular proteins [3]. Simultaneous multi-target binding or serial monovalent binding actions of these ligands can cause high total binding strengths and better mechanistic actions by synergistic interactions of complex network activities [9]. As an example, agmatine is an endogenous ligand at α2-adrenoceptors and imidazoline binding sites with moderate potency (K_d >4 μM and 1 μM, respectively) but shows highly effective biological actions on natriuresis, analgesia and the reduction of blood pressure [10,11]. These characteristics suggest that high affinity and selectivity of ligands are not crucial properties for the development of drug candidates when the interactions between molecules, receptors and the complex mechanisms based on the context of networks are considered [12].

Advantages and practical limitations of the multi-target approach

The low affinity of multi-target drugs is more likely to induce synergistic therapeutic effects by the combination of various mechanistic actions [9] with significantly less toxicity. In fact, many highly efficient drugs, for example nonsteroidal anti-inflammatory drugs (NSAIDs), salicylate, metformin, antidepressants or multi-target antibodies used in cancer therapy [1,3], act on multiple targets. Many NSAIDs transiently bind to receptors and enzymes such as cyclooxygenases (COX) at low affinities (K_d >1 μM) [9]. Moreover, some well-known NSAIDs, such as aspirin and ibuprofen, show good therapeutic effects themselves in addition to synergistic effects when they are administered with opioid analgesics as a combination therapy [13]. NSAIDs and opioid analgesics thus indirectly share multiple sites of action related to pain inhibition and interact to produce the synergistic activities.

Unfortunately, in practice, discovering an optimized molecule that simultaneously modulates more than two molecular targets while balancing other physicochemical and pharmacokinetic parameters is highly challenging [14]. Determining which target pairs to combine tends to be arbitrary, and the application of an *in vitro* screening method is extremely difficult where compound selection is ultimately dictated by phenotypic observations, usually with *in vivo* animal models that have severely limited throughput.

This paper will introduce a practical way to implement the multi-target approach in the development of pain therapeutics as an alternative to overcome the limitations of the current single-target approach. Although the concept of multi-target therapeutics has been proposed in many excellent review articles [1–3,8], the implications for practical methdology to realize the multi-target strategy has received little attention, especially in the early stages of preclinical development. This article will be an initial attempt to bring the multi-target approach into the arena of real-world drug discovery, but further efforts to develop these ideas should be made.

Current drug discovery for the treatment of pain

Failures of single-target analgesics and parallel mechanisms of pain

Despite significant advances in our understanding of pain pathology, therapeutic options for chronic and persistent pain, especially neuropathic pain, remain limited and still rely on multi-decade-old drugs such as opioids, anticonvulsants or antidepressants [15]. The gold standard for the treatment of neuropathic pain, gabapentin, is only effective in less than 30% of patients, and having a safe and effective medication for pain remains a major unmet medical need [16]. With the advent of molecular neurobiology, several new antinociceptive targets have been suggested, and many putative analgesic targets have been studied. However, none has yet emerged as a safe and effective therapeutic option for humans.

As an example, numerous drug discovery companies have made significant efforts since the early 2000s to develop transient receptor potential cation channel subfamily V member 1 (TRPV1) antangonists as a pain therapeutic, but no single compound has successfully shown efficacy in humans. Recently, AZD1386, an antagonist of TRPV1 developed by AstraZeneca, failed in a clinical Phase IIa trial owing to the lack of efficacy (ClinicalTrials.gov ID: NCT00878501). Amgen likewise discovered AMG517 as a TRPV1 antagonist, which failed in a clinical Phase I trial because of undesirable toxicities such as marked hyperthermia [17]. TRPV1 is known to be involved in the transmission and modulation of pain sensation as well as the integration of various nociceptive stimuli. Because TRPV1 is the sole receptor of capsaisin but also is activated by exogeneous stimuli such as heat and low pH, the activation of TRPV1 is caused by many different peripheral pathways and complex networks with other receptors and molecules that exist in the spinal cord [18,19]. TRPV1 could thus be a part of the pain mechanism, not the main center of pain sensation. A TRPV1 antagonist might thus have a limited efficacy [20]. Such an example can also be easily found in reports of recent studies on the development of analgesic drugs based on the single-target approach for NK1 and cannabinoid receptor type 2 (CB2) receptors [21,22].

As shown in the above examples, failure of analgesic development is mostly derived from the logic of targeting a single receptor.

TABLE 1

Selected example of multi-target drugs in CNS therapeutics

Brand name	Companies	Sales	Targets
Seroquel	AstraZeneca	US$5.4B	D_{1-4}, $5HT_2$, $\alpha_{1/2}$, H_1
Zyprexa	Eli Lilly	US$5.0B	$5HT_2$, D_2, $GABA_A$
Abilify	Otsuka/BMS	US$4.7B	D_2 partial agonist, D_3, $5HT_2$
Effexor	Pfizer	US$4.3B	SNRI
Risperdal	J&J	US$3.9B	D_2, $5HT_2$
Cymbalta	Eli Lilly	US$2.8B	SNRI

Notes: SNRI, dual inhibitor of norepinephrine and serotonin reuptake.

The transmission of pain signals, however, involves a multitude of parallel processes, and attenuation can require simultaneous intervention at multiple nodes in different pathways. Nociceptive receptors are classified as peptidergic and non-peptidergic nociceptors, and these two classes have different functions. Peptidergic nociceptors transmit the pain sensation, whereas non-peptidergic nociceptors are involved in modulating the unpleasant properties of pain [23]. These two categories of nociceptors have distinct types of channels and receptors and differentially innervate on central and peripheral terminals of sensory neurons [24]. Peptidergic and non-peptidergic nociceptors are also seperately distributed on lamina I and II of the spinal dorsal horn and differentiate the primary afferent nociceptor populations to form largely independent circuits for pain sensation in the brain [25]. Moreover, numerous receptors and molecules interact with each other for the process of nociceptive sensation. For the peripheral sensitization that is one of the most fundamental processes for pain sensation, numerous peripheral nociceptors, such as TRPV1, tyrosine kinase receptor type 1 (TrkA), acid-sensing ion channels (ASICs), interleukin receptor 1 (ILR1), purinergic receptors (P2X and P2Y), and many subtypes of voltage-gated sodium channels, should be activated by inflammatory sensitizers [23,26]. Because the pain sensation is transmitted by a multitude of signaling cascades, a large number of signaling components, such as G-protein-coupled receptors (GPCRs), ion channels, receptor tyrosine kinases (RTKs) and tumor necrosis factor receptor (TNFR) families, must be activated during the serial cascades [27]. Under these complex parallel mechanisms of pain, the blockade of a single receptor will thus probably fail to induce significant analgesic effects.

Toward multi-targeting analgesics

Given the paralleled complexity of neuronal systems as mentioned above, we believe that a highly potent and selective agent for a single target will not be as efficacious as initially anticipated and could even be detrimental owing to the presence of on-target and off-target side effects. By contrast, moderately potent multi-target drugs are more likely to show clear efficacy because of the integration of simultaneous actions on multiple targets. Most of the approved drugs for central nervous system (CNS) disorders are known to act either on multiple targets or through an obscure molecular mechanism. Table 1 shows selected examples of multi-target drugs in CNS therapeutics working either polypharmacologically or by an unknown mechanism.

Discovering and developing an agent that modulates more than one molecular target is not without precedent; targets for atypical antipsychotics, such as dopamine receptor subtype 2 (D2) and serotonin receptor subtype 2A ($5HT_2A$), are good examples [28,29]. As mentioned earlier, however, optimizing molecules simultaneously against dual or multiple targets is a laborious, time-consuming and expensive process because the selection of efficacious compounds usually depends on *in vivo* animal studies which have severely limited throughput. Therefore, an althernative form of drug screening system including modified processes and tools is required as the initial step to develop multi-target analgesics successfully.

The development of a new process based on a bait-target approach

The assumption of the multi-target approach is that several receptors or targets are involved in the disease or mechanism of disease, and molecules that bind to such receptors can have a significant efficacy without knowing the combination. Figure 1 shows the chemical space of target X. Target X can be any pain-related target, such as TRPV1, CB2 or NK1. Current drug discovery for single targets pursues potent and selective molecules relying on *in vitro* assays. However, our assumption based on multi-targeting [1,8] or on transient-binding drugs [9] is that the chemical space for the *in vivo* efficacy can be less potent and in a less selective area (dotted circle in Fig. 1) that shares chemical space with the chemical space of unknown targets Y and Z. If we can screen the broader range of the chemical space of target X (e.g. IC_{50} or EC_{50} <1 μM or 300 nM) based on the efficacy, we might be able to identify the efficacious chemical space (dotted circle) in which we can identify a lead or optimize the lead continuously within the space relying on the efficacy. Once a lead or optimized lead is identified in the efficacious chemical space, we might identify target Y and/or Z using pan screening supplied by many contract research organizations. Of course, we probably will not be able to identify Y or Z if Y or Z is a new target that the *in vitro* assay is not yet able to recognize. The coverage of pan screening, however, will expand over time.

In bait targeting, target X lures targets Y and/or Z that will synergistically interact and share the chemical space with the bait target. To implement such an approach, the first necessary condition is a screening system for efficacy with a high throughput. Current tools, however, are limited to animal models of pain that are notorious for low throughputs. Unless the throughput of the *in vivo* study is improved, the above approach would be impractical to implement. Even after improving the throughput of the *in vivo* study using various methods such as automation of the animal study [30], the concept of 'reduction' in the guidelines of animal use implemented mostly in the Institutional Animal Care and Use Committee (IACUC) review process would still impede progress. In

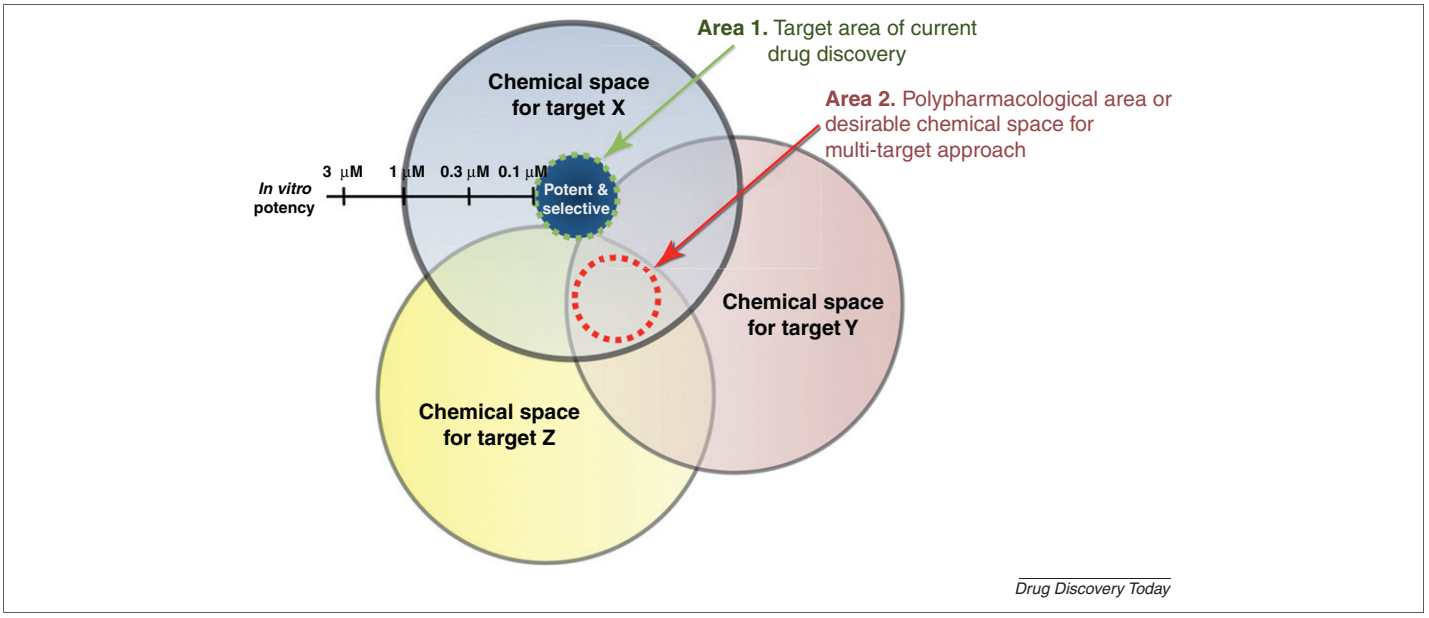

FIGURE 1

The basic concept of the bait-target approach. The chemical spaces for combinatory analgesic targets are represented as a diagram. First, target X, which is already known as a pain target, is established as a bait-target. The area that the single-target approach pursues (area 1) is limited to the specific area of target X because the highly potent and selective ligands of target X are preferentially selected. On the basis of the bait-target approach, the chemical space for the *in vivo* efficacy can be less potent and a less selective area (area 2) that shares the chemical space with the chemical space of unknown targets Y and Z. In other words, the potency against target X is progressively 'de-optimized' as it gains activity against other, unknown targets Y and Z. Finally, polypharmacological targets that are most synergistic with the initial bait-target X are spontaneously selected through the optimization process.

other words, the bait-target approach is almost impossible if relying only on *in vivo* studies using animal models. Also, the reliability and translational value of the animal models has not yet been resolved [31,32]. The solution will be an alternative tool that uses fewer animals and improves throughput yet is as appropriate for efficacy screening as *in vivo* studies. Improving the reliability of *in vivo* animal testing will be another hurdle to the actual implementation of bait-targeting.

An alternative tool for efficacy screening: an *ex vivo* system

To identify a proper methodology, we focused on the technology for displaying neuronal activity of the spinal cord, which is appropriate for demonstrating the transduction of the pain signal at the tissue level. Among the various spinal areas, the dorsal horn of the spinal cord is an essential pathway for the transmission of pain signals through the mechanisms of central sensitization and descending inhibitory and/or facilitatory modulation from the brain [33]. Central sensitization is generally defined as the potentiated synaptic plasticity of nociceptive neurons in the spinal dorsal horn by peripheral noxious stimuli, tissue damage or injury [34,35]. Homosynaptic and heterosynaptic hypersensitization of the spinothalamic tract, which originates from lamina I and II of the dorsal horn, are well known as fundamental mechanisms for the amplification of pain signals and are already widely investigated for the treatment of pain symptoms such as hyperalgesia and allodynia [36]. Numerous recent studies have also focused on spinal long-term potentiation (LTP) elicited in NK1-expressing spinoparabrachial lamina I neurons of the dorsal horn [37]. Although LTP is traditionally studied in the hippocampus as a mechanism of learning and memory, this phenomenon is similarly found in the spinal cord as a long-lasting facilitation of excitatory postsynaptic potentials (EPSP) in response to noxious input stimuli [34].

Spinal LTP is regarded as a distinct type of central sensitization in that it shares the properties of central sensitization such as immediate-onset and activity-dependent changes of synaptic plasticity, but spinal LTP is also usually differentiated from central sensitization because it is a homosynaptic and transcription-independent phenomenon [34]. For these reasons, spinal LTP in the dorsal horn is investigated together with central sensitization as a potential mechanism underlying the amplification of pain in spinal cord tissue *ex vivo* and anesthetized animal models *in vivo* [38]. These facts implicate the neuronal activity of the spinal dorsal horn as a surrogate biomarker for various pain syndromes, specifically for neuropathic pain or postoperative pain.

To monitor the activities in the dorsal horn of the spinal cord as a biomarker, we used optical imaging of brain or spinal-cord slices (*ex vivo*) that enabled the monitoring of intergrated neuronal activity from a broad area using voltage-sensitive dyes [39] (Fig. 2a). The *ex vivo* assay using the optical-imaging system is basically used for electrophysiological studies investigating the neuronal changes in broad areas of the CNS [40–42]. Because traditional electrophysiological methods such as single-cell recording or field-potential recording are suitable only for limited areas of certain synaptic pathways, optical imaging is advantageous in observing widespread signal transductions and their neuronal networks [42]. It can also be more powerful for exploring CNS diseases if the tissues related to the mechanisms of disease are clearly known, for instance the globus pallidus for Parkinson's disease [43] and the hippocampus for Alzheimer's disease [44]. The dorsal horn of the spinal cord is widely investigated by optical imaging because of its usefulness in studying pain mechanisms [45]. Given the advantages of this technique, our effort to establish an *ex vivo* system is noteworthy as the first attempt

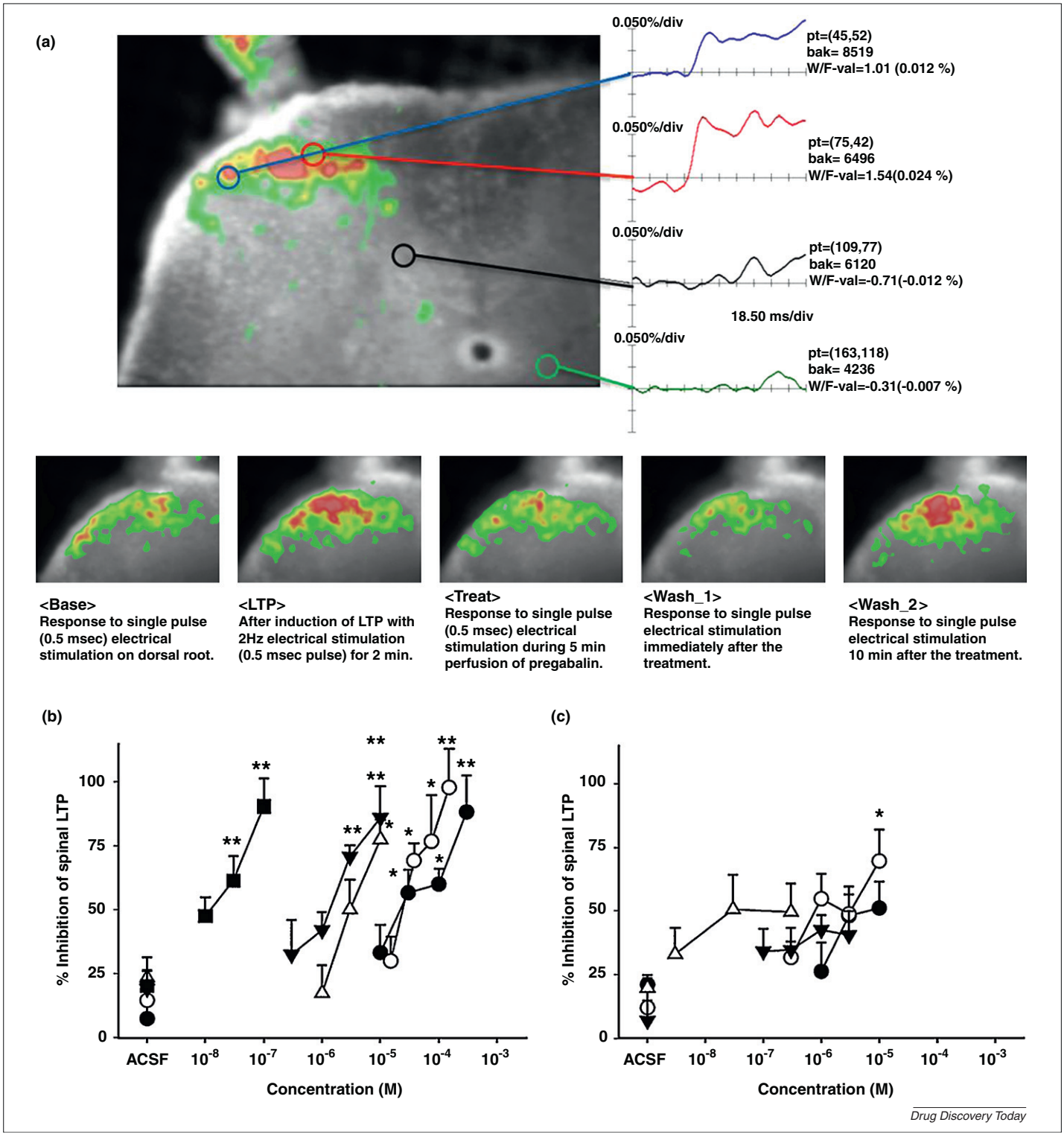

FIGURE 2

An *ex vivo* system. **(a)** The change of optical signal is monitored in the spinal dorsal horn stained with the voltage-sensitive dye (Di-4-ANEPPS). After the induction of spinal LTP, changes in optical signal are continuously obtained while the compounds are treated and washed. The optical signal is analyzed and calculated as % inhibition, the value representing the level of LTP inhibition by the compound treatment. **(b)** The test results of the positive reference compounds. Gabapentin, pregabalin, amitryptiline and mexiletine, and ω-CTx-MVIIA, the reference compounds for blocking neuropathic pain, inhibited the spinal LTP in a dose-dependent manner. ●, gabapentin ($n = 6$); ○, pregabalin ($n = 6$); △, mexiletine ($n = 6$); ▼, amitriptyline ($n = 6$); ■, ω-CTx-MVIIA ($n = 6$). **(c)** The test results of the negative reference compounds. JNJ17203212 (TRPV1 antagonist, IC_{50} = 6–7 nM), alfuzosin (adrenergic receptor antagonist, IC_{50} = 3.5 nM) and L741,626 (D2 antagonist, IC_{50} = 2.2 nM) showed no significant inhibition. ●, alfuzosin ($n = 6$); ○, ICI118,551 ($n = 6$); △, L741,626 ($n = 6$); ▼, JNJ17203212 ($n = 6$).

to apply optical imaging as an assay of efficacy screening for the development of drugs.

The detailed procedure for the *ex vivo* optical-imaging assay has been previously described in many references [42,46]. Briefly, slices of rat spinal cords (0.5 mm) with the dorsal root attached are stained with a voltage-sensitive dye (Di-4-ANEPPS), and changes in the optical signal in the dorsal horn are monitored before and after the induction of spinal LTP (Fig. 2a). At the beginning of the validation study, gabapentin, pregabalin, amitryptiline, mexiletine and ω-conotoxin MVIIA (ω-CTx-MVIIA), which are reference compounds for blocking neuropathic pain, inhibited the spinal LTP in the *ex vivo* assay in a dose-dependent manner (Fig. 2b) (note the *in vitro* potency of the most positive reference compounds is low except for ω-CTx-MVIIA). The negative reference compounds JNJ17203212 (TRPV1 antagonist, IC_{50} = 6–7 nM), alfuzosin (α1 adrenergic receptor antagonist, IC_{50} = 3.5 nM) and L741,626 (D2 antagonist, IC_{50} = 2.2 nM) elicited no significant inhibition (Fig. 2c). Interestingly, one of our negative references, ICI118,551 (β2 adrenergic antagonist, IC_{50} = 1.2 nM) showed a significant inhibition of the spinal LTP at 10 μM; this effect, however, might be non-specific considering the *in vitro* potency (IC_{50} = 1.2 nM).

In addition to the proven effectiveness of the *ex vivo* system, the throughput was approximately 1.5 compounds (n = 4) per day by one full-time employee (FTE), whereas the throughput of an *in vivo* study was less than 0.15 compound (n = 8) per day by one FTE in the rat spinal nerve ligation (SNL) model. The *ex vivo* efficacy screening therefore has at least a ten-fold higher throughput than the *in vivo* study, and we are continuing our efforts to increase the throughput.

The *in vivo* animal model: how to solve the reliability issue

Although we were able to establish a new tool for testing the efficacy of pain treatment via the *ex vivo* technology, the outcome cannot be a final indication that the tested molecule will show the expected efficacy *in vivo*. First, the efficacy *in vivo* also depends on the pharmacokinetic properties of the test molecule. If the plasma exposure of the molecules is extremely low, seeing any efficacy in the *in vivo* study will be difficult even if the *ex vivo* study indicated good efficacy. Second, the spinal cord is located inside the blood–brain barrier and seeing any efficacy in an *in vivo* study will therefore be difficult even if the test molecules show good pharmacokinetic properties unless it is penetrable to the CNS. Many other possibilities exist for not seeing *in vivo* efficacy owing to unknown reasons such as *ex vivo* efficacy by non-specific tissue toxicity or any false positives caused by measurement error or unknown mechanisms. Thus, the *in vivo* efficacy study is unavoidable even after we have the efficacy data from the *ex vivo* study.

The *in vivo* study has problems regarding throughput and reliability. Because the pain measurements in the animal model are mostly subjective, guaranteeing the reliability of the *in vivo* study is difficult unless clear objective methods are properly introduced. The tester provides nociceptive stimuli to the animal and evaluates the responses to the stimuli. The tester, therefore, should not know what the treatment is during the test procedures, otherwise the objectivity of the measurement cannot be guaranteed owing to the subjective bias of the tester. The blind test is a well-known solution, although how often it is used in the protocols of *in vivo* studies is not clear. The blind test should be introduced under complete randomization of the subject animals along with a

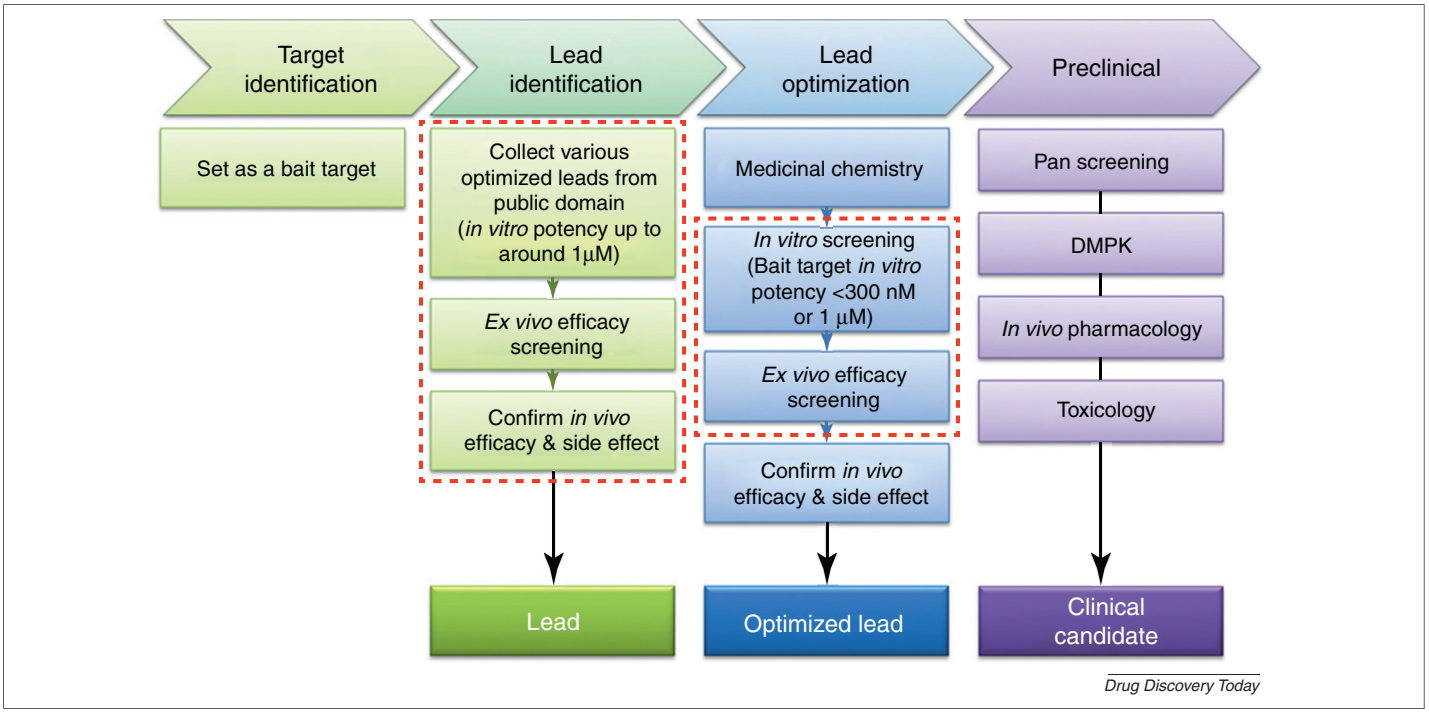

FIGURE 3

Overall scheme of multi-target drug discovery. The series of steps in the process of drug discovery for multi-target analgesics is represented as a schematic diagram. Our process of drug discovery consists of four stages: target identification, lead identification, lead optimization and a preclinical stage. These stages refer to the current process of drug discovery, but some detailed sub-stages are specified. The dotted box indicates those tweaked parts of the current drug discovery. We focused on lead identification and optimization based on efficacy, which is the ultimate goal of drug discovery.

positive control. We believe that this strategy is the best way to guarantee the objectivity and reliability of *in vivo* studies of pain.

As an *in vivo* platform for pain assessment, we used a series of conventional rat models of pain. The rat SNL model [47,48] is widely used as the industry gold standard for neuropathic and chronic pain. The level of pain induced by the SNL surgery is measured with a von Frey test that is applied for the diagnosis of mechanical allodynia in rodents [49]. Another model, the formalin-induced pain model, represents continuous and biphasic (acute and secondary) pain generated by injury to the tissue of a rat's hind paw [30,50]. This model is also widely used as a useful tool in the screening of analgesic and antihyperalgesic drugs because it encompasses most clinical pain, such as inflammatory, neurogenic and central mechanisms of nociception.

The first attempt to find a potential clinical candidate for pain: VVZ-149

Efficacy-based lead optimization

To implement the bait-target approach, we prepared a scheme of drug discovery that slightly tweaked the current platform of drug discovery (Fig. 3). The dotted box in the figure indicates those tweaked parts of the current method of drug discovery, focusing on lead identification and optimization based on efficacy, which is the ultimate goal of the drug discovery. We also tried to use information from the public domain, because many optimized leads for numerous pain targets have been published during the past decade but have mostly failed. Those optimized leads provide a chance to identify easily a new lead that already has druggable properties. The tweaked process focused on efficacy screening further to provide a lead, the *in vivo* efficacy of which has been confirmed during the lead identification. As a result, medicincal chemists can optimize the lead in the efficacious chemical space rather than in the chemical space showing only potency and selectivity. For the lead optimization, ideally we would first need to screen the molecules based on their *in vitro* potency as shown in Fig. 3, but we first started the *ex vivo* efficacy screening to identify efficacious molecules *in vivo* regardless of the *in vitro* activities and confirmed the *in vitro* potency from limited molecules based on the screening results and the molecular structure.

Bait-target approach

We set our first bait target as $GlyT_2$, a type II transporter of glycine that is expressed in limited areas of the brain stem and spinal cord [51]. $GlyT_2$ inhibitors are already being studied in many pharmaceutical companies as well-known and promising analgesic candidates because of regional specificity [52]. The inhibition of glycine reuptake from spinal synapses is known to depress the transmission of pain signals [53,54]. We identified highly (IC_{50} <100 nM, four molecules), moderately (IC_{50} <0.1–3 μM, three molecules) and weakly (IC_{50} >10 μM, one molecule) potent $GlyT_2$ antagonists from the public domain. We then synthesized the selected molecules and tested them with the *ex vivo* efficacy screening system (the endpoint was the percentage inhibition of the spinal LTP). Most of the highly potent molecules greatly inhibited the spinal LTP *ex vivo*, and, surprisingly, one of the moderately potent molecules (VVZ-006, IC_{50} ~1 μM) also showed a large inhibition of the spinal LTP. From following *in vivo* efficacy tests using the rat SNL model of neuropathic pain, the two most potent molecules (VVZ-002 and VVZ-003) showed serious side effects, such as anesthetized behavior [at 40 mg/kg, subcutaneous (s.c.)] or death, but the moderately potent molecule (VVZ-006) showed good *in vivo* efficacy without any serious side effects. No other compounds showed any *in vivo* efficacy or behavioral changes at a certain dose except VVZ-001, which is known to be analgesic.

We started lead optimization with the compound VVZ-006 for which we had clearly confirmed the *ex vivo* and *in vivo* efficacy. Many molecules have shown the appropriate efficacy in the *ex vivo* screening, but only a few had $GlyT_2$ activity (IC_{50} ~1 μM) and *in vivo* efficacy, as expected. Between May and December 2009, we designed, synthesized and screened ~100 new molecules with the *ex vivo* assay followed by *in vivo* studies. Fifty-eight compounds showed efficacy in the *ex vivo* assay based on our internal criteria (>40–60% inhibition of LTP). We tested 43 of the 58 compounds *in vivo* and found significant efficacies from 14 compounds. We also tested $GlyT_2$ activity (Axxam, Italy) on nine of the 58 compounds based on structure and *ex vivo* and *in vivo* efficacies. Six of the nine compounds showed *in vivo* efficacy, two of which (VVZ-149 and VVZ-178) also had $GlyT_2$ activity (IC_{50} <1 μM). Another compound (VVZ-237) had $GlyT_2$ activity but no *in vivo* efficacy. Four of the nine compounds showed *ex vivo* and *in vivo* efficacy but not $GlyT_2$ activity (IC_{50} >1 μM). The solubility of VVZ-178 was low so we chose to develop VVZ-149.

Target profiling

From a functional fluorescent imaging plate reader (FLIPR) assay based on membrane potential (Axxam, Italy), VVZ-149 showed moderately potent (IC_{50} = 0.86 μM) antagonist activity on $GlyT_2$ in a HEK-293 cell line stably transfected with $hGlyT_2$. VVZ-149 also showed moderate antagonistic activity on $rP2X_3$ (IC_{50} = 0.87 μM) from an in-house *in vitro* functional assay (patch-clamp recording using a CHO T-rex cell line stably transfected with $rP2X_3$). $P2X_3$, a ligand-gated ion channel, is one of the purinergic receptor subtypes. It is selectively expressed in peripheral nociceptive sensory neurons and activated by ATP released by inflammation or nociceptive stimulation [55]. Several studies have shown that functional decrease of $P2X_3$ action reduced chronic neuropathic pain by central and peripheral mechanisms [56,57].

For more extensive receptor profiling, VVZ-149 was tested on Millipore's safety panel for 78 GPCRs, eight cardiac voltage-gated ion channels, 39 kinases and two phosphatases (Millipore, USA). No agonist activity was found from a total of 127 receptors, but antagonist activities were identified mainly from $5HT_2A$ receptors (IC_{50} = 1.3 μM, $h5HT_2A$, calcium influx fluorescence assay, Millipore, USA). $5HT_2A$ is a subtype of the $5HT_2$ receptor belonging to the family of serotonin receptors as a GPCR. It is widely expressed in peripheral sensory neurons, mostly nociceptive neurons including peptidergic and non-peptidergic neurons [58], and responds to serotonin released during inflammation or by nociceptive stimulation [59]. $5HT_2A$ is also expressed in the termini of descending facilitatory projection neurons in the midbrain [60] and is involved in the amplification of pain signals in the spinal dorsal horn [61].

All three receptors ($GlyT_2$, $P2X_3$ and $5HT_2A$) are thus already known to have crucial roles in pain. VVZ-149 could therefore be a selective antagonist to multiple pain-related receptors, and our

TABLE 2

Summary of *in vivo* efficacy studies of VVZ-149

Animal models (rat)	Route	ED_{50} (mg/kg unless otherwise stated)	EC_{50} (ng/ml)	Comments	Other effective compounds
Neuropathic pain (SNL model)	p.o.	~80	~1201 (including the active metabolite)	20% HPBCD can lower ED_{50} to around 50 mg/kg	Gabapentin, pregabalin, Duloxetine, etc.
	s.c.	~20	~770 (including the active metabolite)		
Inflammatory and neuropathic pain (formalin model)	s.c.	~20	~770 (including the active metabolite)	Efficacious as much as 3 mg/kg morphine (s.c.)	Morphine or any opioid analgesics, selective $5HT_2A$ antagonist, limited efficacy (20–30%) with NSAID, $P2X_3$ antagonist, etc.
	i.pl. (50 μl)	~12.5 mM	N/A	Complete blockage (first study showing post-treatment effect)	Selective $5HT_2A$ antagonist, ketanserin

Abbreviations: SNL, spinal nerve ligation; HPBCD, hydroxypropyl beta cyclodextrin; NSAID, nonsteroidal anti-inflammatory drugs; p.o., per oral; s.c., subcutaneous; i.pl., intraplantar; N/A, not applicable.

approach is suitable to lure pain-related receptors as a promising combination with $GlyT_2$ as bait.

In vivo *efficacy*

VVZ-149 was orally active in the rat model of SNL, although we identified an active metabolite (VVZ-368) and have shown a dose-dependent efficacy ameliorating mechanical allodynia in the rat SNL model by oral and subcutaneous administration (Table 2). In oral administration in the SNL model, the ratio of VVZ-149 to VVZ-368 (the active metabolite) levels in plasma was ca. 1:9. When we combined the plasma concentration of both compounds after the oral administration, the EC_{50} was ~3 μM. Considering the degree of protein binding in rats (93%), the free-fraction EC_{50} was ~0.21 μM. After the subcutaneous administration, the ratio of plasma VVZ-149 to VVZ-368 was ca. 9:1 and, when combined, the EC_{50} in the SNL model was ~2 μM and the free-fraction EC_{50} was ~0.14 μM. These low values of the expected free-fraction EC_{50} (0.14–0.21 μM) compared with the IC_{50} (0.86–1.3 μM) for the three receptors suggest an additive or synergistic effect from antagonizing the three pain-related receptors, although we cannot rule out the possible influence from unknown receptors or mechanisms.

VVZ-149 has also shown almost complete blockage of the pain induced by intraplantar formalin (5%) in a dose-dependent manner by subcutaneous administration (Table 2). The EC_{50} was ~2 μM, and the expected free-fraction EC_{50} was ~0.14 μM, similar to the SNL model. Intraplantar injection of VVZ-149 (50 μl of 0.25–1.00% solution in saline) 10 min after the formalin injection (post-treatment) dose-dependently blocked the formalin-induced pain, suggesting that the effect of systemic administration of VVZ-149 is not simply a preventive effect. Contralateral injection of the highest dose had no effect, suggesting that the result is not a systemic effect.

A potential synergism between $5HT_2A$ and $GlyT_2$

The rationale of polypharmacology is that pharmacological effects originate from the network of various receptors and their combinations. As a result of combination, we can observe the diversity of the combination effect, such as synergy, additive effects, boosts or coalism [8]. A synergistic effect is when the integrated effect of combination is greater than the effects of the separate components.

Because $5HT_2A$ and $GlyT_2$ are involved in the modulation of pain signals in the spinal cord dorsal horn, we wanted first to confirm the combination effect of these two receptors. The projection neuron of the spinal dorsal horn receives various inputs including sensory inputs from peripheral afferents and inhibitory signals (gamma-aminobutyric acid (GABA) and glycine) from inhibitory interneurons and the medullary descending fibers ($5HT_2A$) [60]. The result of such convergent inputs to the pyramidal projection neuron in the substantia gelatinosa or the deep dorsal horn is a summation of the excitatory and inhibitory modulations. We therefore assumed that the blocking of $GlyT_2$ and $5HT_2A$ modulation would produce at least an additive effect. MDL11,939 is a selective and potent $5HT_2A$ antagonist (K_i values are 0.54 and 2.89 nM at 5 HT_2A of rabbit and rat, respectively) [62,63], and ORG25543 is a highly selective $GlyT_2$ inhibitor (IC_{50} = 16 nM) [64]. We first tested each antagonist in the rat SNL model. ORG25543 showed no significant efficacy up to 20 mg/kg (s.c.). MDL11,939 has shown a slight efficacy at 5 mg/kg (s.c.) without statistical significance. The same dose of ORG25543, however, greatly potentiated the effect of MDL11,939 in a dose-dependent manner. These findings suggest that the effect of VVZ-149 in the rat SNL model is at least partly a combination effect of $GlyT_2$ and $5HT_2A$ antagonism. The drug–drug interaction (MDL11,939 plus ORG25543) could possibly increase plasma exposure levels, but plasma concentrations did not differ between combined treatment and single treatment in our in-house HPLC analysis.

Discussion

We are currently conducting a GLP toxicological study for VVZ-149 aimed at the development of a therapeutic for post-operative pain (intravenous infusion) and to expedite the proof of clinical efficacy of the candidate. If we are successful in showing efficacy in humans, an *ex vivo* surrogate biomarker for pain will become a greater possibility, the translational value of the animal model will be demonstrated and the value of our standard *in vivo* protocol in improving objectivity and reliability will be confirmed. We cannot yet draw definitive conclusions about the value of our proposed

methods of drug discovery that differ from the current platform, such as identifying moderately potent drugs and introducing an *ex vivo* surrogate biomarker for screening purposes. The limited resources of a start-up company have prevented us from expanding our research to include other bait targets and to explore many other series of molecules. Such a multi-target mechanism, however, is not uncommon (US6538008 B1), and we clearly do not have all the tools to identify synergistic or even additive target combinations. We believe that our bait-target approach can provide a viable alternative.

To summarize, we have tried to develop multi-target drugs [8] or transient-binding drugs [9] in therapeutic areas of pain in which we believe parallel mechanisms exist. To achieve such a goal, we have focused on lead optimization based on efficacy using an *ex vivo* surrogate biomarker for pain that optically images neuronal activities in cells of the spinal dorsal horn and the spinal LTP. The throughput for screening of the *ex vivo* system was not comparable to that of the current *in vitro* cell-based functional assay but was sufficiently practical for the purpose of lead optimization, because we could begin optimization from a previously optimized lead (many optimized leads for various pain targets are in the public domain). Many molecules newly designed and synthesized by us showed good efficacies even *in vivo*. One of these molecules has shown good physicochemical properties, acceptable pharmacokinetic properties and good efficacies in rat models of neuropathic and nociceptive pain without noticeable side effects. We still have much to do to confirm the human proof-of-concept of our clinical candidate but we believe that introducing our efforts to the world of drug discovery is worthwhile. We also believe that the system of *ex vivo* efficacy screening using optically imaged spinal LTP could be used for high-throughput screening of candidate compounds enabling the identification of efficacious lead series. We will continue to make significant efforts to improve and elaborate our approach and tool (the *ex vivo* assay) hoping that they will foster a new practical paradigm in drug discovery.

Concluding remarks

The efforts of trying to identify potent and selective molecules acting at a single receptor have not been successful in the world of drug discovery despite the many advantages, such as the beauty of simple logic, screening capacity and reliance of lead optimization on *in vitro* potency. Research in systems biology, network pharmacology and polypharmacology indicates that biological function is the result of complicated interactions of networks and biological systems. The world of drug discovery has been aware of this fact for the past decade from their frequent failures to identify efficacious and safe therapeutics and from incidences of successful marketed drugs often having multiple mechanisms or binding to multiple receptors. Except for a few efforts targeting known dual receptors, no practical efforts have been made to adapt to the world of the complicated system of biological networks. We are trying to establish a new platform for the multi-target approach and have achieved some success, but we do not disregard the possibility of incidental results. We believe that the systemic approach we have taken is worth exploring. We will continue to refine and extend our methodology in hopes of seeing a new era of drug discovery in the near future, armed with a new paradigm and new tools encompassing our new understanding and knowledge in biology and related fields.

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References

1 Korcsmaros, T. (2007) How to design multi-target drugs: target search options in cellular networks. *Expert Opin. Drug Discov.* 2, 1–10
2 Sams-Dodd, F. (2005) Target-based drug discovery: is something wrong? *Drug Discov. Today* 10, 139–147
3 Csermely, P. *et al.* (2005) The efficiency of multi-target drugs: the network approach might help drug design. *Trends Pharmacol. Sci.* 26, 178–182
4 Rupniak, N.M. *et al.* (1993) Antinociceptive activity of NK1 receptor antagonists: non-specific effects of racemic RP67580. *Br. J. Pharmacol.* 110, 1607–1613
5 Garces, Y.I. *et al.* (1993) Lack of potent antinociceptive activity by substance P antagonist CP-96,345 in the rat spinal cord. *Life Sci.* 52, 353–360
6 Dionne, R.A. *et al.* (1998) The substance P receptor antagonist CP-99,994 reduces acute postoperative pain. *Clin. Pharmacol. Ther.* 64, 562–568
7 Goldstein, D.J. *et al.* (2000) Study of the analgesic effect of lanepitant in patients with osteoarthritis pain. *Clin. Pharmacol. Ther.* 67, 419–426
8 Zimmermann, G.R. *et al.* (2007) Multi-target therapeutics: when the whole is greater than the sum of the parts. *Drug Discov. Today* 12, 34–42
9 Ohlson, S. (2008) Designing transient binding drugs: a new concept for drug discovery. *Drug Discov. Today* 13, 433–439
10 Raasch, W. *et al.* (2001) Biological significance of agmatine, an endogenous ligand at imidazoline binding sites. *Br. J. Pharmacol.* 133, 755–780
11 Fairbanks, C.A. *et al.* (2000) Agmatine reverses pain induced by inflammation, neuropathy, and spinal cord injury. *Proc. Natl. Acad. Sci. U.S.A.* 97, 10584–10589
12 Berger, S.I. and Iyengar, R. (2009) Network analyses in systems pharmacology. *Bioinformatics* 25, 2466–2472
13 Kolesnikov, Y.A. *et al.* (2003) The synergistic analgesic interactions between hydrocodone and ibuprofen. *Anesth. Analg.* 97, 1721–1723
14 Morphy, R. *et al.* (2004) From magic bullets to designed multiple ligands. *Drug Discov. Today* 9, 641–651
15 Teasell, R.W. *et al.* (2010) A systematic review of pharmacologic treatments of pain after spinal cord injury. *Arch. Phys. Med. Rehabil.* 91, 816–831
16 Priest, B.T. and Kaczorowski, G.J. (2007) Blocking sodium channels to treat neuropathic pain. *Expert Opin. Ther. Targets* 11, 291–306
17 Gavva, N.R. *et al.* (2008) Pharmacological blockade of the vanilloid receptor TRPV1 elicits marked hyperthermia in humans. *Pain* 136, 202–210
18 Cui, M. *et al.* (2006) TRPV1 receptors in the CNS play a key role in broad-spectrum analgesia of TRPV1 antagonists. *J. Neurosci.* 26, 9385–9393
19 Khairatkar-Joshi, N. and Szallasi, A. (2009) TRPV1 antagonists: the challenges for therapeutic targeting. *Trends Mol. Med.* 15, 14–22
20 Lehto, S.G. *et al.* (2008) Antihyperalgesic effects of (R,E)-N-(2-hydroxy-2,3-dihydro-1H-inden-4-yl)-3-(2-(piperidin-1-yl)-4-(trifluoromethyl)phenyl)-acrylamide (AMG8562), a novel transient receptor potential vanilloid type 1 modulator that does not cause hyperthermia in rats. *J. Pharmacol. Exp. Ther.* 326, 218–229
21 Hill, R. (2000) NK1 (substance P) receptor antagonists – why are they not analgesic in humans? *Trends Pharmacol. Sci.* 21, 244–246
22 Rahn, E.J. and Hohmann, A.G. (2009) Cannabinoids as pharmacotherapies for neuropathic pain: from the bench to the bedside. *Neurotherapeutics* 6, 713–737
23 Woolf, C.J. and Ma, Q. (2007) Nociceptors – noxious stimulus detectors. *Neuron* 55, 353–364
24 Snider, W.D. and McMahon, S.B. (1998) Tackling pain at the source: new ideas about nociceptors. *Neuron* 20, 629–632
25 Braz, J.M. *et al.* (2005) Parallel ''pain'' pathways arise from subpopulations of primary afferent nociceptor. *Neuron* 47, 787–793
26 Tominaga, M. (2007) Peripheral nociceptors. In *Molecular Pain*. Springer, New York pp. 247–263
27 Hucho, T. and Levine, J.D. (2007) Signaling pathways in sensitization: toward a nociceptor cell biology. *Neuron* 55, 365–376

28 Stahl, S. (2003) Describing an atypical antipsychotic: receptor binding and its role in pathophysiology. *Prim. Care Companion J. Clin. Psychiatry* 5 (Suppl. 3), 9–13

29 Kapur, S. *et al.* (1999) Clinical and theoretical implications of 5-HT2 and D2 receptor occupancy of clozapine, risperidone, and olanzapine in schizophrenia. *Am. J. Psychiatry* 156, 286–293

30 Yaksh, T.L. *et al.* (2001) An automated flinch detecting system for use in the formalin nociceptive bioassay. *J. Appl. Physiol.* 90, 2386–2402

31 Qiu, J. (2007) Debates on translational research: a balancing act. *Lancet Neurol.* 6, 208–209

32 Remington, G. (2009) From mice to men: what can animal models tell us about the relationship between mental health and physical activity? *Ment. Health Phys. Act.* 2, 10–15

33 Latremoliere, A. and Woolf, C.J. (2009) Central sensitization: a generator of pain hypersensitivity by central neural plasticity. *J. Pain* 10, 895–926

34 Ji, R.R. *et al.* (2003) Central sensitization and LTP: do pain and memory share similar mechanisms? *Trends Neurosci.* 26, 696–705

35 Woolf, C.J. (1983) Evidence for a central component of post-injury pain hypersensitivity. *Nature* 306, 686–688

36 Woolf, C.J. (2011) Central sensitization: implications for the diagnosis and treatment of pain. *Pain* 152, 2–15

37 Mantyh, P.W. and Hunt, S.P. (2004) Setting the tone: superficial dorsal horn projection neurons regulate pain sensitivity. *Trends Neurosci.* 27, 582–584

38 Sandkuhler, J. (2007) Understanding LTP in pain pathways. *Mol. Pain* 3, 9

39 Chemla, S. and Chavane, F. (2010) Voltage-sensitive dye imaging: technique review and models. *J. Physiol. Paris* 104, 40–50

40 Fujii, R. *et al.* (2008) Imaging of molecular dynamics regulated by electrical activities in neural circuits and in synapses. *Neurosignals* 16, 260–277

41 Maeda, H. *et al.* (2007) Optical and electrophysiological recordings of corticospinal synaptic activity and its developmental change in *in vitro* rat slice co-cultures. *Neuroscience* 150, 829–840

42 Tominaga, T. *et al.* (2000) Quantification of optical signals with electrophysiological signals in neural activities of Di-4-ANEPPS stained rat hippocampal slices. *J. Neurosci. Methods* 102, 11–23

43 Martin, J.P. (1965) The globus pallidus in post-encephalitic parkinsonism. *J. Neurol. Sci.* 2, 344–365

44 Chen, Q.S. *et al.* (2000) Impairment of hippocampal long-term potentiation by Alzheimer amyloid beta-peptides. *J. Neurosci. Res.* 60, 65–72

45 Ikeda, H. *et al.* (2003) Synaptic plasticity in spinal lamina I projection neurons that mediate hyperalgesia. *Science* 299, 1237–1240

46 Ikeda, H. *et al.* (1998) Optical responses evoked by single-pulse stimulation to the dorsal root in the rat spinal dorsal horn in slice. *Brain Res.* 812, 81–90

47 Kim, S.H. and Chung, J.M. (1992) An experimental model for peripheral neuropathy produced by segmental spinal nerve ligation in the rat. *Pain* 50, 355–363

48 Lee, D.H. *et al.* (2003) The role of uninjured nerve in spinal nerve ligated rats points to an improved animal model of neuropathic pain. *Eur. J. Pain* 7, 473–479

49 Chaplan, S.R. *et al.* (1994) Quantitative assessment of tactile allodynia in the rat paw. *J. Neurosci. Methods* 53, 55–63

50 Dubuisson, D. and Dennis, S.G. (1977) The formalin test: a quantitative study of the analgesic effects of morphine, meperidine, and brain stem stimulation in rats and cats. *Pain* 4, 161–174

51 Zeilhofer, H.U. (2005) The glycinergic control of spinal pain processing. *Cell Mol. Life Sci.* 62, 2027–2035

52 Dohi, T. *et al.* (2009) Glycine transporter inhibitors as a novel drug discovery strategy for neuropathic pain. *Pharmacol. Ther.* 123, 54–79

53 Nishikawa, Y. *et al.* (2010) Blockade of glycine transporter (GlyT) 2, but not GlyT1, ameliorates dynamic and static mechanical allodynia in mice with herpetic or postherpetic pain. *J. Pharmacol. Sci.* 112, 352–360

54 Morita, K. *et al.* (2008) Spinal antiallodynia action of glycine transporter inhibitors in neuropathic pain models in mice. *J. Pharmacol. Exp. Ther.* 326, 633–645

55 Abbracchio, M.P. *et al.* (2009) Purinergic signalling in the nervous system: an overview. *Trends Neurosci.* 32, 19–29

56 Barclay, J. *et al.* (2002) Functional downregulation of P2X3 receptor subunit in rat sensory neurons reveals a significant role in chronic neuropathic and inflammatory pain. *J. Neurosci.* 22, 8139–8147

57 Wirkner, K. *et al.* (2007) P2X3 receptor involvement in pain states. *Mol. Neurobiol.* 36, 165–183

58 Van Steenwinckel, J. *et al.* (2009) The 5-HT2A receptor is mainly expressed in nociceptive sensory neurons in rat lumbar dorsal root ganglia. *Neuroscience* 161, 838–846

59 McMahon, S.B. *et al.* (2006) Inflammatory mediators and modulators of pain. In *Pain* (McMahon, S.B., ed.), pp. 49–72, Elsevier

60 Millan, M.J. (2002) Descending control of pain. *Prog. Neurobiol.* 66, 355–474

61 Okamoto, K. *et al.* (2002) 5-HT2A receptor subtype in the peripheral branch of sensory fibers is involved in the potentiation of inflammatory pain in rats. *Pain* 99, 133–143

62 Aloyo, V.J. and Harvey, J.A. (2000) Antagonist binding at 5-HT(2A) and 5-HT(2C) receptors in the rabbit: high correlation with the profile for the human receptors. *Eur. J. Pharmacol.* 406, 163–169

63 Pehek, E.A. *et al.* (2006) Evidence for the preferential involvement of 5-HT2A serotonin receptors in stress- and drug-induced dopamine release in the rat medial prefrontal cortex. *Neuropsychopharmacology* 31, 265–277

64 Caulfield, W.L. *et al.* (2001) The first potent and selective inhibitors of the glycine transporter type 2. *J. Med. Chem.* 44, 2679–2682