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Assay development for the discovery of semaphorin 3B inducing agents from natural product sources

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ABSTRACT

Semaphorins are a class of membrane-bound and secreted proteins. They have been found to regulate basic cell functions such as axonal growth cone guidance and recent studies have focused on their effect on tumor progression. Semaphorin 3B (Sema3B) particularly is a secreted protein that has been known to modulate proliferation and apoptosis, processes that are critical for tumor progression and development. In spite of its importance, there is yet no high-throughput screening assay available to detect or quantify the expression of Sema3B for natural product anticancer drug discovery purposes. Therefore, the development of a new high-throughput bioassay for the discovery of Sema3B inducing agents from natural product sources is described herein. A wide variety of pure compounds and extracts from plants and microorganisms has been found suitable for screening using this Sema3B assay to detect and quantify the effect of Sema3B inducing agents and thereby identify new selective bioactive Sema3B lead compounds for anticancer drug discovery and development. Also, this new bioassay procedure is based on a high-throughput platform using an enzyme-linked immunosorbent assay that involves the optimization of sensitivity and selectivity levels as well as accuracy, reproducibility, robustness, and cost effectiveness.

1. Introduction

Semaphorins (Semas) are membrane-bound and secreted proteins that influence axonal growth cone formation during neuronal development [1]. Also, Semas are known to regulate cell migration, axon guidance factors, immune responses, angiogenesis, and tumor progression [2,3]. Semas are classified into three classes based on their origin, namely, invertebrate (Sema 1, 2, and 5C), vertebrate (Sema 3, 4, 5A and B, 6 and 7), and virus (Sema 8A and B) [4,5]. Among them, there are the two forms of secreted (Sema 2, 3s, and 8s) and membrane-anchored or transmembrane Semas. Sema receptors are divided into two groups based on receptor binding properties: neuropilins and plexins [6–8]. Neuropilins are receptors for Sema3 and require other factors such as plexin A1–4 and plexin D1 to transduce Sema signals [9–12]. In contrast, plexins A, B and C1 are able to bind Semas to transfer signals in cells without binding neuropilins

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[9–12]. Plexin D1 binds to Semas 3E and 4A alone or with Sema 3C and neuropilin 1 or 2 [13]. The functions of Semas are pleiotropic and the signaling of Semas can impact the etiology of various human diseases [14,15]. Structural and functional information on Semas has been well documented [2–4]. Especially, the modulation for the enhancement of tumor progression and the inhibition of tumor progression have been researched based on various perspectives. Semas can also have a positive or a negative effect on cancer proliferation. Fig. 1 shows the relationship between Semas and their binding receptors related to positive and negative effects on tumor progression [13]. Also, the secondary associated receptors such as tyrosine kinases in Semas 4D, 6D, 5A and 3E and plexins in Semas 3A, 3B, and 3F are explained as inducers and inhibitors of tumor progression [8,16–23]. Sema 3B is expressed in both neuronal and non-neuronal cells. In neuronal cells, Sema 3B primarily acts as an axon repellent and at the non-neuronal tissues Sema 3B modulates cell growth, survival, migration and proliferation [24]. In addition to the literature documenting the role of Sema 3B in neuronal cells, there is a growing body of research on Sema 3B as an inhibitor of breast, hepatocellular, lung, and ovarian cancers, and cholangiocarcinoma [25,26]. Sema 3B is able to suppress major leading cancer types and its importance is gradually increasing because its induction on cancer cells is a key way to control malignant cell proliferation and to induce the process of apoptosis during tumor progression and development [26–31]. In order to discover new inducers of Sema 3B, however, optimized methods to economize time-consuming and expensive experimental procedures involved are required.

High-throughput screening (HTS) is used extensively as a core technology in drug discovery and is relevant to the life science fields of research. HTS robots are able to test up to 100,000 compounds per day [32]. HTS is available to select quickly from millions of biologically effective chemicals. The ability of reliable screening to identify active compounds provides the basic foundation for the discovery of new lead compounds to advance into drug design and development. In spite of Sema3B's importance there is not yet a high-throughput screening assay available to detect or quantify the expression of Sema 3B for natural product cancer drug discovery. For this reason, an effective screening method for the selection of active compounds or extracts is required. Therefore, this research focuses on the method development of a new high-throughput bioassay for the discovery of Sema3B inducing agents from natural product sources. The new bioassay is based on a high-throughput platform employing an enzyme-linked immunosorbent assay that involves the optimization of sensitivity and selectivity levels.

2. Materials and methods

2.1. Chemicals

RIPA buffer, *p*-nitrophenyl phosphate, protease inhibitor cocktail, sodium azide, levamisole hydrochloride, actinomycin D, 1,25-dihydroxyvitamin D3, and diethanolamine were purchased for this study (Sigma-Aldrich Co., St. Louis, MO). Other reagents used were of analytical grade. Trypsin EDTA solution (0.5%), the primary antibody of sema3B [SEMA3B(L-20)-R], and the secondary antibody (Goat anti-rabbit IgG-AP) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

2.2. Extraction and isolation of test compounds

All extraction procedures were performed as previously reported [33,34]. Briefly, the procedure employed is described. In case of the fruits of *Elaeocarpus chinensis* [33], the ground material was extracted with methanol three times at room temperature. Extracts were then suspended in a mixture of 80% methanol in H_2O, partitioned with hexane and $CHCl_3$, and then on removal of solvents, separate hexane- and $CHCl_3$-soluble extracts were obtained. The $CHCl_3$-soluble extract exhibited an IC_{50} value of 0.4 μg/mL against HT-29 cells. Then, it was subjected to a LC-MS dereplication procedure. Bioassay-guided fractionation was used to facilitate the isolation process of the active plant isolates. 5Z-7-Oxozeanol was separated initially with 1:1 MeOH/$CHCl_3$. The mixture was shaken, filtered, and the solvent

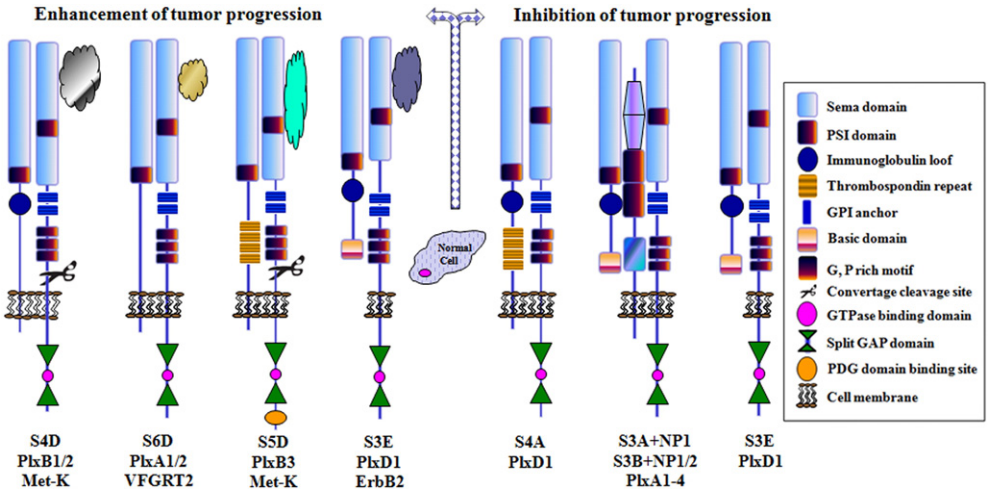

Fig. 1. Schematic views of semaphorin effects based on receptor binding. Primary and secondary bindings with semaphorins play a key role in selectivity and tumor progression [13].