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Effects of Tropomyosin-Related Kinase A Inhibitors on the Proliferation of Human Lung Cancer Cells

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Receptor tyrosine kinases (RTKs) are transmembrane receptors that phosphorylate tyrosine residues in various protein substrates.[1] Included in this small kinase subfamily are the tropomyosin-related kinases (Trks), which play a critical role in the development and maintenance of the central and peripheral nervous systems. Trk has three homologous isoforms, TrkA, TrkB, and TrkC, which are also known as NTRK1, NTRK2, and NTRK3, respectively.[2] Besides the role of maintaining and ensuring the survival of neuronal cells, a line of experimental evidence has also demonstrated that Trks are involved in malignant transformation, chemotaxis, metastasis, and survival signaling in human cancers including prostate,[3] pancreatic,[4] colon,[5] papillary thyroid,[6] and lung cancers,[7] as well as in breast carcinoma,[8] acute myelogenous leukemia,[9] and neuroblastoma.[10] Trks have thus proven to be a promising target for the development of clinical treatments of various human cancers.

Structural investigations of Trks have lagged behind the biochemical and pharmaceutical studies. Three dimensional structures of Trks have not been reported yet. A lack of structural information regarding the nature of Trk-ligand interactions has made it difficult to design the effective Trk inhibitors. Nonetheless, a great deal of effort has been devoted to the discovery of Trk inhibitors, as reviewed comprehensively by Wang *et al.*,[11] with the aim of developing novel anticancer medicines. These scientific endeavors have led to the identification of various ATP competitive inhibitors including 4-aminopyrazolylpyrimidine,[12] indenopyrrolocarbazole,[13,14] isothiazole,[15] oxindole,[16] and 2-aminothiazol[17] moieties as key structural elements. Although most of these inhibitors were discovered using high-throughput screening of chemical libraries or through the structural modifications of known inhibitor scaffolds, virtual screening of a chemical library with docking simulations was also carried out to identify new TrkA inhibitors.[18] This computer-aided drug-design approach resulted in the discovery of 2-(4-((4-hydroxyphenyl)amino)quinazolin-2-yl)phenol (**1**) and 2-(4-aminophenyl)-4-phenylquinazolin-6-amine (**2**), the structures of which are shown in Figure 1. Besides the micromolar inhibitory activities of **1** and **2** against TrkA with the associated K_d values of 4.4 and 3.3 μM, respectively, **1** and **2** were also found to have desirable physicochemical properties

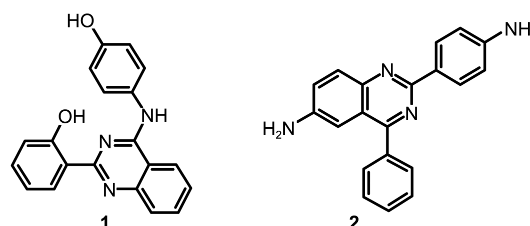

Figure 1. Chemical structures of TrkA inhibitors discovered by virtual screening.

as a drug candidate. Therefore, they deserve further investigation for the development of anticancer medicine.

In the present study, we aim to measure the anticancer activities of **1** and **2** by cell proliferation assays and thereby to address the possibility that they can serve as a new lead compound for anticancer medicine. Among the various cancers caused by Trks, we focus our interest on the lung cancer because Trks are involved in both small cell and non-small cell lung cancers, and therefore can be one of the best targets for the development of therapeutics for lung cancer.[19] More specifically, we examine whether the two TrkA inhibitors can affect the growth of human lung cancer cells in a concentration-dependent manner.

1 and **2** were purchased from InterBioScreen Ltd. (http://www.ibscreen.com) and tested for having antitumor activity against three non-small cell lung carcinoma (NSCLC) cell lines including NCI-H23 (H23), NCI-H522 (H522), and A549. All these cancer cell lines were cultured in RPMI-1640 medium containing 10% fetal bovine serum (FBS) and antibiotic-antimycotic solution. To estimate the selectivity associated with the inhibition of cancer cell proliferation, cytotoxicities of **1** and **2** were also measured using normal lung fibroblast (MRC5) cells cultured in DMEM medium supplemented with 10% heat-inactivated FBS and antibiotic-antimycotic solution.

To prepare each cell line required in the cell-based assays, cells were seeded in 96-well plates at the densities of 2×10^3, 4×10^3, 5×10^3, and 5×10^3 cells/well for A540, H23, H522, and MRC5 cell lines, respectively. After the culture for 24 h, compounds **1** and **2** were added at various concentrations and cultured further for 72 h. Cell counting kit-8 (CCK-8) was then added to each well to estimate the number

Table 1. IC_{50} (in μM) values of **1** associated with the anticellular activities for various lung cancer and normal lung fibroblast cell lines

	H23	H522	A549	MRC5
1	17.6	16.9	10.7	31.1

of viable cells in each cell line. The number of viable cells was determined by measuring the absorbance at the wavelength of 450 nm with a microplate reader. The anticancer activities of TrkA inhibitors for the four cell lines were measured in triplicate at various concentrations to obtain the dose-response curve fits. The IC_{50} values for the four cell lines were then determined from direct regression analysis using the four-parameter sigmoidal curve as implemented in the SigmaPlot program.

At first compounds **1** and **2** were tested for having a significant inhibitory activity against the proliferation of human lung cancer cell lines. **1** was found to inhibit the proliferation of A540, H23, and H522 cell lines by more than 50% at the concentration of 20 μM whereas no inhibitory activity was observed for **2** at the same concentration. Therefore, only **1** was selected for further analysis in cell proliferation assays. Table 1 lists the IC_{50} values of **1** with respect to H23, H522, A549, and MRC5 cell lines determined from direct regression analysis. Also, the dose-response behaviors of **1** measured for the four cell lines are shown in Figure 2. We see that **1** inhibits the growth of H23, H522, and A549 cell lines with the associated IC_{50} values of 17.6, 16.9, and 10.7 μM, respectively. Interestingly, the anticellular activity of **1** with respect to normal MRC5 cell line appears to be lower than those for lung cancer cell lines by a factor of 1.7-2.9. Judging from the moderate anticancer activity and selectivity, **1** deserves consideration for further development by structure-activity relationship (SAR) studies to optimize the anticancer activities.

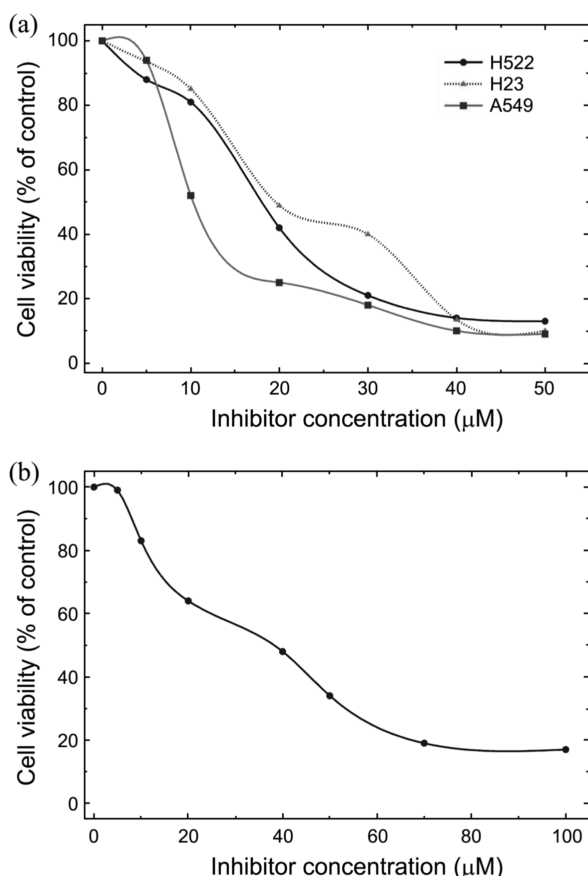

Figure 2. Dose-response curves of **1** for the inhibition of (a) H522, H23, and A549, and (b) MRC5 cell lines. Data points are represented by spline interpolations instead of regression curves to make the data points for a given cell line distinguishable from those for the others.

Because the potencies of **1** and **2** against TrkA are similar,[18] the difference in their inhibitory activities for the proliferation of cancer cells may be attributed to the difference in the permeability for cell membrane. In this regard, ClogP value has been considered a good physicochemical property to estimate the membrane permeability of organic molecules.[20] ClogP values of **1** and **2** are found to be 4.58 and 3.97, respectively, which indicates that the former is more hydrophobic than the latter. This is consistent with the higher cellular activity of **1** than **2** because the former is expected to transmit the cell membrane more easily than the latter. However, the small difference in ClogP values seems to be insufficient by itself to explain the lack of cytotoxic activity for **2**.

Recently it was shown that the intentional introduction of hydrogen bond acceptor-donor pairs in molecules can improve the membrane permeability while retaining other favorable drug-like properties.[21] Therefore we examined the presence of the intramolecular hydrogen bond in the molecular structures of **1** and **2** using quantum chemical calculations. Figure 3 shows the structures of **1** and **2** optimized with density functional calculations at B3LYP/6-31G* level of theory. We note that one of the phenolic groups of **1** establishes a hydrogen bond with the adjacent pyrimidine ring. This intramolecular hydrogen bond seems to play a role in shielding the two polar groups in hydrophobic environment, which would have an effect of increasing the hydrophobicity of **1**. On the other hand, no intramolecular hydrogen bond is observed in the optimized structure of **2** because both NH_2 groups point outward from the central pyrimidine

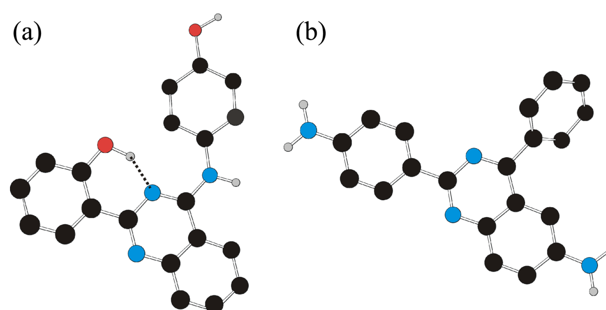

Figure 3. The optimized structures of (a) **1** and (b) **2** with quantum chemical calculations at B3LYP/6-31G* level of theory. The dotted line indicates a hydrogen bond. Hydrogen atoms attached to carbons are omitted for visual clarity.

ring (Figure 2(b)). The difference in structural features is thus consistent with the higher membrane permeability of **1** than **2**, which may culminate in the loss of anticelluar activity in going from the former to the latter. The experimental and computational results found in this study confirm that a proper shielding of hydrophilic moieties by the introduction of intramolecular hydrogen bonding interactions can be a viable means to improve the poor membrane permeability of molecules.

In conclusion, we have demonstrated that TrkA inhibitor **1** has a significant inhibitory activity for the proliferation of human lung cancer cell lines. This anticellular activity can be attributed to the improvement of its membrane permeability by the establishment of an intramolecular hydrogen bond. Because **1** exhibited lower inhibitory activity for normal MRC5 cells than for the cancer cell lines, it deserves consideration for further development by SAR studies to optimize the anticancer activities.

The results found in this study also confirm that TrkA should be an effective target for the development of therapeutics for lung cancer.

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