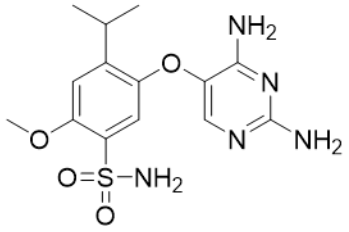
Effect on the Guinea Pig Cough Model

In this test, dextromethorphan (purchased from Shanghai Send Pharmaceutical Technology Co., Ltd.) was used as a positive control compound. A clinical control compound having the following structure was employed, and it was prepared according to the method described in WO 2008/040652 A1:



Test animals were 300-350 g Dunkin-Hartley guinea pigs (purchased from Shanghai Jiagan Biotechnology Co., Ltd). After being purchased and adaptively fed for one week, the animals were randomly divided into groups based on body weight, each test group comprising 10-15 test animals. The vehicle (a 0.5% CMCNa solution), the test compounds, the positive control compound (dextromethorphan) or the clinical control compound were orally administrated (compounds at test dosages were respectively added to a 0.5% CMCNa solution, uniformly dispersed through thorough ultrasonic treatment; the solution was vortex mixed again before administration to each animal, and the test dosage ranged from 1 mg/kg to 100 mg/kg). Administration was performed 30 minutes to 1 hour before inducing cough.

Method of inducing cough: test animals were put in a body pneumotachograph (Buxco) and allowed to stand for several minutes. After the animals were stable, 250 uM ATP was nebulized for 2 minutes or 0.6 mM histamine was nebulized for 2 minutes. After 3 minutes, 0.5 M citric acid was nebulized for 5 minutes. Starting from the nebulization of citric acid, the number of coughs of the animals in 10 minutes were recorded by employing a small animal pulmonary function detector (Buxco).

The data in Figure 1 to Figure 3 showed that compounds 2 and 66 of the present application had a significant antitussive effect, which was better than that of the clinical control compound.

Example 2. Inhibition on the P2X3-mediated current in 1321N1 Cell line Stably Transfected with P2X3

Membrane current was recorded by employing HEKA EPC-10 patch clamp amplifier and PATCHMASTER acquisition system. 1321N1 P2X3 stable transfected cells were transferred to an about 1 ml bath embedded in an inverted microscope platform, and an extracellular fluid (2 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM KCl, 155 mM NaCl, 12 mM glucose and 10 mM HEPES (pH=7.4)) was perfused by using a gravitational perfusion system. The P2X3-mediated current of a single cell was recorded in a whole cell recording mode. After formation of a gigaseal and rapture of the membrane, clamping potential was set at -60 mV. 10 μM Na2ATP was perfused for 5 seconds, and the P2X3-mediated current induced at this point was taken as a control current. The cells were then treated with a solution of compound 66 at a specific concentration (prepared with the extracellular fluid) for 5 minutes. The solution of compound 66 at this concentration and 10 μM Na2ATP were co-applied to induce a cell current (see Figure 4 for the effect of the compound on the current), and an inhibition rate relative to the control current was calculated according to the following formula:

$$\text{Inhibition Rate Relative to the Control Current} = (1-I_2/I_1)*100\%$$

wherein I_1 represents the control current, and I_2 represents the current after application of compound 66. The concentrations of the test compound 66 included 4, 12, 37, 110 and 330 nM, and at least three cells ($n\geq3$) were tested at each concentration.

The concentration of compound 66 (as the horizontal axis) was plotted against the inhibition rate relative to the control current (as the vertical axis) (see Figure 5), and the data were fitted with the Hill equation to obtain that the concentration required for compound 66 to inhibit the P2X3-mediated current induced by 10 μM Na2ATP by 50% (IC_{50}) was 26.16 nM.

Example 3. Inhibition on the P2X3-Mediated Current in *ex vivo* Cultured Rat Dorsal Root Ganglion (DRG)

Membrane current was recorded by employing HEKA EPC-10 patch clamp amplifier and PATCHMASTER acquisition system. The primary cells of rat DRG were transferred to an about 1 ml