The aim of this study was to characterize biological activity of a family of potent PI3Kδ-selective inhibitors and analyze their pharmacokinetics after intratracheal administration. **Compounds and cell lines:** Raji cells were purchased from ATCC. Compounds were synthesized by Celon Pharma Medicinal Chemistry Department. **PI3Kδ kinase assay:** PI3Kδ was purchased from Carna Biosciences and PI3Kα, PI3Kβ, and PI3Kδ from Merck Millipore. The inhibition of examined kinases was evaluated by measuring their ability to concomitantly ATP to ADP in the presence of increasing doses of compounds using ADP-Glo assay from Promega. IC₅₀ values were calculated using GraphPad Prism version 5.03. The curves were fit using a nonlinear regression model with a log (inhibitor concentration) versus response format. Kinase selectivity panel: The selectivity among protein kinases was performed on the in-house panel of enzymes (Carna Biosciences) whose activity was measured at 1 µM concentration of compounds using ADP-Glo assay. No inhibition was observed for any kinase when compounds were tested up to 10 µM concentration. **Western blot analysis:** For Western blot analysis cells were grown on 6-well plates and treated with the compounds for 1h. 15min before cell lysis Raji cells were stimulated with anti-µM antibody (5 µg/ml) [Jackson Immunoresearch]. The proteins were examined by Western blotting according to the protocols provided by the antibodies suppliers. The antibodies against pAKT [Ser473] and Akt were purchased from Cell Signaling Technology. **Metabolic stability assays:** Mouse liver microsomes were purchased from Invitrogen. Donepezil and verapamil (Sigma-Aldrich) were used as stable and unstable control, respectively. The protocol was optimized for testing only phase I metabolic enzymes.

**RESULTS**

**IN VITRO KINASE ACTIVITY**

<table>
<thead>
<tr>
<th>Cpd N.</th>
<th>IC₅₀ PI3Kδ [µM]</th>
<th>IC₅₀ PI3Kα [µM]</th>
<th>IC₅₀ PI3Kβ [µM]</th>
<th>IC₅₀ PI3Kγ [µM]</th>
<th>IC₅₀ PI3Kλ [µM]</th>
</tr>
</thead>
<tbody>
<tr>
<td>302-201</td>
<td>0.0066 12.47 1850</td>
<td>5.47 830 &gt;60 &gt;9000</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>302-215</td>
<td>0.0053 3.14 64</td>
<td>10.69 2000 11.03 2000</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>302-253</td>
<td>0.0007 1.29 1800</td>
<td>3.28 4600 27.07 &gt;3800</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Obtained results indicated that all tested inhibitors blocked PI3Kδ kinase activity at low nanomolar or sub-nanomolar concentration. The compounds had no inhibitory activity on protein kinases from our in-house selectivity panel (data not shown). Although compounds’ properties differ in terms of selectivity within PIK family, selectivity ratios remain high.

**LOSS OF AKT PHOSPHORYLATION IN RAJI CELLS**

Western blot analysis has shown that inhibitors from tested series significantly inhibited phosphorylation of AKT in Raji cell line after anti-µM stimulation. Both 302-201 and 302-215 achieved IC₅₀ values close to 50 nM, with 302-253 having slightly lower effect in this concentration. All three compounds significantly lowered pAKT levels in the concentration of 500 nM.

**METABOLIC STABILITY**

Metabolic stability assays using mouse liver microsomes showed that 302-201 had the highest clearance from tested compounds. That would be preferred when the expected route of administration was by inhalation. 302-215 and 302-253 were much more stable, having clearance values ten times lower than donepezil (3.3 and 7.3 vs 34.2 µL/min/mg, respectively).

**CONCLUSIONS**

- The concentration needed for 90% of inhibition of PI3Kδ was achieved in lungs for at least 2h for 302-201, 7h for 302-215 and 12h for 302-253, after administration of 1mg/kg dose.
- High selectivity of 302-201 translates for low risk of off-target activities: even in higher dose no significant inhibition of other kinases was detected.
- High retention of 302-253 in lung tissue enables possibilities of low-dose, once-a-day administration.
- Novel PI3Kδ inhibitors demonstrate very good absorption after intratracheal instillation, completely blocking PI3Kδ signalling in very low doses. Drug administration by inhalation would be beneficial for low systemic exposure of PI3Kδ inhibitors.