## Discovery of Sisunatovir (RV521), an Inhibitor of RSV Fusion.

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<td>Cockerill, George; ReViral Ltd, R and D&lt;br&gt;Angell, Richard; University College London School of Pharmacy&lt;br&gt;Bedernjak, Alexandre; Reviral Ltd&lt;br&gt;Fraser, Ian; CRL&lt;br&gt;Chuckowree, Irina; University of Sussex&lt;br&gt;Gascon, Jose; University of Sussex&lt;br&gt;Gilman, Morgan; Harvard Medical School, Department of Biological Chemistry and Molecular Pharmacology&lt;br&gt;Good, James; Reviral Ltd&lt;br&gt;Harland, Rachel; Reviral Ltd&lt;br&gt;Johnson, Sarah; University of Michigan Medical School&lt;br&gt;Littler, Edward; Reviral Ltd&lt;br&gt;Ludes-Meyers, John; The University of Texas at Austin&lt;br&gt;Lumley, James; GlaxoSmithKline Research and Development, Molecular Design, Data and Computational Sciences&lt;br&gt;Lunn, Graham; University of Sussex&lt;br&gt;Mathews, Neil; ReViral Ltd&lt;br&gt;McLellan, Jason; The University of Texas at Austin, Molecular Biosciences&lt;br&gt;Paradowski, Michael; Cardiff University, BIOSI&lt;br&gt;Peeples, Mark; The Ohio State University College of Medicine&lt;br&gt;Scott, Claire; Covance&lt;br&gt;Tait, Dereck; Reviral Ltd&lt;br&gt;Taylor, Geraldine; Pirbright Institute&lt;br&gt;Thom, Michelle; Pirbright Institute&lt;br&gt;Thomas, Elaine; Reviral Ltd&lt;br&gt;Villalonga Barber, Carol; University of Sussex&lt;br&gt;Ward, Simon; Cardiff University, Medicines Discovery Institute&lt;br&gt;Watterson, Daniel; Univ Queensland&lt;br&gt;Williams, Gareth; University of Sussex&lt;br&gt;Young, Paul; The University of Queensland, School of Chemistry &amp; Molecular Biosciences&lt;br&gt;Powell, Kenneth; Reviral Ltd</td>
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GRAPHICAL ABSTRACT

Discovery of Sisunatovir (RV521), an Inhibitor of RSV Fusion.


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Columbus, Ohio 43205, USA.  "The Pirbright Institute, Ash Road, Pirbright, Surrey, GU24 0NF, UK.  "Department of Molecular Biosciences, The University of Texas at Austin, Austin, TX 78712, USA.
Discovery of Sisunatovir (RV521), an Inhibitor of Respiratory Syncytial Virus Fusion.

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ABSTRACT: RV521 is an orally bioavailable inhibitor of Respiratory Syncytial Virus (RSV) fusion that was identified after a lead optimization process based upon hits that originated from a physical property directed hit profiling exercise at Reviral. This exercise encompassed collaborations with a number of contract organizations, with collaborative medicinal chemistry and virology during the optimization phase in addition to those utilized as the compound proceeded through pre-clinical and clinical evaluation. RV521 exhibited a mean IC$_{50}$ of 1.2 nM against a panel of RSV A and B laboratory strains and clinical isolates with antiviral efficacy in the Balb/C mouse model of RSV infection. Oral bioavailability in preclinical species ranged from 42% to >100%, with evidence of highly efficient penetration into lung tissue. In healthy adult human volunteers experimentally infected with RSV, a potent
antiviral effect was observed with a significant reduction in viral load and symptoms compared to placebo.

**INTRODUCTION**

Respiratory syncytial virus (RSV) is an RNA virus of the Pneumoviridae family and is responsible for a seasonal and global respiratory tract infection. Patient populations most at risk of severe disease from RSV are specifically patients with weakened immune function due to old age or immature immune and physical development. Populations at risk are premature infants, immunocompromised adults, COPD (chronic obstructive pulmonary disease), CHF (chronic heart failure) patients and the elderly. The viral infection can progress to lower respiratory tract infection (LRTI) and result in airway inflammation, bronchiolitis, pneumonia and in extreme cases, respiratory failure. Premature infants and young children with underlying heart disease or pulmonary dysplasia have the highest risk of severe disease resulting from RSV LRTI. RSV is associated with significantly more deaths and infant hospitalizations than influenza, parainfluenza, or human metapneumovirus. Additionally severe RSV infection in infancy is linked to the later development of asthma.

Despite the clear impact of the virus and associated economic burden, limited treatments exist for RSV disease. No vaccine exists
and only palivizumab, a monoclonal antibody (mAb), approved for prophylaxis and ribavirin, a broad-spectrum nucleoside antiviral drug of low efficacy have been approved. The standard of care for RSV-infected patients remains supportive, including fluids and oxygen.

RSV contains a single stranded linear RNA genome with 10 genes encoding 11 proteins. Proteins of interest to drug discovery programs have been the L (RNA polymerase), the N (nucleoprotein), M (Matrix protein), M2.1 and the F (fusion protein). This paper describes our approach to inhibitors of the fusion protein, given its fundamental importance in viral infectivity and also its conserved nature.

RESULTS AND DISCUSSION

A perspective on the position of anti-RSV drug discovery in 2018 described the progression of several direct acting antivirals for the treatment of RSV into clinical trials, targeting both fusion and replication. When this particular project was initiated in 2011, the field was far less mature, indeed only RSV604, an RSV nucleocapsid protein inhibitor had demonstrated efficacy, and that in a subanalysis of stem cell transplant patient data. Recently data has been disclosed for both presatovir and JNJ-53718678 in adult and pediatric patient populations respectively. Additionally, an inhibitor of RSV fusion, ziresovir and EDP-
an N protein inhibitor (structure not disclosed), have progressed into the human challenge model and phase 2 trials (Figure 1).

Figure 1. Clinical stage RSV fusion inhibitors.

**Hit Identification.**

We focused our attention on the fusion target, not least because in addition to its key role in the infectivity process, there were small molecule fusion templates that possessed inherent developability advantages, specifically in terms of molecular size, physical properties and potency that could lead to a hit compound worthy of progression. We focused our attention on a cyclic urea/benzimidazole scaffold previously described. This series looked to have beneficial optimization properties over other fusion archetypes known at that time. Although it was unclear why compounds had failed to progress in 2011, subsequent
summaries have appeared.\textsuperscript{16} As part of our analysis, we looked at the physical properties of compounds prepared in this series from a number of sources. A typical output is shown in Figure 2. We examined a number of patents pertaining to RSV fusion inhibitors\textsuperscript{17} - \textsuperscript{19} and identified a clear trend toward higher clogP values, with the majority of compounds possessing values greater than 3.5. The detrimental effects of higher clogP values have been documented.\textsuperscript{20, 21} Our intention was to focus our modifications to restrict our clogP figure to less than 3.5 to enhance the development potential of any candidate clinical compound.
Figure 2. A plot of numbers of compounds disclosed in three benzimidazole patents against clogP.\textsuperscript{16-18} clogP figures represent the central point of each bin covering clogP values +/- 0.25. ClogP values were calculated using Biobyte version 5.4 (http://www.biobyte.com/index.html). Patent example data was extracted from Surechembl (https://www.surechembl.org/search/). Examples with MWt outside of the range 300-700 were removed and structures were checked manually with reactive compounds removed.

In what was an increasingly complex patent space,\textsuperscript{9} structural novelty was a key consideration. Despite this complexity, we had analyzed the patent space associated with the area and identified spirocyclic systems as possessing the required novelty. We therefore focused our initial synthetic program on a novel range of spirocyclic ring containing heterocycles, by constructing spirocyclic systems anchored to a bicyclic oxindole or the corresponding tetrahydroquinolinone component of the fusion inhibitor (Figure 3).
Figure 3. Summary of spirocyclic structural scan, with cores A–G used in the initial hit finding exercise. The five and six membered lactam cores are shown in red and the proposed three to five membered spirocycles in blue.

<table>
<thead>
<tr>
<th>Cmpd</th>
<th>Core</th>
<th>RSV A2 PRA EC_{50} (nM)</th>
<th>CC_{50} (µM)</th>
<th>cLogD_{7.4}</th>
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<tr>
<td>4</td>
<td>A</td>
<td>580</td>
<td>9.94</td>
<td>2.84</td>
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<tr>
<td>5</td>
<td>B</td>
<td>240</td>
<td>13.75</td>
<td>2.39</td>
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Table 1. Spirocyclic compounds prepared during the hit finding exercise. Cores and R group as defined in Figure 3. The plaque reduction assay (PRA) was performed in Vero cells with the RSV A2 strain. CC<sub>50</sub> refers to Vero cell cytotoxicity and was assessed by MTT staining. EC<sub>50</sub> and CC<sub>50</sub> values are an average of at least 2 testing occasions. clogD<sub>7.4</sub> were calculated using Marvin software 18.16.0, 2018, ChemAxon (http://www.chemaxon.com.)

This limited structural scan, of three to five membered ring containing spiro systems, allowed the observation of a structure activity pattern, which was most apparent with an amino methylene benzimidazole scaffold (Figure 3 and Table 1). Potency against the virus was measured using an RSV A2 strain plaque reduction assay (PRA) in Vero cells with cellular cytotoxicity measured in parallel. Significant potency was observed with a 6,5 oxindole containing a spirocyclopropyl (compound 6), whilst oxindoles containing larger spirocyclic ring systems (4, 5 and 7) were less potent in this smaller oxindole system. The larger 6,6 systems, either tetrahydroquinolinone or tetrahydropyranone 8-10 were observed to be markedly less potent (Table 1). A comparative study
with unsubstituted benzimidazole series demonstrated analogous trends but with poorer activities generally (data not shown); in this less active series the corresponding spirocycllobutyl oxindole was the most potent analogue (EC$_{50}$ >7.5µM). We were encouraged that the structure activity trends were in line with our physicochemical targets; clogP and clogD values were acceptable at this stage, particularly for the smaller ring sizes.

**Table 2a.**

<table>
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<tr>
<th>Cmpd</th>
<th>Kinetic solubility$^a$ (µM)</th>
<th>P$_{app}$$^b$ (10$^{-6}$ cm s$^{-1}$)</th>
<th>PPB F$_u$ (%) rat, dog, human</th>
<th>Microsomal CL$_{int}$ (µL/min/µg protein) rat, dog, human.</th>
<th>Microsomal t½ (min) rat, dog, human</th>
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<td>6</td>
<td>155</td>
<td>12</td>
<td>10, 10, 7</td>
<td>&lt;19, &lt;28, 592</td>
<td>&gt;95, &gt;100, 5</td>
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<tr>
<td>7</td>
<td>160</td>
<td>11</td>
<td>24, 14, 17</td>
<td>&lt;31, &lt;28, 488</td>
<td>&gt;89, &gt;100, 6</td>
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**Table 2b.**

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<tr>
<th>Cmpd</th>
<th>C$_{max}$ (oral) (ng/mL)</th>
<th>Clearance (i.v.) (mL/min/kg)</th>
<th>Vd$_{ss}$ (i.v.) (L/kg)</th>
<th>F%</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>6</td>
<td>98</td>
<td>34</td>
<td>4</td>
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**Table 2a and 2b.** In vitro (2a) and in vivo (2b) pharmacokinetic data for leading compounds from the hit finding exercise. $^a$ Kinetic solubility determined by the addition of the compound in DMSO to 0.1 M PBS at pH 7.4; precipitation of the compound was measured at a range of concentrations. $^b$ Apparent permeability (P$_{app}$) in a wild type unidirectional MDCK cell assay. $^c$ The
The compound was dosed as a suspension of the amorphous solid at 1 mg/kg iv and 5 mg/kg po.

Profiling of leading compounds 6 and 7 from this exercise in an in vitro ADME and physicochemical screen showed compounds with good solubility, permeability and plasma protein binding unbound fractions (Table 2a). Clearance data was acceptable in rodent and dog microsomes although half-lives were low in human microsomes. We progressed the most potent compound 6 into a mouse in vivo pharmacokinetic study. We observed a low level of exposure (F, 4%, \(C_{\text{max}}\) 6 ng/mL) with high clearance (98 mL/min/kg). In light of this high clearance, we employed a microsomal ID study in rat and human microsomes to investigate the metabolic susceptibility of the molecule. In this study, the lead compound 6 was incubated at 10 \(\mu\)M with microsomes isolated from liver hepatocytes and sites of metabolism were subsequently proposed from mass spectral analysis of fragmentation patterns (Error! Reference source not found.). Significantly, greater turnover was observed in incubations with human microsomes than the rat system. This correlated with the previously observed microsomal clearance data (Table 2a). Most interestingly, both human specific metabolites and those observed in both species were confined structurally to the cyclopropyl oxindole fragment of the molecule.22 Although hydroxylation metabolites were proposed in the rat microsomal
incubation, a specific mono-hydroxylation metabolite was far more prevalent in human microsomes, double hydroxylation metabolites were only observed in the human incubation (see supplementary information). This focused our attention on the cyclopropyl oxindole.

![Chemical structure with reactions]

**Figure 4.** Summary of metabolite ID study for compound 6 following incubation with microsomes derived from rat and human liver cells. Most likely sites of metabolism are indicated by arrows.

This point in time represented a key moment for the project and ReViral; we had identified a potent hit compound, with pharmacokinetic properties that demonstrated some level of oral bioavailability, albeit with the aforementioned metabolic liability (Tables 2a and 2b). This series of compounds we believed possessed potential for good oral absorption properties. Additionally, a high volume of distribution was observed in this compound *in vivo*. The link between compound distribution and
clearance could not be ignored. Indeed, a positively distributing compound would be expected to attain higher concentrations in target tissue than those observed in the plasma compartment, with a potential beneficial effect for efficacy studies. The metabolite study itself provided us with a clear plan and the potential for a productive optimization process. Initial studies in the next phase of the project were directed towards blocking metabolism on the oxindole.

**Lead Optimization.**

The project employed a design, make and test framework whereby compounds were tested initially in an RSV fusion reporter-based cell-cell fusion assay (in human embryonic kidney 293T cells) before subsequent antiviral evaluation in the whole virus RSV A2 plaque assay (**Figure 5**). This fusion assay was a higher throughput assay that allowed an efficient generation of initial activity data and was importantly for us co-located with the medicinal chemistry group. We progressed compounds with fusion IC₅₀ ≤5 nM into the plaque assay which was run at distance by a collaborator. Compounds were then evaluated in physicochemical and pharmacokinetic assays on contract. Some changes took place at this point, specifically the contractor supplying in vitro ADME/physical property determination assays. At a later stage in the project, the development of a company “in house” antiviral
capability led to the use of additional cell lines. For example, HepG2 cells and Hep2 cells were added in the cell toxicity screen. Where this affects data in this paper the alternate cell lines used are indicated. Despite these changes, toxicity data for compounds retained a consistent profile in these assays.

**Figure 5.** Project flowchart depicting the design, make and test cycle for compounds prepared in the project. The design, make, test cycle allowed a progression to in vivo evaluation in the mouse BalbC model. Detailed virology and toxicity analysis prior to progression into pre-clinical evaluation followed. Generalized progression criteria are shown in italics below or alongside each assay.
Compounds satisfying our basic criteria for progression would progress into two key antiviral assays: the human airway epithelial (HAE) model,\textsuperscript{25} whereby differentiated human epithelial cell cultures were infected with a luciferase expressing RSV, and the use of the Balb/C mouse RSV infection model to study \textit{in vivo} efficacy (\textit{vide infra}).\textsuperscript{26}

Detailed analysis followed for a compound satisfying our potency and pharmacokinetic requirements; principally this comprised testing against a range of RSV A and B laboratory strains and low passage clinical isolates of RSV A and B. Consistent activity against these strains was an important requirement for any compound to progress given that RSV A and B strains co-circulate during an infectious season. Additionally, there was now a longer-term commitment to perform experiments to raise and subsequently sequence the RNA of resistant mutants to such a compound. The complexity of these later-stage virology studies restricted them to the analysis of a compound moving towards clinical evaluation and were performed as an integral part of the company’s in-house capability as it evolved.
Figure 6. Structural modification strategies employed in the lead optimization of hit 6.

The medicinal chemistry strategy employed during this phase of the project was to target three key areas of the lead compound 6 depicted in Figure 6. An initial focus on introduction of substituents into the oxindole portion of the molecule to help block metabolism was followed by targeted modifications of the chain substituting the benzimidazole and modifications to the basic aminomethylene functionality. Approaches to analogues of the aza-oxindole 7 were of lower priority within the research team, due to the lower potency of this lead compound and practical issues associated with the synthesis of the spirocyclic aza-oxindole fragment at this stage in the project. Compounds subsequently derived from lead 7 are reviewed briefly at the end of this section (Figure 7).

Spirocyclopropyl oxindole ring modification
The initial LO studies were aimed at the introduction of stabilizing groups into the oxindole portion of the lead compound as directed by our earlier metabolite ID study. Table 3 shows a selection of substituents that were successfully introduced as part of this exercise. The emphasis at this stage was focused on small halogen substituents that would represent as small a physical property change as possible whilst functioning in a metabolic blocking role. Potency could be retained for 5- and 6- chlorine substituted compounds (13 and 15 Table 3) relative to compound (6), 4- and 7- chlorine substitution had a deleterious effect (11 and 16), whereas 5- and 6-fluorine substitution produced the most potent analogues (12 and 14); with the synthetically more accessible 6-regioisomer 14 marginally more potent. Cell assay variability has to be taken into account with this data and it was seen that the fusion assay grouped analogues into tighter groups of potent and less potent modifications. In the light of the later crystal structure of RV521 bound to the trimeric pre-hairpin binding pocket (PDB code 7KQD); the binding of the oxindole component into a small well-defined pocket is evident (Figure 9, particularly panel b). One would suspect a chlorine substituent would explore the steric limitations of this pocket with direct ligand protein steric effects and conformational effects upon the ligand itself (particularly in the 7 position). 6-Fluoro
substitution direct ligand/protein interactions are harder to discern but there could be beneficial binding effects.

The evaluation of compounds in *in vitro* ADME studies (Table 4) provided new data for us at this stage. The permeability assay was changed to a bi-directional assay in MDCK cells, as opposed to the previously employed unidirectional system in the same cell line. Additionally, the solubility assay method was changed to assess thermodynamic solubility, amorphous solid compound was now administered to aqueous buffer and solubility assessed over 24 hours as opposed to an administration of compound in DMSO to buffer. The lead compounds 12 and 14 exhibited good solubility in this assay and maintained reasonable protein binding unbound fraction figures. They were profiled alongside the 7-fluorine substituted regioisomer 17. There was some variability observable in unbound fraction between species for these compounds and microsomal clearance was still significant but did seem to show an improvement for compound 12 in human microsomes over compound (6).

\[ 
\text{structural diagram of compound 12} 
\]
<table>
<thead>
<tr>
<th>Cmpd</th>
<th>Substituent</th>
<th>RSV Fusion IC$_{50}$ (nM)</th>
<th>RSV A2 Plaque EC$_{50}$ (nM)</th>
<th>CC$_{50}$ (nM)</th>
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<tr>
<td>6</td>
<td>H</td>
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<tr>
<td>11</td>
<td>4-Cl</td>
<td>11.1</td>
<td>180.7</td>
<td>5,200</td>
</tr>
<tr>
<td>12</td>
<td>5-F</td>
<td>0.7</td>
<td>4.2</td>
<td>7,600</td>
</tr>
<tr>
<td>13</td>
<td>5-Cl</td>
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<td>710</td>
</tr>
<tr>
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<td>6-F</td>
<td>0.5</td>
<td>0.8</td>
<td>3,000</td>
</tr>
<tr>
<td>15</td>
<td>6-Cl</td>
<td>1.2</td>
<td>6.7</td>
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<tr>
<td>16</td>
<td>7-Cl</td>
<td>46.7</td>
<td>2384.3</td>
<td>21,700</td>
</tr>
<tr>
<td>17</td>
<td>7-F</td>
<td>1.3</td>
<td>40.8</td>
<td>31,100</td>
</tr>
</tbody>
</table>

Table 3. Lead optimization of hit spirocycle (6): antiviral activity and cell toxicity for compounds prepared as a result of the metabolite ID study. The RSV cell fusion assay was performed in human embryonic kidney 293T cells co-transfected with RSV F protein from the RSV A2 strain or an expression plasmid encoding a transcriptional transactivator fusion protein. The plaque reduction assay (PRA) was performed in Vero cells with the RSV A2 strain. CC$_{50}$ refers to Vero cell cytotoxicity and was assessed by MTT staining. IC$_{50}$, EC$_{50}$ and CC$_{50}$ values are an average of at least 2 testing occasions.

The permeability of the 7-fluoro example 17 was low, and both of these regioisomeric compounds 14 and 17 exhibited significant efflux ratios. No advantage was observed with the 5-fluoro analogue 12 as this compound exhibited a very high clearance in human microsomes and a low to moderate permeability in the unidirectional permeability assay (CL$_{int}$: 344 µL/min/µg protein, t$_{1/2}$ = 8 minutes; P$_{app}$ = 7.3 $10^{-6}$ cm/s$^{-1}$). When evaluated in in vivo
pharmacokinetic studies (Table 4b), the 6-fluoro compound 14 was more promising with a bioavailability of 25% versus 8.8% for 17 in rats (10 mg/kg oral, 1 mg/kg iv), however clearance remained high. The disconnect observable between in vitro microsomal clearances and the in vivo clearance for all these compounds has to be taken in context with the large volumes of distribution observable for these compounds (6, 14 and 17, Table 4b). The consensus at this point was that we were indeed in a position whereby we had modified the metabolic lability of the compounds; however, the distributive properties of the compounds clouded the overall picture. A high volume of distribution, since it relates the total body concentration of drug to the drugs plasma concentration, would actually be reflected in enhanced clearance figures.\textsuperscript{23a} We progressed the optimization of these compounds with the view that retaining good distribution properties was a favorable characteristic, indeed the presystemic extraction of drugs that can be classed as lipophilic amines in organs such as the lung is documented\textsuperscript{23b}. This strategy was borne out ultimately in the properties of RV521 (20, vide infra). At this point we turned our attention to chain modifications.

\textbf{Table 4a}

<table>
<thead>
<tr>
<th>Thermo. Solubility\textsuperscript{a}</th>
<th>$P_{\text{app}}$\textsuperscript{b} (10$^{-6}$ cm s$^{-1}$) $A_2/B_2A;$ ER</th>
<th>PPB $F_u$ (%) rat, dog, human</th>
<th>Microsomal CL\textsubscript{int} (µL/min/µg protein)</th>
<th>Rat Liver Hepatocyte CL\textsubscript{int}</th>
</tr>
</thead>
</table>

\textsuperscript{a}Thermo. Solubility

\textsuperscript{b}Microsomal CL\textsubscript{int} (µL/min/µg protein)
Table 4b

<table>
<thead>
<tr>
<th>Cmpd</th>
<th>C&lt;sub&gt;max&lt;/sub&gt; (oral) (ng/mL)</th>
<th>Clearance (iv) (mL/min/kg)</th>
<th>V&lt;sub&gt;dss&lt;/sub&gt; (iv) (L/kg)</th>
<th>F%</th>
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<tbody>
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<td>6</td>
<td>6</td>
<td>98</td>
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<tr>
<td>14</td>
<td>44.2</td>
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<td>17</td>
<td>31.3</td>
<td>107.7</td>
<td>11.9</td>
<td>8.8</td>
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Table 4.  

**in vitro** (Table 4a) and **in vivo** (Table 4b) pharmacokinetic data for leading compounds from lead optimization of the oxindole.  

<sup>a</sup> Thermodynamic solubility at pH 7.4 was determined by addition of aqueous solvent to solid compound and mixed overnight. The saturated solution was filtered and quantified against a DMSO stock solution using LC-UV.  

<sup>b</sup> Permeability assay in MDCK (MDR1) cells. Direction is indicated by A<sub>2</sub>B or B<sub>2</sub>A. ER denotes efflux ratio.  

<sup>c</sup> Permeability data from a wild type unidirectional MDCK cell assay.  

<sup>d</sup> Compounds were dosed as a suspension of the amorphous solid at 1 mg/kg iv and 5 mg/kg po.
Benzimidazole chain modifications

The benzimidazole chain substituent was a major lipophilic component of the structure, in addition there was some level of evidence for this chain as a target of metabolism (Figure 4). Our strategy was to reduce the lipophilicity of this chain substituent without a significant increase in polar surface area. This approach would allow us to reduce the overall lipophilicity with the benefit of improving solubility and the metabolic profile. We would hope to whilst retain the acceptable permeability observed thus far.

![Chemical structure](image)

<table>
<thead>
<tr>
<th>Cmpd</th>
<th>R¹</th>
<th>R²</th>
<th>RSV Fusion IC₅₀ (nM)</th>
<th>RSV A2 Plaque EC₅₀ (nM)</th>
<th>CC₅₀ (nM)</th>
<th>ΔclogD₇.₄ᵃ</th>
<th>PSA (Å²)</th>
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<td>20</td>
<td>6-F</td>
<td>4-&lt;sub&gt;CF₃&lt;/sub&gt;</td>
<td>0.9</td>
<td>1.3</td>
<td>5,588</td>
<td>-0.1&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>2.3</td>
<td>&gt;25,000</td>
<td>-0.1</td>
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<td>6-F</td>
<td>4-&lt;sub&gt;O&lt;/sub&gt;</td>
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<td>2.4</td>
<td>&gt;25,000</td>
<td>-1.65</td>
<td>73.4</td>
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Table 5. Chain modification antiviral activity and calculated physicochemical properties. The plaque reduction assay (PRA) was performed in Huh-7 cells with the RSV A2 strain. \( \text{IC}_{50} \) and \( \text{EC}_{50} \) values are an average of at least 2 testing occasions. \( \text{CC}_{50} \) refers to cell cytotoxicity measured in HepG2 cells. \( ^{a} \Delta \text{clogD}_{7.4} \) values are calculated by comparison with the parent iso-pentyl substituent compounds 14 and 17 (clogD\(_{7.4} \) 2.09 for both compounds). Values were calculated using Marvin. (Marvin 16.5.16.0, 2016, ChemAxon [http://www.chemaxon.com].) \( ^{b} \) clogD\(_{7.4} \) for compound 20 is 2.00.

In general, as observed in other reported inhibitors of RSV F protein,\(^{12} \) chains with four to five atoms were acceptable in terms of potency and most interestingly, cyclic systems, such as cyclohexanol (22) or pyran (23) were also potent in the RSV plaque and fusion assays (Table 5). In the case of the pharmacokinetics of cyclohexanol 22 (Tables 6 and 8), low permeability and low clearance was observed in vitro (\( P_{\text{app}} \) MDCK-MDR1A2B/B2A; ER 0.13/2.8; 21 \( 10^{-6} \) cm s\(^{-1} \), 0.13 mL/min/mg protein in rat microsomes), and high clearance and low bioavailability in mice (82.7 mL/min/kg, F 4.4%).

Low permeabilities were observed with the methylsulfonylpropyl chain compound 18, perhaps as a result of increased polar surface
area and lowered clogP. This low permeability was reflected in poor pharmacokinetics in the mouse, no oral exposure was seen for this compound (data not shown). The introduction of either the pyran or trifluorobutyl chain provided acceptable permeabilities in addition to reduced clogPs for the pyran (23) and a polar surface area figure below 70 Å² for the trifluorobutyl substituted systems (20) and (21). Conventionally viewed as a lipophilic substituent, the trifluorobutyl system can be viewed in a different perspective. A matched pair analysis reported by Böhm et al described the contextual dependence of the fluorine as a logP lowering or raising substituent. They described how the polarity increase of the fluorine atoms overcompensated for their lipophilic nature functioning as logP lowering substituents in their study.27

<table>
<thead>
<tr>
<th>Cmpd</th>
<th>Thermo. Sol. a (µM)</th>
<th>P app b (10⁻⁶ cm s⁻¹) A5B/B2A; ER</th>
<th>PPB c F u (%)</th>
<th>Microsomal c CL int (µL/min/µg protein) r/d/h</th>
<th>Microsomal t½(min) r/d/h</th>
<th>Hepatocyte c CL int (µL/min/10⁶ cells) r/d/h</th>
<th>Hepatocyte t½ (min) r/d/h</th>
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<tr>
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<td>2,595</td>
<td>0.24/0.7; 9; 3.26</td>
<td>ND</td>
<td>3.63/5.34/3.78</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>20</td>
<td>417</td>
<td>0.5/73; 146</td>
<td>52/27/67</td>
<td>17/2.6/44</td>
<td>80/532/32</td>
<td>14.8/2.4/6.58</td>
<td>93/580/21</td>
</tr>
<tr>
<td>21</td>
<td>1178</td>
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<td>0.7/2.0/2.9</td>
<td>ND</td>
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<td>ND</td>
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<tr>
<td>22</td>
<td>313</td>
<td>0.1/2.8; 21</td>
<td>ND</td>
<td>9.4/5.02/4.6</td>
<td>147/276/300</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>
Table 6. in vitro ADME data for selected examples prepared as part of the chain modification process. \(^a\) Thermodynamic solubility at pH 7.4 was determined by addition of aqueous solvent to solid amorphous compound and mixed overnight. The saturated solution was filtered and quantified against a DMSO stock solution using LC-UV. \(^b\) Permeability assay in MDCK (MDR1) cells. Direction is indicated by A\(_2\)B or B\(_2\)A. ER denotes efflux ratio. ND denotes not determined. \(^c\) r/d/h denotes rat, dog or human derived plasma protein/microsomes/hepatocytes respectively.

The trifluorobutyl 20 and pyran 23 both exhibited acceptable permeability and microsomal half-lives (Table 6). In the case of compound 20, acceptable hepatocyte half-lives were observed in all species. Unbound protein fractions were good for both compounds and thermodynamic solubility was improved versus the iso-pentyl 14. As a result, both these compounds were progressed into in vivo pharmacokinetic evaluation, this data is shown in Table 8 vide infra. The trifluorobutyl substituted 7-fluorooxindole system 21
had shown favorable in vitro ADME properties and acceptable potency (Table 5). In this case, low oral bioavailability observed in both mouse and rat (12.9% and 14% respectively, Table 8). The high clearance in mice and rats for 21 was not predicted by the in vitro data. Compound 20 showed lower clearances in mice and dogs indicative of an improved metabolic stability in these species. High volumes of distribution were observed for both compounds 20 and 21.

Aminomethylene substituent modifications

To complete the investigation into substituent effects in this system, a range of modifications were introduced at the 6 position of the benzimidazole. Polar, non-polar, basic and acidic groups were all investigated at this position (Table 7). The potential for the interaction of this group in this class of molecule to bind to target site acidic amino acid residues has been highlighted in earlier publications.28 Our strategy at this stage was to investigate alternatives to the aminomethylene, mostly with hydrogen bonding potential that could be associated with a different pharmacokinetic absorption or stability profile. To summarize the output from this program of work, the structure activity pattern observed within our system clearly demonstrated that basic groups, such as a simple amine or the strongly basic amidine functionality, were the most potent (e.g. 25) and potency
in the fusion assay for these compounds was maintained in the plaque assay. Neutral groups such as amide and methanesulphonyl (28 and 30) demonstrated significantly poorer levels of activity whilst acidic groups were inactive in this specific scaffold (29). In the case of the 6-chlorine substituted analogue 33, reduced potency in the plaque assay was compounded by low permeability and high clearance in microsomes (table 6). In addition, there was a clear trend for simplicity of substituent, with the introduction of alkyl groups into the amidine functionality either as a spacer group or nitrogen substituent negatively impacting upon activity (26 and 27)29. The potency of the amidine containing analogue was counteracted by the low absorption characteristics of this strongly basic group:30 permeability was low in both directions (25: MDCK (MDR1) A2B:B2A \( P_{app} = 0.413:0.876 \times 10^{-6} \) cm s\(^{-1}\)). Subsequently the potential for interaction of such basic functionality with a hydrophilic region within the fusion protein was supported by the crystal structure obtained for our clinical compound (Figure 9).

\[
\text{Figure 9}
\]
<table>
<thead>
<tr>
<th>Cmpd</th>
<th>R</th>
<th>RSV Fusion IC\textsubscript{50} (nM)</th>
<th>RSV A2 Plaque EC\textsubscript{50} (nM)</th>
<th>CC\textsubscript{50} (nM)</th>
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<td>NH\textsubscript{2}</td>
<td>0.9</td>
<td>1.3</td>
<td>5,588 &gt;2000* &gt;4,000**</td>
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<td>&gt;20,000*</td>
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<tr>
<td>26</td>
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<td>640.5</td>
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<td>&gt;25,000</td>
</tr>
<tr>
<td>27</td>
<td>NH\textsubscript{2}</td>
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<td>21.1*</td>
<td>&gt;20,000*</td>
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<tr>
<td>28</td>
<td>O</td>
<td>71.0</td>
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</tr>
<tr>
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<td>O</td>
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<tr>
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<td>3,636.0</td>
<td>&gt;25,000</td>
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<tr>
<td>31</td>
<td>H</td>
<td>364.5</td>
<td>106.7*</td>
<td>&gt;20,000*</td>
</tr>
<tr>
<td>32</td>
<td>Cl</td>
<td>37.2</td>
<td>17.9</td>
<td>&gt;25000**</td>
</tr>
</tbody>
</table>

Table 7. Antiviral activity and cell cytotoxicity data for 6-substituted benzimidazoles. The plaque reduction assay (PRA) was performed in Vero cells or Hep2 cells* with the RSV A2 strain. CC\textsubscript{50} refers to cell cytotoxicity in HepG2 cells and was assessed by MTT staining. Alternatively, compound cell toxicity was performed in either Hep2 cells* or Vero cells** as described...
earlier. IC₅₀, EC₅₀ and CC₅₀ values are an average of at least 2
testing occasions.

**Aza-oxindoles and heterocyclic spiro-oxindole systems**

Introduction of nitrogen into the oxindole system had been of
interest through this optimization period, due to an expectation
of improved potency associated with the cyclopropyl variant of 7
(Figure 3) that was identified as a lead compound during the hit
finding exercise. Synthesis of useful quantities of material to
allow full profiling of compounds containing this substructure
proved difficult and these difficulties relegated the priority of
this work within the optimization program. Two exemplary aza-
oxindoles (34 and 35, Figure 7) were prepared which demonstrated
potency in plaque assays (34: EC₅₀ = 2.1 nM and 35: EC₅₀ = 4.1 nM).
Compound 35 displayed a low intrinsic permeability (MDCK (MDR1)
Pₐₚₚ A₂B:B₂A = 0.1:0.0 × 10⁻⁶ cms⁻¹) and high clearance (Table 6)
that translated to low bioavailability in the mouse (18%) with
high clearance (81 mL min⁻¹ kg⁻¹, Table 8).

Our program strategy had from the start utilized the structurally
novel approach of incorporating a spirocyclic ring system into a
bicyclic template (Figure 3). Although this annotation has focused
on the spirocyclopropyl output from this exercise, a number of
other substituted spirocyclic ring systems were prepared and evaluated in this project, and single figure nanomolar potency was achievable with heterospirocycles of a variety of ring sizes. Azetidine and pyran lead compounds 36 and 37 that arose from this work are shown in Figure 7. Subsequent patent filings by others dictated that we moved away from optimization of these compounds. The SAR and synthetic approaches to these compounds will be described elsewhere.

![Aza oxindoles and Heterospirocyclic oxindoles](image)

**Figure 7.** Spirocyclopropyl aza-oxindoles and heterocyclic spirocyclic oxindoles identified at ReViral Ltd.

**RV521 evaluation**

Compound 20 (RV521) and subsequently its related pyran analogue 23 were evaluated in rodent and dog pharmacokinetic studies (Table 8). RV521 preceded its pyran analogue 23 through the project evaluation process by several months and although 23 demonstrated similar pharmacokinetic properties, it was never in a position to
catch up and overtake the evaluation of RV521. As such the compound was held as a short term back up compound in the event of issues occurring with RV521. The remainder of the discussion will focus on the properties of RV521.

<table>
<thead>
<tr>
<th>Entry</th>
<th>Species</th>
<th>Dose; iv/po, (mg/kg)</th>
<th>t½ (po/iv, h)</th>
<th>tmax (h) po</th>
<th>Cmax po (ng/mL)</th>
<th>Cl (iv) (mL/min/kg)</th>
<th>Vdss (iv) (L/kg)</th>
<th>F (%)</th>
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<td>18.1</td>
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Table 8. *in vivo* Pharmacokinetic data for leading compounds in multiple species. ND indicates not determined; NT indicates not tested. The compound was dosed as a suspension of the amorphous solid at the doses indicated iv and po.

RV521 showed a moderate to long intravenous half-life (4.2 h in mice, 1.8 h in rats and 12.3 h in dogs) with variable clearance in
these species, approximately 53% to 61% of hepatic blood flow in mice and dogs and in contrast, approximately 200% of hepatic blood flow in rats. The compound had a large volume of distribution in all the species tested, suggestive of extensive tissue distribution. Subsequently, the relevance of this property of RV521, and other molecules in this series, was reinforced by repeat oral dosing studies in rats that showed a high lung to plasma ratio for RV521 (see supplementary information). Lung and plasma samples were taken on day seven (4 hr and 24 hr timepoints) of a repeat dosing study, QD at 43.5mg/kg. Analysis of these samples showed a 100-fold and 500-fold lung to plasma ratio. RV521 was present in the lung at a concentration of 11.8 mg/g at this dose at the 24-hour timepoint on day 7.

Oral exposure was demonstrated in rats at doses of 10 and 50 mg/kg with bioavailability at 102% and 132%, respectively: the apparent availability >100% most likely due to dose-dependent non-linear kinetics. Plasma concentrations in rats declined with a half-life of 3.1 hours and the compound was absorbed relatively slowly with a $t_{\text{max}}$ of 5.3 hours. When dogs were dosed at 3 mg/kg, the bioavailability was shown to be 44.2%, with an oral $t_{\text{eq}}$ of 12.3 hours and the compound was absorbed relatively slowly with a $t_{\text{max}}$ of 4 hours. An evaluation of metabolism in primary hepatocytes suggested that hepatic metabolism was a major pathway of elimination across all these species.
The activity of RV521 in RSV plaque assay was evaluated against a panel of laboratory strains and low passage clinical isolates of RSV A and B strains. RV521 showed potent activity, with an average IC\textsubscript{50} of 1.4 nM for RSV A (n=20) and 1.0 nM for RSV B (n=16) (see supplementary information for strains tested against and IC\textsubscript{50}s).

The inhibition of RSV by RV521 was further investigated in an in vitro primary human airway epithelial cell (HAE) infectivity model.\textsuperscript{25} The use of HAE cells provided a closer clinical context for RSV evaluation than that possible by standard plaque assay formats in continuous cell lines. Further, the use of trans-well plate inserts in this system allows for apical infection of the system with virus whilst allowing dosing the compound basolaterally, a system reflective of systemic exposure (\textbf{Figure 8}). Differentiated HAE cell cultures mimic certain aspects of infection pathology and unlike rodent models which are only semi-permissive to infection, retain replicative capacity. This model has been used previously in the testing of RSV N protein inhibitors.\textsuperscript{33} Recently the differential activities of RSV inhibitors in the human airway epithelium model has been reported.\textsuperscript{34}
Figure 8a. RV521 inhibition of RSV infection of HAE cells. Treatment of HAE cells with 10 nM RV521 2 h prior to infection (shaded bars) with recombinant luciferase-expressing RSV reduced the titre (as measured by luminescence) at 7 and 8 days post infection versus untreated controls (white bars). Data are presented as mean ± SD (n=3).

Figure 8b. Representation of the insert cell system that allows viral infection to and sampling of the apical surface of the epithelial layer and compound administration (blue arrows) to the basolateral surface of the cells via the cell nutrient (salmon).
Treatment of HAE cells with 10 nM of RV521 resulted in a significant reduction in RSV shed from the apical surface compared to untreated controls. As shown in Figure 8, 2.30 log$_{10}$ and 2.35 log$_{10}$ reductions in RSV titre compared with untreated controls were observed on day 7 and day 8 post infection, respectively. Histological analysis of the control and compound treated HAE cell cultures, at 8 days post infection, revealed that control and compound treated cells retained tight junctions, suggestive of a healthy epithelial cell layer and RV521 treatment did not elicit any apparent change in cell morphology or viability.

In order to characterize the mode of action of RV521 and to identify the viral determinants conferring reduced susceptibility to RV521, we performed an in vitro resistance selection study.

An RSV stock resistant to RV521 was generated by serial passage of RSV in increasing concentrations of RV521. We utilized Hep-2 cell cultures inoculated with RSV strain Memphis 37b in the presence of RV521 (at IC$_{50}$ concentration) or no inhibitor (control). Once an 80–90% cytopathic effect was observed, or after a maximum of 7 days, RSV was passaged into fresh HEp2 cells and the concentration of RV521 increased 2-fold. Serial passaging in the presence of increasing concentrations of RV521 was continued for ten passages. The resistant RSV stocks were used for EC$_{50}$ determinations by plaque assay, and RNA was extracted for RSV F gene sequencing. This
identified an amino acid substitution at position 489 in the RSV F protein, aspartic acid (D) to tyrosine (Y) (D489Y) with resistance to RV521. EC<sub>50</sub> values for RV521 against the RV521 resistant virus and passage control virus were 88.98 nM and 1.18 nM, respectively, representing a 76-fold shift in IC<sub>50</sub> in response to the acquisition of viral resistance to RV521.

D489Y is a mutation in the RSV F protein observed in in vitro resistance studies with other RSV fusion inhibitors<sup>35-37</sup> and cross resistance with these inhibitors of RSV fusion was observed (see supplementary information). The resistant virus remained sensitive to treatment with known RSV replication inhibitors, the N-protein inhibitor RSV604 and the nucleoside pro-drug ALS-8112. However, most importantly, RSV containing D489Y has shown to have reduced fitness, with the virus exhibiting poor growth characteristics, in culture.<sup>37, 38</sup>

We were able to obtain a crystal structure of RV521 bound to the pre-fusion conformation of the F protein (<strong>Figure 9</strong>). This structure placed the compound into the central binding region defined by subunits of the trimeric protein as described for other fusion inhibitors, notably that described for JNJ-53718678.<sup>37</sup> Key interactions observed are interactions with the three key phenylalanine residues in this trimer defined pocket. Phe140 and Phe488 from one monomer shown in gold in Figure 9a and Phe140 from
a second monomer define these interactions. The aminomethylene functionality was observed to form a potential H bond with Thr397 from the third monomer (Figure 9b). The mutable aspartic acid D489 was observed as a close neighbor of the amino group of RV521 with an N-H to C=O distance of around 2.6Å. This observation was strongly supportive of the requirement for basic group in the inhibitor.

Figure 9. Crystal structure of RV521 bound to the F-protein (PDB code 7KQD) in its trimeric pre-fusion conformation. Views are shown of the binding site created by the trimer. Amino acid residues are shown as gold, pink or green filled sticks with the backbone shown as a ribbon in the corresponding color. Each colored region corresponds to each individual monomer. a) Binding mode in a top-down view of RV521 depicting \( \pi \) bonding interactions with phenylalanine 488 and 140 of the gold monomer. b) Binding
mode showing the orientation of the aminomethylene into a hydrophilic region created by Asp489 and Thr397 with the H bonding interaction with Thr397 backbone carbonyl indicated by the dotted line. The proximity of the mutable Asp489 is depicted most clearly in panel b. Amino acid residue labels are shown in the colour of the monomer.

Final support for the progression of the compound to the clinic was provided by an \textit{in vivo} efficacy evaluation of RV521 in Balb/C mice inoculated with RSV A2 (Figure 10). A number of animal models exist for this evaluation of compounds \textit{in vivo} however they are of varying utility, most notably in terms of compound requirement.\textsuperscript{26} In our case, mice received two oral doses of RV521 (0, 1, 10 or 50 mg/kg) 2 hours pre- and 24 hours post intranasal inoculation with RSV strain A2. Animals were euthanized 5 days post RSV inoculation and lung homogenates prepared. Viral titres in lung homogenates were evaluated by plaque assay on Vero cells. Virus titres in the lungs of mice treated with 50, 10 and 1 mg/mL RV521, compared to the control group, were reduced by $1.6 \log_{10}$ (98% reduction), $1.09 \log_{10}$ (92% reduction) and $0.67 \log_{10}$ (79% reduction), respectively. Concentrations of RV521 were measured in plasma samples taken 5 hours after the first dose and immediately prior to the second dose of the compound. Concentrations of RV521 measured in the plasma were vastly in excess of EC$_{50}$ figures (see supplementary
information) except at the lowest dose. The activity of the compound observed in this model at the 1mg/kg dose has to be taken in the context of the distributive properties of the compound *vide infra*.

![Graph showing RV521 dose vs % of control](image)

**Figure 10.** RV521 reduced lung virus titres in a Balb/C mouse model of RSV infection. Balb/C mice were treated with RV521 (1, 10 or 50 mg/kg) 2 hours pre- and 24 hours post intranasal inoculation with RSV strain A2. RSV viral titres in lung homogenates were measured 5 days post infection. Data are expressed as a percentage of control group and shown as mean ± sd (n=6 animals per group).

**Synthesis Overview**

In general, compounds prepared within this program utilized the same convergent synthetic strategy with benzimidazole and
spirocyclic oxindole fragments prepared as key intermediates (Scheme 1). These two fragments were then linked via alkylation of the spirocyclic lactam.

Scheme 1. Disconnection of generalized target compound structure into chlorobenzimidazole and spirocyclic intermediates. Blue and red rings indicate variable sized spirocyclic ring systems. X = F, Cl, H. Y = H, CH₂NHBoc, Cl, CN. R is a variable alkyl chain.

The discovery phase synthesis of RV521 is representative of the route employed for the preparation of the benzimidazole intermediates (Scheme 2 and Scheme 3). This proceeded via an established sequence which started with an SₘAr displacement of the chlorine of 4-chloronitrobenzonitrile 38 by trifluorobutylamine. Nitro reduction, benzimidazole formation with the chloroacetyl chloride and acetate hydrolysis provided nitrile 40. Nitrile reduction and subsequent chlorination provided the key chloromethyl intermediate 41 (Scheme 2).¹⁸ Cyclopropanation of 6-fluorooxindole 42 was performed utilizing lithium diisopropylamide as base in an alkylation methodology with 1,2-dibromoethane to provide the spirocyclopropyl oxindole 43.
Both halves of the molecule (41 and 43) were then linked via a simple displacement reaction (Scheme 3). Removal of the BOC protecting group and hydrochloride salt formation afforded a clean preparation of the HCl salt of RV521.39

Scheme 2. Synthesis of benzimidazole intermediate for RV521.

Reagents and conditions: i) 4,4,4-trifluorobutan-1-amine, NEt₃, MeCN, rt, 16 h, 92%. ii) Pd/C, H₂, rt, 16 h, 88%. iii) acetoxyacetyl chloride, NEt₃, CH₂Cl₂, rt, 2 h, then AcOH, 80 °C, 16 h, 69%. iv) K₂CO₃, MeOH, rt, 1 h, 91%. v) Pd(OH)₂/C, HCl, H₂, MeOH/THF, rt, 16 h, then BOC anhydride, DIPEA, CH₂Cl₂, rt, 3 h, 83%. vi) MsCl, DIPEA, THF, rt, 16 h, 96%.
Scheme 3. Synthetic sequence used in the preparation of RV521.

Reagents and conditions: i) n-BuLi, diisopropylamine, 1,2-dibromoethane, -40 to 18 °C, 47%. ii) NaH, DMF, rt, 16 h, 75%. iii) HCl (2 M in Et₂O), CH₂Cl₂, rt, 6 h, then aq. NaHCO₃ workup, 63%. iv) HCl (2 M in Et₂O), CH₂Cl₂, 30 min, 81%.

Other benzimidazole precursors were prepared via an analogous sequence to that shown for the synthesis of 20 (and are detailed in the supporting information), while the spirocyclic lactams employed in the hit finding exercise could be prepared via an alkylation methodology to construct the exocyclic ring last, with a complementary approach enabling preparation of a spirocyclic 6,6-system (details in supporting information). Recently
developed cyclopropanation methodologies have been reported that enable facile one-pot access to the spirocyclopropyl oxindoles of compounds 11-17 and 35 without N-protection.41, 42 Of particular note, the nucleophilic alkylation methodology of Andreasson et al. was exploited to enable construction of the spirocyclopropyl azaoxindole 49 (Scheme 4).43 Addition of a methylene to the pyridyl acetoacetate 46 afforded 47. Subsequent cyclopropanation to 48 was followed by nitro reduction and ring closure to give the target oxindole 49. 6-Substituted benzimidazole target compounds were assembled either directly from 20 or via manipulation of the 6-cyano analogue 24 (Scheme 5).

Scheme 4. Synthesis of spirocyclic azaoxindole 49. Reagents and conditions: i) Ethyl chloroacetate, KOtBu, 0 °C to rt, 2.5 h, 80%. ii) K$_2$CO$_3$, benzyltriethylammonium chloride, paraformaldehyde, toluene, 90 °C, 1.25 h, 77%. iii) DBU, trimethylsulfoxonium chloride, CH$_3$CN, 60 °C, 45 min, 59%. iv) Pd/C, EtOH, H$_2$, 16 h. v) 1,5,7-triazabicyclo[4.4.0]dec-5-ene, 80 °C, 1 h, 90%.
Scheme 5. Synthesis of 6-aminomethylene derivatives from 6-cyanobenzimidazole 24 (Synthesis described in supplementary information). Reagents and conditions: 25: i) acetyl chloride, EtOH, rt, 16 h. ii) NH₃, methanol, rt, 48 h, 21%. 26: iii) acetyl chloride, EtOH, rt, 16 h. iv) EtNH₂, methanol, rt, 16 h, 57%. 27: v) 5% Pd/C, H₂, HCl/MeOH, 77%. vi) (Boc)₂C=N(SO₂CF₃), Et₃N, CH₂Cl₂, rt, 23 h. vii) TFA, CH₂Cl₂, rt, 19 h. 78%. 28: viii) NaOH, dioxane, reflux, 36%. 31: ix) 5% Pd/C, H₂, HCl/MeOH, 77%. x) acetyl chloride, NEt₃, CH₂Cl₂, rt, 16 h, 71%.

RSV521 Progression into Clinical Trials

RV521 progressed into the pre-clinical evaluation phase utilizing a virtual development model employing external contract synthesis, pharmacology and toxicology. In summary, in vitro broad receptor
pharmacology was acceptable and the cardio toxicology profile exhibited no QT effects in an anaesthetised guinea pig model or subsequently in any in vivo (dog telemetry) studies. Ames and rat micronucleus tests were negative. Single dose safety pharmacology showed no evidence of any toxicology and repeat dose studies in rats and dogs determined a NOAEL of 45 mg/kg/day with all events observed at higher doses found to be reversible. Rats were dosed up to 240mg/kg and dogs to 120mg/kg in single dose studies. Repeat dose studies were for 28 days, dosing once daily at 120mg/kg/day in rats and 45mg/kg/day in dogs. The dose predictions for the first study in man were based on in vitro clearance values in rat. On this basis, and assuming a one-compartment first-order absorption model, daily oral doses of 50 mg were predicted to achieve steady state trough concentrations equivalent to a 3-fold margin above the in vitro EC$_{90}$ value for total (free and bound) plasma concentration of RV521.

Subsequent evaluation of PK from the initial Phase 1 study in healthy volunteers indicated that the predictions based on rat clearance overestimated RV521 plasma exposure and doses of 200mg and 350mg bid were required to meet or exceed the target of a 3-fold margin above the in vitro EC$_{90}$ value.

The path through the initial stages of clinical development has become established for inhibitors of RSV primarily because of the
availability of an RSV challenge virus. In general, small-molecule inhibitors of RSV have progressed through the clinic according to draft EMEA guidelines.\textsuperscript{44, 45} In general, after Phase 1 single and multiple ascending dose studies in healthy volunteers to demonstrate safety, tolerability, and indicate pharmacokinetics, clinical compounds have entered a Phase 2a proof of concept study in the human challenge model. This is a trial whereby cohorts of healthy volunteers are quarantined in a specialist unit and intranasally infected with RSV strain Memphis 37b, the only GMP quality RSV virus currently available for human challenge. Viral load, as assessed by assay of nasal washes, has been shown to correlate with disease severity in the challenge model\textsuperscript{46} and treatment is delayed until volunteers are shedding virus, this model therefore formed a basis whereby the effect of antiviral agents can be studied in a well-controlled patient group.

Data from RSV fusion inhibitors studied in this clinical model has been published. Presatovir and JNJ-53718678 (1 and 2, Figure 1) were shown to effectively reduce viral load and reduce symptom scores.\textsuperscript{11, 47} Subsequently with this proof of concept in hand, inhibitors have moved into patient population studies. The initial patient studies being in pediatric populations, targeting infants up to the age of 24 months. In cases where toxicology of a compound has not supported progression in infants,\textsuperscript{48} the next stage of
evaluation has been in vulnerable adult populations such as hematopoietic cell transplant (HCT) recipients with RSV infections of the upper or lower respiratory tract (URTI or LRTI), lung transplant patients with RSV infection and adults hospitalized with RSV infections.\textsuperscript{49-51}

Significant challenges are associated with both of these clinical evaluation routes. Aside from recruitment issues, adult populations can be highly variable due to multiple clinical sites being required to ensure patient numbers. Pediatric clinical studies require a revisiting of ascending dose studies prior to any patient study and the disease course in pediatric patients is quite different to that in adults. Essentially infants struggle to control primary RSV infections and lower age trends toward slower viral clearance and a greater viral load, as measured by viral AUC.\textsuperscript{52}

RV521 progressed through phase 1 single ascending and multiple ascending dose studies in a total of 76 healthy volunteers. At doses between 175 mg and 350 mg, exposures could be achieved that provided trough levels greater than a multiple of three times the plasma protein adjusted EC\textsubscript{90}. (this was the median EC\textsubscript{90} from multiple (n=16) RSV plaque assays with RSV strain A2). No serious adverse events were reported at the doses investigated and this allowed the dosing regimen to be formulated for the progression of the compound into the human challenge model.
The Phase 2a clinical study\textsuperscript{53} was performed to establish proof-of-concept for the antiviral activity of RV521 in the treatment of RSV, using a virus challenge model per regulatory guidance. Additional aims for this study were to determine the safety, tolerability, and pharmacokinetic profile of RV521 and to perform mutation detection analyses to test for potential development of RSV resistance to RV521.\textsuperscript{45}

The study was conducted in healthy male or female adult volunteers (aged between 18 and 45 years), with low serum levels of pre-existing specific antibodies to RSV, which indicated that the subject would be sensitive to RSV infection and would likely become infected following inoculation with the challenge virus. This challenge virus was the RSV-A Memphis 37b strain and approximately $4 \log_{10}$ plaque forming units (PFU) were administered on day zero. Subjects were then randomly assigned to three equivalently sized groups and dosed with 200 mg, or 350 mg of RV521, or placebo twice daily for 5 days following confirmation of infection. Nasal wash samples were taken twice daily from day 2 through to discharge (day 12), to allow measurement of viral load via reverse transcriptase quantitative RT-qPCR and a cell-based infectivity assay.\textsuperscript{53} The occurrence and severity of symptoms were reported, and nasal mucus weighed. Pharmacokinetic assessments were based on venous blood samples, taken from day zero through to discharge. Safety assessments including electrocardiogram recordings,
physical examination and urinalysis were taken at prespecified
timepoints throughout the study. Adverse events were monitored
daily.
Figure 21. Mean viral load by nasal wash RT-qPCR (A) and by nasal wash cell-based infectivity assay (B) by day relative to dosing. Once RSV infection was confirmed treatment was initiated at 200 mg or 350 mg b.i.d. Viral load (RT-qPCR) appeared to rebound after day 8.5 in the placebo arm. However, this apparent increase resulted from the staggered randomization of subjects (the mean viral load at day 9 was calculated from just four subjects, three of whom had consistently high viral loads throughout the study). Figure reprinted under a Creative Commons Attribution 4.0 International Licence (CC BY 4.0).


Efficacy data for mean viral loads versus time are shown in Figure 11a and Figure 11b. There was a significant reduction in the mean AUC of viral load as assessed by RT-qPCR and cell-based infectivity assay with RV521 versus placebo for both doses of RV521. The percentage reduction highlighted the scale of this effect with the mean reductions in RT-qPCR AUC for RV521 at 200 mg and 350 mg relative to placebo calculated at 55.25% and 63.05%, respectively,
and 76.42% and 68.60% reduction by cell-based infectivity assay. RV521 at these doses significantly reduced the AUC of total symptom score compared with placebo and a post-hoc analysis of nasal mucus weight data showed that the mean daily nasal mucus weight was significantly lower in the RV521 treatment groups relative to placebo group. Target group mean trough levels (3 × plasma protein adjusted in vitro EC\textsubscript{90}) were achieved and were maintained at a level equivalent or greater than this for the duration of the study. The target exposure level was achieved in both dose groups with no significant differences in efficacy observed between the 200 mg and 350 mg dose groups, although it should be noted that the study was not designed to assess differences in treatment effect between the two doses. The most common treatment-emergent adverse events (nausea and diarrhea) were mild, transient and resolved. No treatment-related serious adverse events were observed. In summary, this clinical human challenge study demonstrated proof of concept for clinical efficacy of RV521.

CONCLUSION

Herein we have described the properties of and process behind the identification of 20, known as RV521 and subsequently named sisunatovir, as a potent and clinically efficacious inhibitor of RSV fusion in the human challenge model of RSV infection.

RV521 was shown to be potent against a panel of RSV A and B clinical isolates. Resistance studies confirmed the mode of action
with key mutations identified and in vivo antiviral efficacy was demonstrated in the Balb/C mouse model of RSV infection at levels comparable with those seen for other inhibitors\textsuperscript{53}. Additionally, an effective demonstration of activity was seen in human airway epithelial cells. Oral bioavailability in preclinical species ranged from 42\% to >100\%, with evidence of efficient penetration into lung tissue. Following a successful pre-clinical phase, the compound progressed through phase one healthy volunteer single and multiple dose studies. In healthy human volunteers experimentally infected with RSV, a potent antiviral effect was observed with a significant reduction in viral load and reduction in symptoms compared to placebo. In conclusion, a potent, oral RSV fusion inhibitor with the potential to treat RSV infection in infants and adults is reported.

This program to identify RV521 has been core to the progress of the ReViral Ltd. and was supported by a number of funding initiatives. A discussion of the background to the process is of some worth at this stage in the article. The plans for the project were laid by the founding team of ReViral and this led to the initial lead compound 6. The properties of this compound were sufficient, in terms of absorption and distribution particularly, to allow progression into the lead optimization phase. Identification of RV521 was supported by Reviral and allowed the
elevation of the compound into full clinical evaluation. As the compound continues to move through clinical evaluation, the company has continued to grow, building a pipeline of projects in addition to this initial flagship project.

EXPERIMENTAL

General Synthetic Protocols. All reagents were purchased from commercial suppliers and were of the highest available purity. Unless otherwise stated, chemicals were used as supplied without further purification. Anhydrous solvents were purchased from Acros (AcroSeal™) or Sigma-Aldrich (SureSeal™) and were stored under nitrogen. Petroleum ether refers to the fraction with a boiling point between 40 °C and 60 °C. All reactions were carried out under a nitrogen atmosphere, unless otherwise specified. Flash column chromatography was performed using an automated chromatography system on silica gel. $^1$H NMR and $^{13}$C NMR spectra were typically recorded on a Varian VNMRS 500 MHz spectrometer at 30 °C, using residual isotopic solvent as an internal reference. The chemical shift data for each signal are given as δ in units of parts per million (ppm). LC-MS data was recorded on a Shimadzu Prominence Series coupled to a LCMS-2020 ESI mass spectrometer. Samples were eluted through a Phenomenex Gemini C18 (pore size: 110 Å; particle size: 5 μm; dimensions: 250 mm × 4.6 mm column), using water and acetonitrile acidified by 0.1% formic acid at 1 mL/min, a run time
of 7 min unless otherwise noted and detection at 254 nm. HRMS (ESI) was recorded on either a Bruker Daltonics, Apex III, ESI source: Apollo ESI, with methanol as spray solvent or a QToF Premier, Waters machine (diode array detection: 210-500 nm, oven temperature: 40 °C, MassLynx v4.1 data handling) equipped with a Waters BEH C18 column (particle size: 1.7 μm; 2.1 × 100 mm) eluting with 0.1% formic acid in water and methanol gradient. Only molecular ions, fractions from molecular ions and other major peaks are reported as m/z ratios. The purity of final compounds was determined to be >95%, using UHPLC analyses performed on a Waters Acquity UPLC system with a Kinetex C18 column (pore size: 100 Å; particle size: 1.7 μm; dimensions: 2.1 × 50 mm).

3-Amino-4-(4,4,4-trifluorobutylamino)benzonitrile (39)

A solution of 4-chloro-3-nitro-benzonitrile (10 g, 54.78 mmol), 4,4,4-trifluorobutan-1-amine (6.91 mL, 60.25 mmol) and NEt₃ (9.16 mL, 65.73 mmol) in MeCN (130 mL) was stirred at rt for 16 h. Further 4,4,4-trifluorobutan-1-amine (0.6 mL, 5.23 mmol) and NEt₃ (0.9 mL, 6.46 mmol) were added, and the reaction was stirred at rt for 5 h. The volatiles were removed in vacuo, the residue was taken up in water, and the resultant precipitate filtered, washed with water, and dried. Trituration with petroleum ether afforded 3-nitro-4-(4,4,4-trifluorobutylamino)benzonitrile as a bright yellow solid (13.71 g, 92%), which was used without further purification. ¹H NMR (500 MHz, CDCl₃) δ 8.54 (d, J = 2.1 Hz, 1H),
8.41 (s, 1H), 7.65 (dd, \( J = 9.0, 2.1 \) Hz, 1H), 6.92 (d, \( J = 8.9 \) Hz, 1H), 3.49 (q, \( J = 6.7 \) Hz, 2H), 2.28 (ddt, \( J = 15.3, 10.6, 5.5 \) Hz, 2H), 2.05 (p, \( J = 7.5 \) Hz, 2H).

A solution of 3-nitro-4-(4,4,4-trifluorobutylamino)benzonitrile (13.71 g, 50.18 mmol) in MeOH (250 mL) was flushed with N\(_2\), charged with 10 wt.% palladium on carbon (534 mg, 0.50 mmol) and purged with hydrogen. The reaction mixture was stirred under an atmosphere of hydrogen (balloon) at rt overnight. The reaction mixture was filtered through a pad of Celite and washed with MeOH until the filtrate was colourless. The filtrate was evaporated in vacuo to give the crude product as a dark solid (12.6 g). Recrystallization from EtOAc/petroleum ether afforded 2× batches of product as a grey solid (batch 1: 2.56 g; batch 2: 2.17 g). The filtrate from the recrystallization was evaporated under reduced pressure, and purified (SiO\(_2\), 40-70% EtOAc in petroleum ether) to afford a purplish dark grey solid (6.02 g). Combined yield: 10.75 g, 88%.

\(^1\)H NMR (600 MHz, CDCl\(_3\)) \( \delta \) 7.19 (dd, \( J = 8.3, 1.8 \) Hz, 1H), 7.02 (d, \( J = 1.8 \) Hz, 1H), 6.60 (d, \( J = 8.3 \) Hz, 1H), 3.27 (t, \( J = 7.1 \) Hz, 2H), 2.24 (tdt, \( J = 16.0, 10.7, 5.7 \) Hz, 2H), 1.95 (p, \( J = 7.2 \) Hz, 2H). LCMS (ESI+) \( m/z \) 244.3 [M+H]\(^+\) at 3.28 min.

**[5-Cyano-1-(4,4,4-trifluorobutyl)benzimidazol-2-yl]methyl acetate**

Acetoxyacetyl chloride (2.21 mL, 20.56 mmol) was added dropwise via a syringe to a stirred and cooled (0 °C) solution of 3-amino-4-
(4,4,4-trifluorobutylamino)benzonitrile (5.0 g, 20.56 mmol) and NEt₃ (5.73 mL, 41.11 mmol) in anhydrous CH₂Cl₂ (75 mL). The reaction mixture was allowed to attain rt and stirred for 2 h. The volatiles were removed in vacuo, and the residue was dissolved in acetic acid (30 mL) and stirred at 80 °C for 16 h. The volatiles were removed under reduced pressure and the residue taken up in CH₂Cl₂ (200 mL), then washed with saturated solution of Na₂CO₃ (3× 100 mL), dried (MgSO₄) and the solvent removed under reduced pressure. Purification by flash chromatography (SiO₂, 40-80% EtOAc in petroleum ether) followed by trituration afforded an off-white solid (4.61 g, 69%). ¹H NMR (500 MHz, DMSO-d₆) δ 8.27 – 8.15 (m, 1H), 7.89 (dd, J = 8.4, 0.7 Hz, 1H), 7.71 (dd, J = 8.4, 1.5 Hz, 1H), 5.37 (s, 2H), 4.40 (t, J = 7.7 Hz, 2H), 2.46 – 2.32 (m, 2H), 2.09 (s, 3H), 2.05 – 1.90 (m, 2H). LCMS (ESI+) m/z 326.1 [M+H]+ at 3.26 min.

2-(Hydroxymethyl)-1-(4,4,4-trifluorobutyl)benzimidazole-5-carbonitrile (40)

A suspension of [5-cyano-1-(4,4,4-trifluorobutyl)benzimidazol-2-yl]methyl acetate (7.44 g, 22.87 mmol) and potassium carbonate (6.32 g, 45.74 mmol) in MeOH (120mL) was stirred at rt for 1 h. The reaction mixture was concentrated in vacuo, CH₂Cl₂ (200 mL) added, the mixture stirred for 10 min, then filtered, washing with CH₂Cl₂ and the filtrate concentrated in vacuo. Purification by flash chromatography (SiO₂, 0-5% MeOH in CH₂Cl₂) followed by trituration with Et₂O afforded a white solid (3.90 g). The Et₂O tritrate was concentrated in vacuo and purified by flash.
chromatography (SiO$_2$, 0-5% MeOH in CH$_2$Cl$_2$) to afford a second crop of product (2.02 g, pink solid). Combined yield: 5.92 g, 91%. $^1$H NMR (600 MHz, DMSO-$d_6$) $\delta$ 8.14 (dd, $J = 1.5$, 0.7 Hz, 1H), 7.84 (dd, $J = 8.4$, 0.7 Hz, 1H), 7.66 (dd, $J = 8.4$, 1.5 Hz, 1H), 5.80 - 5.70 (m, 1H), 4.75 (d, $J = 5.4$ Hz, 2H), 4.40 (t, $J = 7.6$ Hz, 2H), 2.44 - 2.28 (m, 2H), 2.00 (dq, $J = 12.0$, 7.8 Hz, 2H).

LCMS (ESI+) m/z 284.2 [M+H]$^+$ at 2.06 min.

tert-Butyl N-[[2-(hydroxymethyl)-1-(4,4,4-trifluorobutyl)benzimidazol-5-yl]methyl]carbamate

Palladium hydroxide on activated carbon (20 wt.%; 773 mg, 5.51mmol) was added to a solution of 2-(hydroxymethyl)-1-(4,4,4-trifluorobutyl)benzimidazole-5-carbonitrile (4.33 g, 15.29mmol) in MeOH (150 mL) and THF (77 mL). The reaction was purged with H$_2$, hydrogen chloride (0.4M solution in water; 37 mL, 15.26 mmol) added and stirred under an atmosphere of hydrogen via a balloon at rt overnight. The reaction was filtered through a pad of Celite, washing with MeOH (3× 30 mL), and the filtrate concentrated in vacuo. The residue was diluted with water (50 mL) and basified to pH ≈ 9.5 with saturated aqueous Na$_2$CO$_3$ solution. The resulting suspension was then concentrated to dryness under reduced pressure. The residue was suspended in MeCN/Et$_2$O (1:1, 2× 50 mL) and filtered. The precipitate was washed with EtOH (6× 50 mL), and the solvent removed under reduced pressure to afford a white solid.
(4 g). The precipitate was washed further with EtOH (3× 30 mL) and the solvent removed under reduced pressure. The filtrate from the MeCN/Et₂O wash was also concentrated under reduced pressure, and the combined residues purified by flash chromatography [SiO₂, eluting with CH₂Cl₂:MeOH:NH₃ (100:0:0 to 90:10:2)] afforded a further 380 mg of off-white solid. The combined material (~4.38 g) was taken to the next step without further purification.

Di-tert-butyl dicarbonate (3.26 g, 14.92 mmol) was added portionwise to a solution of crude 5-(aminomethyl)-1-(4,4,4-trifluorobutyl)benzimidazol-2-yl]methanol (4.38 g, 15.25 mmol) and N,N-diisopropylethylamine (4.82 mL, 27.67 mmol) in CH₂Cl₂ (150 mL) under N₂ and the reaction stirred at rt for 3 h. The volatiles were removed under reduced pressure, the crude suspended in petroleum ether:EtOAc (~8:2; 50 mL) and filtered. The precipitate was washed with petroleum ether:EtOAc (~8:2; 3× 50 mL), and the filtrate discarded. The precipitate was washed with water H₂O (2× 50 mL) and dried in a vacuum oven. The solid was then triturated with MeOH (30 mL), and dried to afford a white solid (4.93 g, 83%).

¹H NMR (500 MHz, DMSO-d₆) δ 7.52 (d, J = 8.4 Hz, 1H), 7.44 (s, 1H), 7.39-7.32 (m, 1H), 7.14 (d, J = 8.3 Hz, 1H), 5.60 (t, J = 5.7 Hz, 1H), 4.70 (d, J = 5.5 Hz, 2H), 4.33 (t, J = 7.4 Hz, 2H), 4.20 (d, J = 6.2 Hz, 2H), 2.43 - 2.26 (m, 3H), 2.00 (p, J = 7.6 Hz, 2H), 1.39 (s, 9H). LCMS (ESI+) m/z 388.1 [M+H]+ at 0.57 min.
tert-Butyl N-[[2-(chloromethyl)-1-(4,4,4-trifluorobutyl)benzimidazol-5-yl]methyl]carbamate (41)

Methanesulfonyl chloride (193 µL, 2.50 mmol) was added dropwise via syringe to a cooled (0 °C) solution of tert-butyl N-[[2-(hydroxymethyl)-1-(4,4,4-trifluorobutyl)benzimidazol-5-yl]methyl]carbamate (0.88 g, 2.27 mmol) and N,N-diisopropylethylamine (1.19 mL, 6.81 mmol) in THF (15 mL). The reaction mixture was allowed to warm to rt and stirred overnight. The reaction was quenched with water (10 mL) and the volatiles removed under reduced pressure. The residue was diluted with water (25 mL) and extracted with EtOAc (2× 25 mL). The combined organic extracts were washed with saturated citric acid solution, then saturated aqueous NaHCO$_3$ solution, dried (MgSO$_4$) and the solvent removed under reduced pressure to afford a light orange oil (880 mg, 96%) which was used without further purification. $^1$H NMR (500 MHz, DMSO-$d_6$) $\delta$ 7.58 (d, $J$ = 8.3 Hz, 1H), 7.48 (s, 1H), 7.38 (t, $J$ = 6.4 Hz, 1H), 7.21 (d, $J$ = 8.3 Hz, 1H), 5.06 (s, 2H), 4.36 (t, $J$ = 7.6 Hz, 2H), 4.21 (d, $J$ = 6.3 Hz, 2H), 2.38 (ddt, $J$ = 14.8, 8.6, 3.2 Hz, 2H), 2.06 – 1.98 (m, 2H), 1.39 (s, 9H). LCMS (ESI+) $m/z$ 406.0 [M+H]$^+$ at 3.74 min. HRMS (ESI): $m/z$ [M+H]$^+$ calcd. for C$_{18}$H$_{24}$ClF$_3$N$_3$O$_2$ 406.1504, found 406.1503.

6′ Fluoro-1′,2′-dihydrospiro[cyclopropane-1,3′-indole]-2′-one (43)
A solution of 6-fluoroindolin-2-one (1.63 g, 10.79 mmol) and
diisopropylamine (3.17 mL, 22.65 mmol) in anhydrous
tetrahydrofuran (18 mL) under N₂ was cooled down at -40 °C using
a dry ice/acetonitrile bath. n-Butyllithium solution (2.5 M in
hexanes; 17.26 mL, 43.14 mmol) was added dropwise via syringe
over 15 min. Upon complete addition, the dry ice/acetonitrile
bath was changed for an ice bath and the reaction allowed to
warm to 0 °C. A solution of 1,2-dibromoethane (2.79 mL, 32.35
mmol) in THF (7 mL) was then added dropwise and the reaction
mixture stirred at rt overnight. The reaction was quenched with
saturated aq. NH₄Cl (60 mL) and diluted with EtOAc (100 mL) and
the phases separated. The aqueous layer was extracted with EtOAc
(50 mL) and the combined organic layers with brine (2× 75 mL),
dried (MgSO₄) and the solvent removed under reduced pressure.
The crude was purified by flash chromatography (SiO₂, 0-100%
EtOAc in petroleum ether) to afford an orange solid (902 mg,
47%). ¹H NMR (500 MHz, CDCl₃) δ 8.49 (s, 1H), 6.79 - 6.67 (m, 3H),
1.76 (q, J = 3.9 Hz, 2H), 1.53 (q, J = 4.2 Hz, 2H). LCMS (ESI+)

\[ m/z \text{ 178.2 [M+H]⁺ at 1.31 min.} \]

tert-butyl

\[ \text{N-([2-((6'-fluoro-2'-oxo-1',2'-dihydrospiro[cyclopropane-1,3'-indole]
-1'-yl)methyl]-1-(4,4,4-trifluorobutyl)-1H-1,3-benzodiazol-5-yl]methyl)carbamate (44)} \]
Sodium hydride (60% dispersion in mineral oil; 0.11 mL, 2.75 mmol) was added in one portion to cooled (0 °C) solution of 6'-fluoro-1,2-spiro[cyclopropane-1,3'-indole]-2'-one (487 mg, 2.75 mmol) in DMF (10 mL) under nitrogen. The reaction was allowed to attain rt and stirred for 1 h. A solution of crude N-[2-(chloromethyl)-1-(4,4,4-trifluorobutyl)-1H-1,3-benzodiazo[5-yl]methyl]carbamate (1.01 g, 2.48 mmol) in DMF (4 mL) was then added by dropwise addition over 5 minutes, and the reaction mixture stirred at rt for 16 h. The reaction was quenched with water (100 mL) and extracted with EtOAc (3× 75 mL). The combined organics were washed with water (100 mL), brine (120 mL), then dried (MgSO₄) and evaporated under reduced pressure. The crude oil was purified by flash chromatography (SiO₂, 0-100% EtOAc in petroleum ether) followed by trituration with petroleum ether/EtOAc (4:1; 10 mL) to afford a yellow solid (1030 mg, 75%). ¹H NMR (500 MHz, CDCl₃) δ 7.74 – 7.70 (m, 1H), 7.34 (dd, J = 9.1, 2.1 Hz, 1H), 6.77 – 6.66 (m, 2H), 5.30 – 5.26 (m, 2H), 4.91 (s, 1H), 4.45 (d, 2H), 4.33 (t, J = 7.9 Hz, 2H), 2.20 – 2.06 (m, 2H), 1.91 – 1.81 (m, 2H), 1.81 – 1.72 (m, 2H), 1.63 (d, J = 1.0 Hz, 1H), 1.59 – 1.53 (m, 2H), 1.48 (s, 9H). ¹³C NMR (100 MHz, CDCl₃) δ 177.1, 162.3 (d, J_CF = 245 Hz), 148.4, 143.1 (d, J_CF = 12 Hz), 142.6, 134.5, 133.8, 127.0, 125.3, 123.5, 119.1 (d, J_CF = 9 Hz), 118.96, 109.6, 109.1 (d, J_CF = 23Hz), 99.6 (d, J_CF = 30 Hz), 77.2, 42.7, 38.2, 31.1 (q, J_CF = 28 Hz), 26.7, 22.7, 22.6, 22.58, 19.6. LCMS m/z [M+ H]+ 547.2 at 4.55 min.
1’-[(5-(Aminomethyl)-1-(4,4,4-trifluorobutyl)-1H-1,3-benzodiazol-2-yl)methyl]-6’fluoro-1’,2’-dihydrospiro[cyclopropane-1,3’-indole]-2’-one (20)

To a solution of tert-butyl N-[(2-[(6’-fluoro-2’-oxo-1’,2’-dihydro[spirocyclopropane-1,3’-indole]-1’-ylmethyl]-1-(4,4,4-trifluorobutyl)-1H-1,3-benzodiazyl)methyl]carbamate (1030 mg, 1.88 mmol) in CH₂Cl₂ (3.5 mL) under nitrogen was added hydrogen chloride solution (2 M in Et₂O; 12.54 mL, 25.08 mmol). A pink/white solid precipitate formed almost immediately, and the mixture was stirred at rt for 6 h. The reaction mixture was concentrated under reduced pressure at ambient temperature and azeotroping with CH₂Cl₂ (3× 20 mL) to avoid HCl concentration. The crude product was sonicated and triturated with Et₂O (2× 15 mL, then 4× 10 mL). The mixture was filtered, washing with (Et₂O (3× 10 mL) and dried in a vacuum oven to give the crude HCl salt of the desired product as an off-white solid (851 mg, 89% crude yield).

The crude HCl salt was partitioned between EtOAc (80 mL) and saturated aqueous NaHCO₃ solution (80 mL). The organic phase was separated, and the aqueous layer extracted with EtOAc (3× 30 mL). The organics were combined, dried (MgSO₄), and concentrated under reduced pressure. The residue was triturated with Et₂O (15 mL), then purified by flash chromatography on [SiO₂, 0-50% CH₂Cl₂:MeOH:NH₃ (9:1:0.2) in CH₂Cl₂] to afford the free base as a white solid (539 mg, 63%). ¹H NMR (400 MHz, DMSO-d₆) δ 7.58 (d, J =
1.5 Hz, 1H), 7.54 (d, J = 8.3 Hz, 1H), 7.24 (dd, J = 8.4, 1.6 Hz, 1H), 7.17 (dd, J = 9.6, 2.4 Hz, 1H), 7.06 (dd, J = 8.3, 5.4 Hz, 1H), 6.81 (ddd, J = 10.5, 8.2, 2.4 Hz, 1H), 5.29 (s, 2H), 4.36 (t, J = 7.7 Hz, 2H), 3.79 (s, 2H), 2.39–2.26 (m, 2H), 1.88–1.80 (m, 2H), 1.67 (q, J = 4.1, 3.4 Hz, 2H), 1.59 (q, J = 4.5, 3.9 Hz, 2H). $^{13}$C NMR (100 MHz, DMSO-d$_6$) δ 176.3, 161.4 (d, J$_{CF}$ = 239.7 Hz), 148.8, 143.65 (d, J$_{CF}$ = 12.2 Hz), 142.0, 137.7, 133.9, 127.2 (q, J$_{CF}$ = 276.7 Hz), 125.6 (d, J$_{CF}$ = 2.4 Hz), 122.5, 120.2 (d, J$_{CF}$ = 9.7 Hz), 117.4, 109.8, 108.0 (d, J$_{CF}$ = 22.5 Hz), 98.2 (d, J = 28.2 Hz), 45.7, 41.9, 37.2, 30.1 (q, J$_{CF}$ = 28.4 Hz), 26.3, 22.2, 19.9. HRMS (ESI): m/z [M+H]$^+$ calcd. for C$_{23}$H$_{23}$N$_4$O$_4$F$_4$: 447.1808, found: 447.1816. LCMS m/z [M + H]$^+$ 447.3 at 0.58 min.

1’-([5-(Aminomethyl)-1-(4,4,4-trifluorobutyl)-1H-1,3-benzodiazol-2-yl]methyl)-6’fluoro-1’,2’-
dihydrospiro[cyclopropane-1,3’-indole]-2’-one hydrochloride (20·HCl)

HCl (2.0 M in Et$_2$O; 0.6 mL, 1.21 mmol) was added dropwise to a solution of 1’-([5-(aminomethyl)-1-(4,4,4-trifluorobutyl)-1H-1,3-benzodiazol-2-yl]methyl)-6’fluoro-1’,2’-
dihydrospiro[cyclopropane-1,3’-indole]-2’-one (539 mg, 1.21 mmol) in CH$_2$Cl$_2$ (10 mL) and the reaction mixture stirred for 30 min. The solvent was then evaporated under vacuum. The residue was dissolved in MeOH (20 mL), concentrated under vacuum at rt, and dried further in a vacuum oven at 40 °C, affording the
hydrochloride salt as a white solid (480 mg, 81%). $^1$H NMR (600 MHz, DMSO-d$_6$) δ 8.39 (br. s, 3H), 7.75 (d, $J = 1.5$ Hz, 1H), 7.68 (d, $J = 8.3$ Hz, 1H), 7.40 (dd, $J = 8.4$, 1.6 Hz, 1H), 7.14 (dd, $J = 9.6$, 2.4 Hz, 1H), 7.08 (dd, $J = 8.3$, 5.4 Hz, 1H), 6.82 (ddd, $J = 10.3$, 8.3, 2.4 Hz, 1H), 5.33 (s, 2H), 4.40 (t, $J = 7.7$ Hz, 2H), 2.40 – 2.27 (m, 2H), 1.88-1.83 (m, 2H), 1.69 (q, $J = 3.9$ Hz, 2H), 1.58 (q, $J = 3.8$ Hz, 2H). $^{13}$C NMR (150 MHz, DMSO-d$_6$) δ 176.35, 161.4 (d, $J = 240$ Hz), 149.8, 143.6 (d, $J = 12$ Hz), 141.8, 135.1, 127.2 (q, $J = 276$ Hz), 127.8, 125.65 (d, $J = 2$ Hz), 123.8, 120.3 (d, $J = 10$ Hz), 120.0, 110.4, 108.05 (d, $J = 22.5$ Hz), 98.1 (d, $J = 28$ Hz), 42.6, 42.0, 37.2, 30.0 (q, $J = 28$ Hz), 26.35, 22.2, 18.9. HRMS (ESI): m/z [M+H]$^+$ calcd. for C$_{23}$H$_{23}$N$_4$OF$_4$: 447.1808, found: 447.1805.

**RSV fusion assay**

The inhibition of RSV F protein mediated cell-cell fusion was investigated in an *in vitro* RSV F protein cell-cell fusion assay based on the method of Branigan et al.$^{24}$ Briefly, human embryonic kidney 293T cells (ATCC, CRL-11268) were co-transfected with an expression plasmid encoding the RSV F protein from the RSV A2 strain (pCDNA3.1-A2-F; codon optimised F protein sequence from RSV strain A2 was synthesised (GeneArt) and subcloned into pCDNA3.1(+)) (Invitrogen, V79020) and a reporter plasmid containing the
luciferase gene under the control of a GAL4 responsive promoter (pFR-Luc, Stratagene 219050). A second set of 293T cells were transfected with an expression plasmid encoding a transcriptional transactivator fusion protein consisting of the GAL4 DNA binding domain fused to the activation domain of NF-κB (pCDNA3.1 GAL4/NFκB: GAL4-NF-κB sequence was synthesised (Genscript) and subcloned into pCDNA3.1(+) (Invitrogen, V79020). After 24 h, the 2 cell populations were mixed in the presence of 3-fold serial dilutions of test compound and the RSV F protein-mediated fusion between the 2 cell populations measured by quantifying the luciferase activity induced by the co-localisation of the GAL4-NF-κB transactivator fusion protein and the GAL 4 responsive luciferase reporter expression plasmid after 24 h.

**Plaque Reduction Assay:**

The inhibition of RSV infection of cells was investigated in an in vitro RSV plaque assay. African green monkey kidney (Vero) or HepG2 cell cultures were infected with the RSV strain A2 or low passage clinical strains of RSV for 48 h in the presence of a range of test compound concentrations (0.01 nM – 100 μM) prior to detection of distinct foci of infection (plaques) by immunostaining.

**Cell Cytotoxicity Assay:**

Cell cytotoxicity was assessed in parallel to plaque reduction assays. African green monkey kidney (Vero) cells, HepG2 or Hep2
cells were cultured in the presence of a range of test compound concentrations (0.01 nM - 100 μM) prior to measurement of cell viability by the addition of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), CellTox green (Promega) or CellTitre-Glo (Promega).

**Inhibition of RSV in primary human airway epithelial cells**

The inhibition of RSV was investigated in an in vitro primary HAE cell infectivity model. Briefly, HAE cells were differentiated on Type IV collagen coated trans-well plate inserts for 61 days. Differentiated HAE cell cultures (n=3) were pre-incubated with compound (10 nM) for 2 hours, prior to apical inoculation with $4 \times 10^5$ plaque forming units (pfu) recombinant luciferase-expressing RSV (lucRSV). Samples of lucRSV shed by the infected HAE cells were obtained daily, for eight consecutive days, by washing the HAE apical cell surface for 2 hours. Analysis of RSV yield in the HAE apical samples was conducted by infecting human lung epithelial A549 cells and determining the luciferase activity 24 hours post infection (pi). On day 8 pi, the HAE cells were fixed and stained with H&E for histological examination.

**Animal experiments**

All animal experimentation was covered under the UK Animals (Scientific Procedures) Act (1986) and EU directive 86/609/EEC.
All such work was monitored by regular inspections of procedures and facilities by the on-site Veterinarian and UK Home Office inspectors.

**in vivo Pharmacokinetics**

The pharmacokinetics of the compounds were studied in vivo in male Sprague Dawley rats at doses of 1 mg/kg (IV) and 10 mg/kg (PO). Sprague Dawley rats were treated with experimental compounds via intravenous and oral administration. Three animals for each route of administration were used with serial blood sampling at ten time points post dosing of compound.

An intravenous bolus was administered at a dose of 1 mg/kg and at a concentration of 1 mg/ml in 40:60 dimethyl acetamide/saline (0.9% w/v saline). Animals were weighed and used if between 200-250 g. Serial blood samples were collected at 0.02, 0.08, 0.25, 0.50, 1, 2, 4, 6, 8 and 24 h post dosing. Animals were observed for any overt clinical signs or symptoms. Blood samples were delivered into an anticoagulant (sodium heparin) and centrifuged at 4 °C. Plasma samples were subsequently stored frozen at less than -20 °C prior to analysis.

Following protein precipitation with acetonitrile, samples were analysed with tandem liquid chromatography/mass spectrometry using electrospray ionisation. A full matrix curve with internal standards was employed and PK parameters were calculated.
In a similar manner, oral administration was performed by gavage at doses of 5 or 10 mg/kg at a concentration of 5 mg/mL in 1% methyl cellulose (Sigma M7140), 0.1% Tween 80 in water. Serial samples were taken as described above.

Acclimatized male CD-1 mice were group housed in a temperature and light controlled facility on a 12-hour light/dark cycle with food and water available ad libitum. Mice included in the study were individually housed until completion. All animals were subjected to health monitoring in accordance with the above guidelines by the onsite Home Office registered veterinarian. Animals were anaesthetized and terminal blood samples were collected by cardiac puncture. Samples were transferred into heparinized polypropylene tubes on wet ice and centrifuged at 4°C within 15 minutes, to yield plasma which was snap-frozen and stored at -20°C. Sampling time points were 2, 5, 15 min. and 1, 2, 4, 8, 12, 24 hours for the in vitro arm and 5, 15, 30 min, 1, 1.5, 2, 4, 6, 8, 12 and 24 h for the oral arm. Samples were analyzed by UHPLC-TOF that comprised an Agilent 1290 HPLC pump with an Agilent 1290 autosampler, coupled with an Agilent 6550 quadrupole-TOF mass spectrometer. The system was controlled by MassHunter software vB.05.01

Six male Beagle dogs (non-naive, 12-13 kg) were housed in pairs and acclimatized in a temperature and light controlled on a 12-hour light/dark cycle, with food offered each morning and water
available ad libitum. On dosing days dogs were fed 2 hours after
dose administration with water available ad libitum. Prior to
commencement of each dosing session each dog was examined by a
qualified Veterinary Surgeon for suitability for the study.
Serial blood samples were collected into heparinized polypropylene
tubes and immediately centrifuged at 4°C for 10 min at 3,000 G to
yield plasma which was snap-frozen and stored at -20°C. Sampling
times were 2, 5, 15 min. and 1, 2, 4, 8, 12, 24 h. for the in vitro
arm and 15, 30 min., 1, 1.5, 2, 4, 6, 8, 12 and 24 h for the oral
arm.
Samples were analyzed by UHPLC-TOF that comprised an Agilent 1290
HPLC pump with an Agilent 1290 autosampler, coupled with an Agilent
6550 quadrupole-TOF mass spectrometer. The system was controlled
by MassHunter software vB.05.01

**Efficacy in a Balb/C Mouse Model of RSV**

Briefly, Balb/C mice (6 per treatment group, 7-8-week-old females)
received two PO doses of compound (0, 1, 10 or 50 mg/mL) 2 hours
pre- and 24 hours post intranasal inoculation with 5 x 10⁶ pfu of
RSV strain A2. Animals were euthanized 5 days post RSV inoculation
and lung homogenates prepared. RSV titres in lung homogenates were
evaluated by plaque assay on Vero cells.

**ASSOCIATED CONTENT**
Supporting Information

Procedures for in vitro ADME assays, virology data, synthetic procedures. Synthetic procedures and characterization of all other compounds and key intermediates, molecular strings. This material is available free of charge via the Internet at http://pubs.acs.org.

Crystallographic data and procedures.

For PDB code 7KQD (RSV Fusion protein with compound 20 bound), the authors will release the atomic coordinates and experimental data upon article publication.

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Notes

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ABBREVIATIONS
$A_2B$, $B_2A$, direction of compound permeability; BID, twice daily dosing; CHF, chronic heart failure; $C_{\text{int}}$, intrinsic clearance; DIPEA, $N,N$-diisopropylethylamine ERD, enhanced respiratory disease; $f_u$, fraction unbound; $\Delta \text{clogD}_{7.4}$, change in calculated log D at pH 7.4; $\Delta \text{tPSA}$, change in total polar surface area; ERD, enhanced respiratory disease; F protein, fusion protein; GLP, good laboratory practice; G protein, glycoprotein; HAE, human airway epithelial; HBSS, Hanks balanced salt solution; HCT, hematopoietic
cell transplant; Hep2, human epithelial type 2 cells; lucRSV, luciferase expressing respiratory syncytial virus; LO, lead optimization; mAb, monoclonal antibody; LRTI, lower respiratory tract infection; M protein, matrix protein; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NOAEL, no observed adverse event level; N protein, nucleoprotein; P<sub>app</sub>, apparent permeability; P protein, phosphoprotein; pi, post infection; PFU, plaque forming units; PRA, plaque reduction assay; RSV, respiratory syncytial virus; RSV A2, respiratory syncytial virus A2 strain; SAD, single ascending dose; t<sub>max</sub>, time to maximum absorption; URTI, upper respiratory tract infection;

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