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Description of the main S&T results/foregrounds

Screening approach

First a follow up on interesting hit compounds discovered in the two previous projects, FluDrugStrategy and FLUINHIBIT, was performed. Various techniques were used, including multi-step organic synthesis of many derivatives, to decipher crucial structure-activity relationship (SAR) trends and discover improved hit compounds. The definition of a Hit is a compound exhibiting binding to the proteins in the ribonucleoprotein (RNP) complex, the core of influenza virus replication. It consists of the PB2, PB1, PA subunit and the nucleoprotein (NP). Hit optimization involves improving the binding interaction between the hit and its target. A lead compound demonstrates antiviral activity and an acceptable toxicity profile. Lead optimisation included ADME-tox profiling and animal studies for selection of potential clinical candidates.

Hits Identification

The initial hit series that were elaborated in the NP-NP and PB1 project branches suffered from drawbacks that could not be overcome in the course of the FLUCURE project. Therefore, structural modifications of the original hits and the search for alternative structures were pursued.

Computational strategies were employed with the aim of identifying new small molecules able to affect the PB-PA protein-protein interaction. To this aim, a Molecular Dynamic simulation (MD) were first performed on the PA-PB_1 complex to identify the crucial residues for the interaction between the two proteins. On the basis of results from molecular dynamic simulation, next calculations were focused on the wide region of the identified hot-spots to discover small molecule inhibitors of PA-PB1 protein-protein interaction.

Asinex commercial databases were virtually screened using two different approaches: 1) a pharmacophore-based virtual screening; 2) high throughput docking. A structure-based pharmacophore was constructed on the basis of the PA-PB1 complex resulting in a complex model consisting of many features.

Based on the existing pharmacophore models part of the consortium started to develop a strategy for the extension of the available small hit molecule “anchors” along the hydrophobic helical part of the PB1-N-terminus by synthesizing small-molecule-peptide-hybrids.

Several different approaches were used; 1) biological screening on the commercially available analogues of the most active hits previously identified 2) systematic alterations of the scaffold of the most active hit candidate from the screening campaign 3) introduction of short peptidic side chains to the scaffold and evaluation of several linkers/side chains 4) use of peptidomimetic approaches ranging from introduction of non-natural amino acids to linkage of amino acid side chains (stapling) for mimicry of the hydrophobic helical part of the PB1-N-terminus

In conclusion, three compounds were selected for biological investigations in a competitive ELISA and in MDCK cells that had previously been infected with the FluA virus (WSN strain). Treatment with one of the compound, MR37 led to complete lack of expression of the viral NP nucleoprotein with inhibition of virus replication at low micromolar concentrations.

Finally, because of the large size of PB1 binding site within PA, it is very difficult to identify small molecules able to inhibit this protein-protein interaction. However, useful insight into PB1 binding site of PA by computational analysis have been discovered. Future work will focus on identify unexplored binding sites analyzing protein conformations derived from enhanced-sampling MD simulations in addition to the available crystal structures.

Mechanistic characterization & structure-based design

Detailed Protein-Ligand interaction data for optimized hit compounds determined (NP-NP)

A new way of grouping hits from the primary screen, based on qualitative analysis of SPR biosensor sensorgrams were introduced. Compounds identified as hits by Differential Scanning Fluorimetry (DSF) have an influence on the thermal stability of NPs detected as a shift in T_m. Together; the two methods were able to identify hits that lowered or increased T_m, as well as compounds that had no effect on T_m. Altogether, 177 hit analogues obtained from partners and a collection of 80 compounds obtained during FluDrugStrategy (the predecessor project) were analyzed once again by Biacore and DSF.

Characterization of hits

In parallel with the follow-up of the screening hits, re-evaluation of old experiments has identified a handful of compounds for further exploration. To interfere with oligomerization is a lead candidate property that the NP branch of FLUCURE aims for.

Hit validation

ITC (Isothermal calorimetry) has been evaluated as a possible complement to Biacore and DSF. However, ITC was not a suitable technique for detecting NP-fragment interactions. Analytical Size Exclusion Chromatography (SEC) has also been tested since a compound that binds to the tail-loop binding pocket could affect the oligomerization of NP. However, the experimentally observed differences in the chromatograms for NPs in presence and absence of compound were very small. Microscale thermophoresis, a novel technique for detection of interactions in solution and at equilibrium, was also evaluated as a method for analysis of interactions with NP

To summarize, results from 3 independent techniques (SPR biosensor analysis, DLS and microscale thermophoresis) support a specific interaction between NP and IOTA0781. DSF neither supports nor excludes an interaction since the T_m is essentially unchanged.

Detailed Protein-Ligand interaction data for optimized hit compounds determined (PA-PB1)

An SPR-biosensor based assay for the characterization of the interactions between low molecular weight compounds and Influenza PA_C (C-terminal domain) has been established. The performance of the assay was adequate for the estimation of the kinetic parameters of the interactions with four peptides. The analysis revealed that it is the association rate constant that differs between the different peptides.

An SPR- biosensor based assay for determining affinity by competition with an immobilized peptide was also developed. This assay can be used to estimate the affinities of compounds binding to the same binding site as the immobilized peptide.

Interactions between the Influenza PA and peptides were also analyzed by DSF. All peptides stabilized the protein while PKE 138 did not.

Experiments involving the endonuclease PA_N (N-terminal domain) were also performed. The aim was to establish an interaction assay and involved exploratory work in order to find a suitable immobilization strategy using pH-scouting and different detergents.

Structures of Ligand-Protein complexes

The crystallization of target protein complexes was initiated but the structural heterogeneity of the target protein and lack of stabilizing ligands prevented the project from obtaining diffracting crystals. Unfortunately, the crystallization attempts made at SARomics did not work. The conclusion drawn

by SARomics was that the potential to succeed was very small and it was decided to put further crystallization experiments on hold.

Modelling of target protein

Molecular dynamics calculations started in the second reporting period. The performed simulations of the trimeric NP demonstrated that even after 10 ns in water the conformational changes are negligible, loops stays stable in the cavities and the cavities SAS (Solvent Accessible Surface) shapes are almost unchanged. The main structure movements remain in the loop and in the most protuberant parts of the trimer. Contrary, when the molecular dynamics were carried out with a separate monomer, after 10-15 ns in water the cavity (as in the example of the C chain depicted above) closes completely. The analysis of the trajectory reveals that this is due to the hydrophobic interaction inside the cavity. Having in mind the results above we concluded that the creation of an adequate drug model must be based on the understanding how one hydrated monomer enters the "closed" cavity of another monomer.

Modelling of target protein-inhibitor complexes

The structure of NP and its interaction with inhibitors was modelled to generate design hypotheses.

Functional protein variants

Polymerase for screening of library were made e.g. a new variant of the polymerase was designed, cloned and expressed. Preparation of fusion proteins for complementation assay were made, which is used for detection of complex of Flu virus polymerase subunits PA and PB1. Screening system components needed to track the interaction between Flu virus subunits PA and PB1. The purified proteins have been successfully used for complementation assay. PA protein has been purified from yeast for competitive ELISA binding assay. mABs against subunit PA for competitive ELISA binding assays have been amplified.

Peptidomimetic Approaches

Several compounds mimicking the sequence of PB1 (5-11) were synthesized and tested in a competitive ELISA. The activity of the most potent compound, MR37 exhibited an IC_{50} value of 10 µM (Figure 5). Two other compounds, MR39 and MR41, showed a 30-40% inhibitory effect at 200 µM. As a next step, we tested the effect of all three compounds in MDCK cells that had previously been infected with the FluA virus (WSN strain). Treatment with MR37 led to complete lack of expression of the viral NP nucleoprotein (Figure 6). MR41 also resulted in loss of NP signal, although to a lesser degree, whereas the effect of MR39 in the NP signal production was weak (Figure 2). We finally assessed the binding of MR37 to the PA protein using differential scanning fluorimetry (DSF). Results show that 10 µM of peptide cause a thermal shift in the melting temperature of PA by 2 °C.

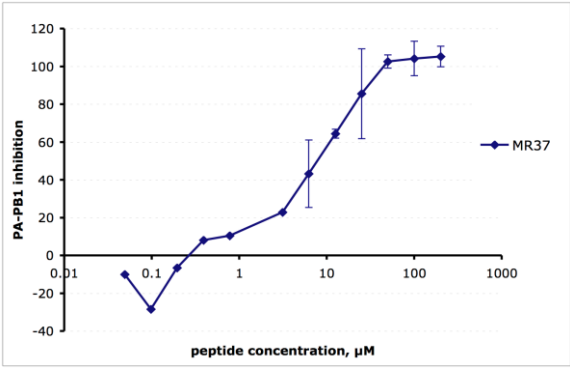

Figure 1. ELISA dose response of MR37.

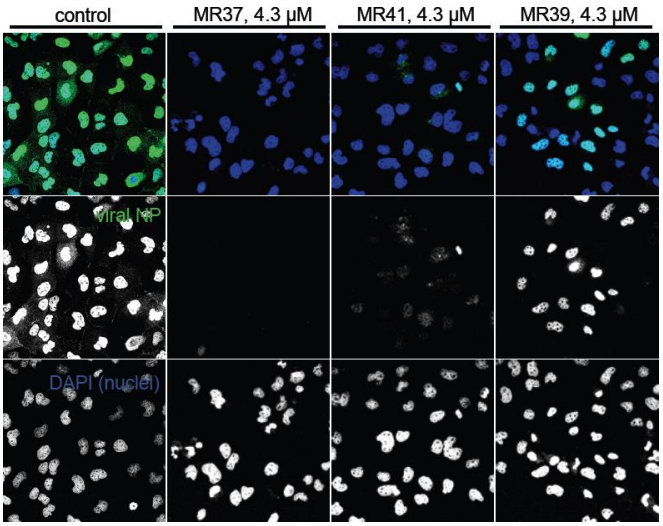

Figure 2. M37 inhibits virus replication in MDCK cells. Cells were infected with influenza A virus (WSN) for 1h before treatment with compound for 4h. Subsequently, cells were fixed and immunostained for viral NP

The complete inhibition of virus replication at low micromolar concentrations resembles an approximately 20-fold improvement of the activity compared to the first generation of compounds and we are confident that the fusion with the small molecule anchor will lead to even more potent inhibitors of virus replication in the near future.

Lead optimization

Series of candidates that fulfil clinical candidate selection criteria

The first criteria for selecting a lead compound are a demonstrated antiviral activity and an acceptable toxicity profile.

A majority of the consortium concentrated the synthetic chemistry resources on the early screening compound series that was developed for the PA-PB1 project. Although combined efforts resulted in considerable results and new insights, the criteria to allow leads for further preclinical optimisation was not obtained in time. The same constellation continued early screening even when time and resources were supposed to focus on already generated leads.

Partenr 1 (Vironova) focused the second part of the project with optimizing the lead compound for antiviral activity, solubility and ADME tox parameters in order to perform animal model experiments. This work included experiments with Prof. Peter Staheli at Freiburg University and substances were tested by Pharmacelsus for ADME/ tox properties.

Analysis of virus-infected cells and model animals treated with test substances

The main task has been testing of compounds for antiviral activity in cell-based systems and preparation for testing in animal models.

Antiviral activity-cell setup

Antiviral assays in which MDCK cells were infected with various influenza A virus strains. For most experiments the H1N1 lab strain A/Texas/91 or the H3N2 lab strain A/Panama were used at a multiplicity of infection of 0.01. To demonstrate specificity of the compounds, control experiments were performed in the same manner with the pandemic 2009 H1N1 virus strain A/HH/2009 that carries mutations in NP which were previously shown to confer nucleozin resistance [1].

Isoxazole nucleozin derivatives

(VNFC009, VNFC014, VNFC015, VNFC029, VNFC030, VNFC031, VNFC032, VNFC035 and VNFC037 table 3.1)

Previously we have reported that thionating the isoxazole derivatives gave good and sometimes improved antiviral activity compared with the parent compounds. We were able to determine from these first studies that the addition of the methoxy group in the R_1 position improved the solubility property which had also a direct effect on improving the antiviral activity of the compounds. Another interesting observation was the importance of the presence of the nitro group in the R_3 position (Figure 3). This was established when we searched for alternatives for replacing the aromatic nitro group which is known as a structural alert in drugs that can cause a potential carcinogenic effect [2].

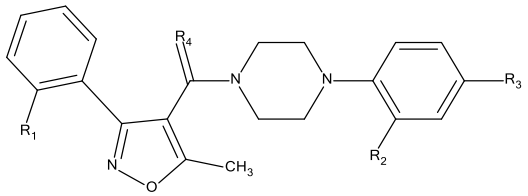

Figure 3. Structure of Isoxazole nucleozin derivatives

Compounds VNFC014, VNFC015, VNFC029, VNFC030, VNFC031 and VNFC032 were synthesized to purposely eliminate the potential risks of toxicity. Unfortunately, replacement of the nitro with the CF_3 substituent resulted in reduced or no reduction in viral titers. Based on these studies it appeared that the nitro substituent was vital to the antiviral nature of the compound. Furthermore, Gerritz et al [3] reported that there is a face-to-face π–π interaction between the 2-Cl-4-NO2-Ph ring of "compound 3" (VNFC035) and Y289 of NP_A The NO_2 group may be necessary for the correct face-to-face alignment when interacting with the OH group of the Y289 amino acid.

Conclusion

Replacement of the nitro group in the R_3 position of nucleozin with the CF_3 substituent resulted in reduced or no reduction in viral titers.

VNFC035 and VNFC037

VNFC035 and VNFC037 indicated to have the best antiviral activity and more detailed studies with several concentrations of the compounds were conducted in virus yield reduction assays. Again both compounds inhibited viral titers at concentrations 0.3, 1, and 3 µM. VNFC037 eliminated the Texas virus yield more dramatically than VNFC035 (Figure 4). At lower concentrations (0.03 and 0.1 µM) both VFNC035 and VNFC037 more efficiently reduced viral titers compared to nucleozin. Therefore, VNFC035 and VNFC037 were selected for further studies in WP4.

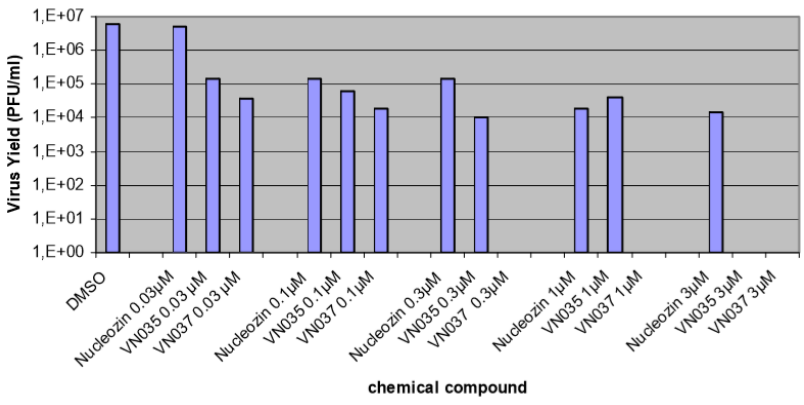

Figure 4. Antiviral activity of isoxazole nucleozin derivatives. MDCK cells were infected with the Texas strain of influenza A virus at an MOI of 0.1 for 1 h at room temperature. The inoculum was removed, the cells were washed and incubated at 37° with fresh medium containing the indicated concentrations of the listed compounds. After 24 h, virus content of the various culture supernatants was determined by plaque assay. Medium containing solvent, only (DMSO), served as negative control.

Conclusion: *Both VFNC035 and VNFC037 more efficiently reduced viral titers compared to nucleozin. This is directly related to the addition of the methoxy group in the R1 position where we know there is an improvement in the solubility of the compound as well as the antiviral effect.*

Triazole building blocks

In recent years, several groups have identified and published NP-interacting molecules that are able to inhibit virus replication [3,4,5]. Among them, Gerritz et al [3] demonstrated that optimization of a related series of nucleozin compounds displayed potent antiviral activity both *in vitro* and *in vivo*. They were able to determine that the isoxazole derivatives metabolized quickly in mouse liver microsomes (MLM), but by bioisoterically replacing them with a 1-methyl, 4,5-phenyl trisubstituted [1,2,3] triazole, the derivatives metabolized slower in MLM. In addition, further studies conducted by Ding and coworkers [4] confirmed that 1-phenyl,4-methyl, 5-trisubstituted [1,2,3] triazole analogues also showed anti-influenza activity against influenza A/HK/8/68 H3N2 with an IC_{50} 1,97µM and influenza A/WSN/33 H1N1 with an IC_{50} 0,68 µM. Therefore, synthesis of triazole variations of the nucleozin pharmacophore and the thionation of these with crystalline P_2S_5.2(C5H5N) were carried out.

1-phenyl, 4, 5-methyl trisubstituted 1,2,3 triazole nucleozin (VNFC021, VNFC022, VNFC023, VNFC024, VNFC27, VNFC028, VNFC038 and VNFC039 table 3.2)

1,4,5-trisubstituted [1,2,3] triazoles were synthesized using the Huisgen azide-alkyne 1,3-dipolar cycloaddition. However, this method yields a mixture of the two regioisomeres, **A** and **B** (Scheme. 3.1). The regioisomer situation can be controlled by using a catalytic approach. 1,4-disubstituted triazoles can be synthesized by copper-catalyzed azide-alkyne cycloaddition (CuAAC) and 1,5-disubstituted triazoles can be formed by ruthenium-catalyzed azid-alkyne cycloaddition (RuAAC). We continued with the synthesis of the 1-phenyl 4, 5-methyl trisubstituted 1,2,3 triazole nucleozin derivatives and tested them against influenza A Texas strain virus in MDCK cells. It was determined that these isomers were antivirally inactive.

Conclusion: *1-phenyl, 4, 5-methyl trisubstituted 1,2,3 triazole nucleozin derivatives (VNFC021, VNFC022, VNFC023, VNFC024, VNFC027, VNFC028, VNFC038 and VNFC039) are inactive against influenza A strain Texas.*

1-phenyl, 4-methyl, 5 trisubstituted 1,2,3 triazole nucleozin (VNFC025, VNFC026, VNFC042, VNFC043, VNF046, VNFC047, VNFC048 and VNFC049)

The correct regioisomer is instead prepared, as the minor product, via the traditional 2+3 Huisgen cycloaddition as reported by Ding et al **[4]**. The phenylazides are easily available from the anilines via a diazotisation and treatment with sodium azide.

Conclusion : *The most active compounds in this series of 1-phenyl, 4-methyl, 5 trisubstituted 1,2,3 triazole nucleozin derivatives are VNFC025 and VNFC026. The dimethoxy derivatives do not seem to be as antivirally active as the single methoxy derivatives.*

1-methyl, 4-phenyl, 5 trisubstituted 1,2,3 triazole nucleozin (VNFC040, VNFC041, VNFC044, VNFC045, VNFC050 and VNFC051 table 3.4)

Repeating Ding and coworkers [4] method for the preparation of the N-methyl triazole derivatives via the Huisgen cycloaddition involved heating methylazide in a sealed tube at high temperatures for

several hours. Methylazide is a low boiling compound that is highly energetic and shock sensitive and at decomposition gives rise to toxic degradation products. For that reason it was desirable to find an alternative route to the desired regioisomer of the triazole derivative. Alkylation of an unsubstituted triazole will give a mixture of the three isomers with the N-2 alkylated regioisomer as the major product. Hence, an alternative to methylazide was desired. Using the trimethylsilyl (TMS) substituted methylazide, which have better physical properties than methylazide e.g. higher boiling point, less shock sensitive, made this approach safer. The TMS-group is easily removable by adding fluoride salts e.g. tetrabutylammoniumflouride (TBAF).

As illustrated below (Fig 5), compound **4** was synthesized via 2 + 3 cycloaddition using trimethylsilyl methylazide as the key step. Iodoanisidine was coupled with methyl propiolate in presence of Cu(I) to provide **1**.[7] Compound **1** was reacted with trimethylsilyl-methyl azide giving a mixture of the two regioisomers, **2a** and **2b**, which were separated by chromatography on SiO_2 yielding the desired **2a** as the minor product. The removal of the TMS-group proceeded in good yields as well as the hydrolysis of the ester, **3**, to the corresponding carboxylic acid, **4**. The overall yield for this route was 7.0 % (Figure 5).

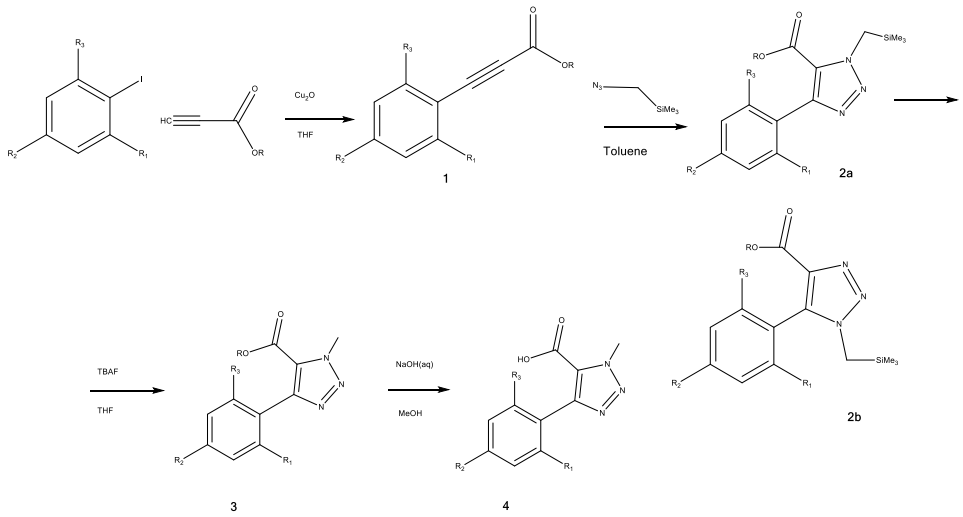

Figure 5. Route with Huisgen's cycloaddition to 4.

An alternative approach to the desired isomer, **4**, is by using "Click"-chemistry. The key intermediate in this approach was the monosubstituted acetylene, **5**. The procedure is expected to proceed regioselective and only gives the desired 1,4-substituted triazole isomer which can be substituted under basic conditions in the 5-position giving the 1,4,5-trisubstituted triazole.

A direct approach to **5** by using a Negishi coupling of the *o*-iodoanisidine with the zinc Grignard reagent of acetylene proceeded in moderate yields.[8] Using the Sonogashira coupling of ethynyl trimethylsilane with the iodobenzene and subsequently removal of the TMS-group with TBAF gave the monosubstituted acetylene in good yields. The copper mediated cycloaddition of **5** with trimethylsilyl-methylazide followed by the removal of the TMS-group gave **7**, exclusively. The carboxyl group was introduced by quenching the lithiated **7** with carbon dioxide at low temperatures (-70^{o}C) which gave the desired carboxylic acid, **4**. The overall yields were 15.5% (via Negishi coupling) and 34% (via Sonogashira coupling) to the 1,4,5-trisubstituted [1,2,3] triazole (Figure 6).

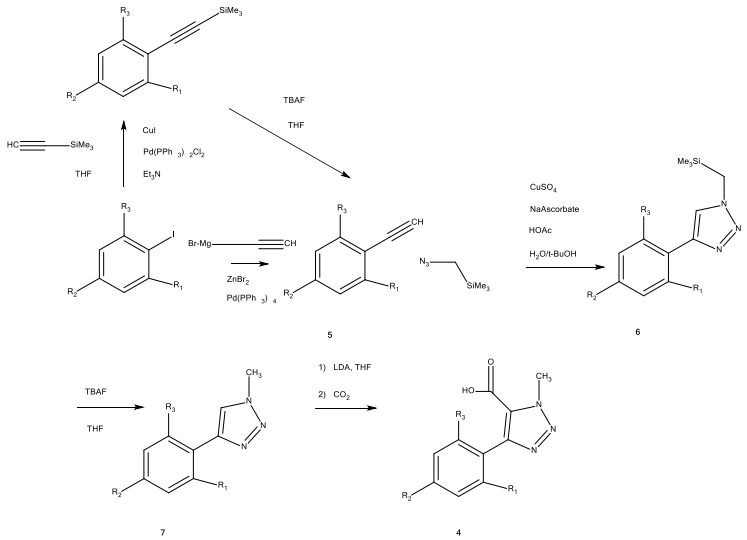

Figure 6 A regioselective route to building block 4

Several 1-methyl, 4-phenyl, 5 trisubstituted 1,2,3, triazole nucleozin derivatives (VNFC040, VNFC041, VNFC044, VNFC045, VNFC050 and VNFC051 (Figure 7) have been synthesized and tested for antiviral activity against influenza (Table 1).

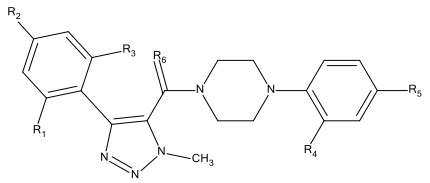

Figure 7 1-methyl, 4-phenyl, 5 trisubstituted 1,2,3 triazole nucleozin derivatives

Compound	R1	R2	R3	R4	R5	R6	Antiviral activity
VNFC040	OMe	H	H	Cl	NO_2	O	n/a
VNFC041	OMe	H	H	Cl	NO_2	S	+++
VNFC044	OMe	H	OMe	Cl	NO_2	O	+++
VNFC045	OMe	H	OMe	Cl	NO_2	S	+++
VNFC050	OMe	H	F	Cl	NO_2	O	+++
VNFC051	OMe	H	F	Cl	NO_2	S	+++

Table 1 1-methyl, 4-phenyl, 5 trisubstituted 1,2,3 triazole nucleozin derivatives with their antiviral activities. Compounds n/a have been synthesized but not tested for antiviral activity. Antiviral activity was determined using the antiviral assays described above. Symbols: (+++) = very potent, (++) = moderate, (+) = low and (-) = no detectable antiviral activity.

Conclusion: *All derivatives of the 1-methyl, 4-phenyl, 5 trisubstituted 1,2,3 triazole nucleozin (VNFC040, VNFC041, VNFC044, VNFC045, VNFC050 and VNFC051) indicated anti-viral activity. The addition of a fluorine or methoxy substituent in the R_3 position does not affect the antiviral activity of the compounds in comparison to the 1-phenyl,4-methyl, 5 trisubstituted 1,2,3 triazoles (VNFC021, VNFC022, VNFC023, VNFC024, VNFC27, VNFC028, VNFC038 and VNFC039). The strategy to include these substituents is thought to block these positions and prevent the formation of undesirable metabolites.*

VNFC041, VNFC045 and VNFC051

Figure 7 depicts the antiviral assay with Texas virus-infected cells treated with some of the compounds described in table 1 and in the text. Compounds VNFC041, VNFC044, VNFC045, VNFC050 and VNFC041 showed better antiviral activity than nucleozin at 0.1 µM. In conclusion, the 1-methyl, 4-phenyl, 5 trisubstituted 1,2,3 triazole nucleozin derivatives (VNFC041, VNFC044, VNFC045, VNFC050 and VNFC051) have more antiviral activity than 1-phenyl, 4-methyl, 5 trisubstituted 1,2,3, triazole nucleozin derivatives (VNFC046, VNFC047, VNFC048 and VMFC049) and nucleozin. The three thionated compounds VNFC041, VNFC045 and VNFC051 showed excellent antiviral effects and were selected for further studies at lower concentrations (figure. 8). Under these experimental conditions, compound VNFC045 and VNFC051 were active at all concentrations higher than 0.03µM.

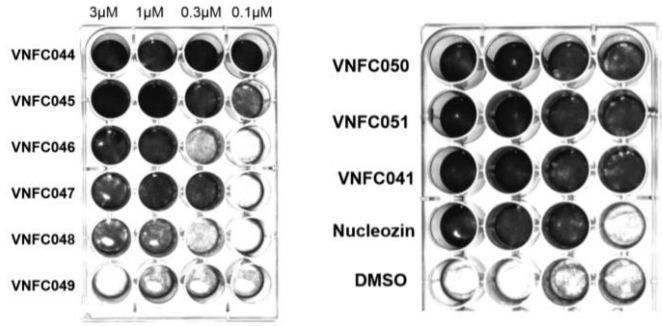

Figure 8 Anti-influenza activity of various compounds measured by cytopathic effect (CPE) assay. Semi-confluent monolayers of MDCK cells were infected with A/Texas /36/1991 at an MOI of 0.01 for 1 h at room temperature, before the inoculum was removed and medium containing the listed compounds at the indicated concentrations was added. The plates were then incubated at 37° for 24 h before the cell monolayers were stained with crystal violet. Intense staining of a well indicates that cells remained largely intact, whereas light staining indicates virus-induced cell destruction.

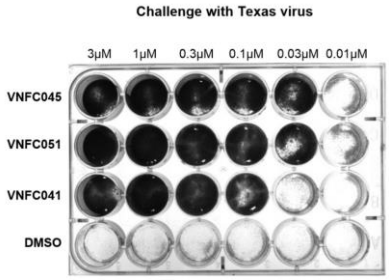

Figure 9 Anti-influenza activity of selected compounds at very low concentrations. The CPE assay was performed exactly as described above in figure 3.4.

Conclusion: *The 1-methyl, 4-phenyl, 5 trisubstituted 1,2,3 triazole nucleozin derivatives (VNFC041, VNFC044, VNFC045, VNFC050 and VNFC051) have more antiviral activity than 1-phenyl, 4-methyl, 5 trisubstituted 1,2,3, triazole nucleozin derivatives (VNFC046, VNFC047, VNFC048 and VNFC049) and nucleozin.*

VNFC026 and VNFC041

A selection of compounds was used to test the antiviral activity with various virus strains (Table 2). Nucleozin had a good antiviral effect against Texas (H1N1), WSN (H1N1) and Panama (H3N2) viruses with no effect against the 2009 pandemic H1N1 influenza. VNFC025 and VNFC026 were also having a good effect against the same viruses with the addition of an interesting result of an antiviral effect against the 2009 pandemic H1N1 influenza.

Antiviral testing Oct. 18 2012

				Antiviral activity against Texas (CPE assay)			
				Texas (H1N1)	WSN (H1N1)	Panama (H3N2)	pandemic H1N1 2009
VNFC 025	23,8mg	456,7 g/mol	C21H21ClN6O4	+++	+++	+++	-
VNFC 026	21,8mg	472,8 g/mol	C21H21ClN6O3S	+++	+++	++	+
VNFC 029	46,2mg	450g/mol	C22H19ClF3N3O2	+	+/-	-	-
VNFC 031	26,7mg	480g/mol	C23H21ClF3N3O3	++	++	-	-
VNFC 032	32,4mg	496g/mol	C23H21ClF3N3O2S	+	+	-	-
Nucleozin				+++	+++	+++	-

Table 2 Anti-influenza activity of various compounds measured by cytopathic effect (CPE) assay with Influenza virus strains Texas (H1N1), WSN (H1N1), Panama (H3H2) and pandemic H1N1 2009. Symbols: (+++) = very potent, (++) = moderate, (+) = low and (-) = no detectable antiviral activity.

Further studies with VNFC025 and VNFC026 were conducted in separate experiments to understand the extent of the antiviral effect against 2009 pandemic H1N1 influenza (Figure 10). First of all we were able deduce that VNFC026, the thionated derivative of VNFC025, was the most active of the compounds.

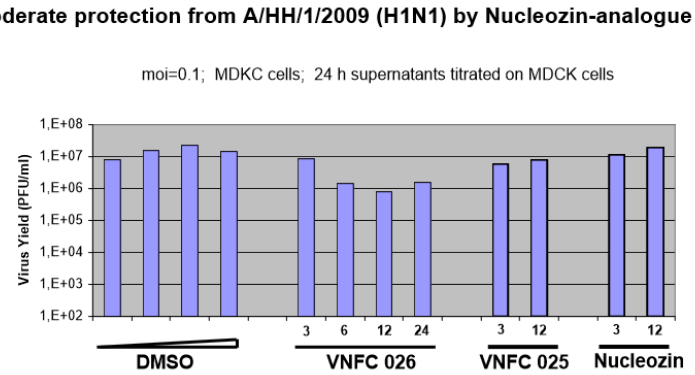

Figure 10 Antiviral activity of VNFC025 and VNFC026 against the pandemic H1N1 2009 influenza A virus. The assay was performed essentially as described in legend to figure 4, except that larger concentrations of compounds (up to 24 µM) were used. Moderate activity of VNFC025 was observed if used at 6, 12 or 24 µM.

A second study indicated that a second compound VNFC041, also a thionated derivative, had shown antiviral effect against 2009 pandemic H1N1 influenza (Figure 11). Only a moderate antiviral effect against 2009 pandemic H1N1 influenza with a viral yield reduction assay. It required 30 µM of VNFC026 and VNFC041 to reduce the virus yield (PFU/ml) from more than 10^7 to over 10^5.

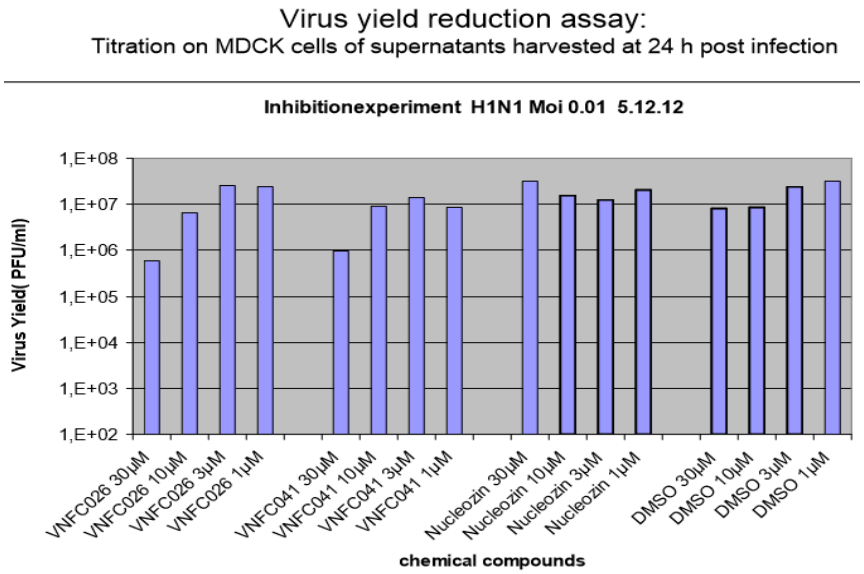

Figure 11 VNFC026 and VNFC041 but not nucleozin are active against pandemic H1N1 2009 at high concentrations. The virus yield reduction assay was performed essentially as described in legend to figure 10 (MDCK cells were infected with the pandemic H1N1 2009 virus at an MOI of 0.01 for 1 h at room temperature. The inoculum was removed; the cells were washed and incubated at 37° with fresh medium containing the indicated concentrations of the listed compounds. After 24 h, virus content of the various culture supernatants was determined by plaque assay. Medium containing solvent, only (DMSO), served as negative control.) This experiment confirmed that unlike parental nucleozin, VNFC026 and VNFC041 exhibit low but significant activity against the pandemic virus.

Conclusion: *VNFC026 and VNFC041 are thionated nucleozin derivatives that have indicated moderate antiviral activity against pandemic H1N1 2009 influenza A.*

Overall conclusions from the lead optimization

In summary using the trimethylsilyl substituted methylazide in the method for the preparation of the N-methyl triazole derivatives via the Huisgen cycloaddition proved to be a safer approach than using methylazide. A regioselective route to the building block compound 4, an important precursor for 1-methyl, 4-phenyl, 5 trisubstituted 1,2,3 triazole derivatives, has been established.

It has been previously established that thionated nucleozin derivatives have antiviral activity against influenza virus strain Texas (H1N1). Several triazoles derivatives in different configurations have been synthesized to replace the isoxazole moiety in nucleozin which was known to have a disadvantage of rapid metabolism in MLM. Most of these compounds have been screened and tested for anti-influenza activity and it seems that the 1-methyl, 4-phenyl, 5 trisubstituted 1,2,3 triazole derivatives show a more potent inhibition. Furthermore, VNFC026 and VNFC041, the thionated triazole derivatives, have shown a moderate anti-influenza activity against the H1N1 2009 pandemic influenza A. It would be interesting to understand the selectivity and why the thioamide is more active than the amide of these derivatives and establish the interactions that are part of the H1N1 2009 pandemic influenza A inhibition. Both the thionated VNFC045 and VNFC051 (1-methyl, 4-phenyl, 5 trisubstituted 1,2,3 triazole derivatives) have shown the most potent anti-influenza result at 0,03 µM against influenza virus strain Texas (H1N1). In conclusion, we were able to provide that there is substantial evidence to indicate that the thionated derivatives are valid candidates for further research and development against influenza A virus in future projects.

Animal models

Gerritz et al [3] reported pharmacokinetic results for "compound 5" the equivalent compound in the FLUCURE project is VNFC040. The authors conducted a proof-of-concept study in a mouse model of influenza and were able to establish that a t.i.d. dosing of 10 mg/kg (30 mg/kg per d) of compound

5 completely protected the mice from H1N1 influenza A/WSN/33 virus (A/WSN) infection. Not only did all of the mice survive without any observable weight loss relative to the healthy controls, but the viral titers were below the lower limit of detection. (500 pfu) at both the 5 and 8 dpi. Post-mortem inspection of the lung tissue indicated that, although the lungs of the 10 mg/kg t.i.d. group showed evidence of infection, the virus did not appear to have spread to neighbouring lung tissue.

An antiviral screening (Figure 8 and 9) of the two triazole series has consequently led to the identification of VNFC045 and VNFC051 (Figure 12) as good candidates for *in vivo* antiviral activity studies in mice. Although VNFC045 with dimethoxy substituents in the ortho-positions in the phenyl ring of the 1-methyl, 4-phenyl, 5 trisubstituted 1,2,3 triazole derivatives show equal potent anti-influenza inhibition as VNFC051 (methoxy and fluorine in ortho-positions), VNFC051 was chosen for further *in vivo* studies due to the known chemical and biological nature that the fluorine atom can deliver when it is incorporated into a drug candidate.

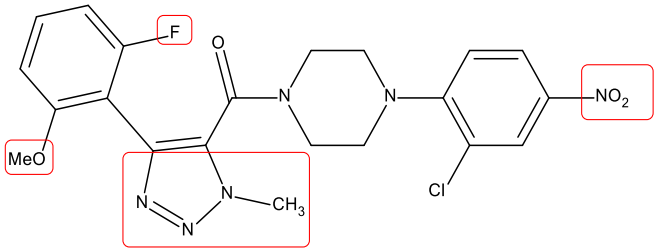

Figure 12 VNFC051

We were able to establish that the presence of a triazole ring was necessary for preventing rapid metabolism, the methoxy substituent improved the solubility of the compound, the fluorine substituent blocks the labile site where undesirable metabolites could form and the nitro substituent was largely responsible for the antiviral activity. A permit from the Regierungspräsidium Freiburg (Germany) for mouse experiments in which compound VNFC051 could be tested for anti-flu activity was achieved. A scaled-up the synthesis method to provide 250mg of VNFC051 for the *in vivo* studies were performed. The quality of this compound was controlled by a GLP-certified analytical laboratory that could validate that the compound was more than 98% pure.

Another fundamental decision for the animal studies was using the same vehicle combination that was administered in the Gerritz et al. *in vivo* studies. The combination mixture of the vehicle was Labrafil: N-methyl-2-pyrrolidone, 9:1 vol/vol. NMP is generally good for compounds with solvating problems and the micro-emulsion formed from the NMP:Labrafil combination is thought to reduce the thionated derivatives instability issues of rapid oxidation.

In vivo study

A study for testing antiviral activity of VNFC051 in mice was designed and performed. However, the experiment was stopped after 72 h due to strong side effects of treatment. Unexpectedly, the compounds did not cause the trouble. Rather, the vehicle solution itself caused heavy irritations of the peritoneum. The animals assumed non-physiological positions for 1-2 min after the injections. When opening up the animals at the end, the peritoneum was oily. Due to the unexpected complications, it was decided that all animals were killed at 72 hpi. Unfortunately, we experienced problems with the solubility of this compound in solvents that may be used for in vivo applications. By the time this project was stopped, the solubility issue had not been solved. As a consequence, no reliable data regarding the in vivo potential of VNFC051 are available at this time.

Cytotoxicity assays have started with the more recent compounds that have been developed and plans to perform antiviral assays in-house are underway. A collaboration with another antiviral research groups have been initiated and an *in vivo* mouse study for the efficacy of the most promising candidate VNFC051 is planned later this year.

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ADME/Tox Profiling

The work has been focused on coordinating the optimization and toxicity studies of promising lead compounds and to provide these compounds for anti-influenza screening and to perform in vivo pharmacokinetics.

Physicochemical property determination

Solubility (nucleozin, VNFC009, VNFC035 and VNFC037)

Since all *in vitro* assays such as metabolic stability, Caco-2, and plasma protein binding etc. are affected by poor solubility of test compounds, aqueous solubility of new chemical entities is an important parameter in the lead optimization process to be investigated as early as possible.

Prior to conduction of ADMET assays, all FLUCURE compounds were tested for their solubility in aqueous buffer using the semi-thermodynamic shake-flask method and applying LC/MS analysis for quantification of the dissolved portion in the supernatant. Results, expressed as µM solubility, are summarized in the table below:

The solubility for NP-NP inhibitors VNFC009, VNFC035 and VNFC037 was measured by the traditional way of determining the equilibrium solubility in buffer (Table 3).

Compound name (Cut-off conc ≤ 250 µM)	Solubility in PBS buffer w/ 2.5% DMSO (n=3)	
	Mean solubility [µM]	SD [µM]
Nucleozin (VNFC007)	0.9	0.04
VNFC009	0.6	0.1
VNFC035	71.5	5.6
VNFC037	13.1	0.5

Table 3 Solubility results for VNFC035 and VNFC037

Previous results from the earlier progress report indicated poor solubility concentrations with nucleozin (VNFC007) and VNFC009. An addition of a methoxy group in position R_1 (see Figure 3) of the nucleozin pharmacophore i.e. compounds VNFC035 and VNFC037 has remarkably improved the solubility while still maintaining the anti-viral activity of the compounds.

Permeability Caco-2 assay (nucleozin (VNFC007), VNFC009, VNFC035 and VNFC037)

In the drug discovery screening process a key success factor is oral absorption of a lead compound. One of the barriers that restrict the bioavailability of orally administered drugs is the epithelium of the intestinal mucosa that lines the surface of the gastro-intestinal tract. The intestinal mucosa is both a physical and a biochemical barrier, separating the external environment form the internal milieu of the body. The physical barrier arises from cell membranes and the intracellular junctions between the cells, i.e. tight junctions. Permeation of drugs across the intestinal epithelium is restricted to paracellular and transcellular pathways depending on their physicochemical properties. Only lipophilic molecules may directly pass across the lipid bilayer of the cell membranes by passive diffusion. In addition to the physical barrier, the intestinal epithelium also possesses various metabolic enzymes, such as intestinal peptidases, cytochromes P450, as well as polarized efflux systems such as p-glycoprotein (P gp), which act as biochemical barriers further limiting intestinal drug absorption. Consequently, many drug candidates are restricted from oral dosing in clinical development owing to this biological barrier. Therefore, in vitro assays, which assess transport across the intestinal epithelial, cell barrier and thereby predict absorption rates of drug candidates, are widely used. The Caco-2 cell line is the best-characterized and most widely used culture system.

The Caco-2 bidirectional permeation assay, which is an accepted method of the FDA for preclinical drug screening, provides a well-established in vitro model to predict the human intestinal absorption. In their differentiated stage Caco-2 cells exhibit morphological and physiological properties of the human small intestine, e.g. barrier function, active transport systems and efflux systems.

FLUCURE compounds were tested at a concentration of 0.5 µM in presence of 0.5% DMSO. According to their apparent permeation (P_{app} values) in Caco-2 cells from apical to basolateral, compounds can be ranked into 3 categories. Compounds with a $P_{app}*10^6$ <1 are predicted to be poorly absorbed, compounds with $P_{app}*10^6$ between 1 and 10 moderately and compounds with a $P_{app}*10^6$ >10 are predicted to be well absorbed.

Test compound	**Direction**	**P_{app} *10 $^{-6}$ (cm/sec)**	**SD *10 $^{-6}$**	**Mass balance (%)**	**SD**	**Efflux ratio b->a/a->b**
Experiment 1						
Atenolol	a-b	0.3	0.3	99.7	4.1	3.5
	b-a	1.1	0.3	107.8	10.5	
Erythromycin	a-b	0.06	0.0	79.1	2.8	99.96
	b-a	5.89	0.2	82.4	2.0	
Testosterone	a-b	17.2	0.68	74.6	1.1	1.4
	b-a	23.5	1.0	89.7	1.0	
VNFC007	a-b	39.8	8.5	139.3	10.1	0.7
	b-a	28.9	0.3	142.4	12.0	
VNFC009	a-b	51.8	5.1	390.9	19.7	0.1
	b-a	4.3	0.8	136.7	6.7	
Experiment 2						
Erythro-mycin	a-b	0.1	0.1	87.5	6.3	111.3
	b-a	12.4	0.5	85.1	2.1	

Testosterone	a-b	17.1	2.8	81.4	2.3	2.3
	b-a	39.6	2.4	75.8	6.4	
VNFC035	a-b	26.6	9.2	120.5	8.0	1.7
	b-a	44.8	10.3	155.3	38.8	
VNFC037	a-b	1.0	1.6	110.6	15.7	0.7
	b-a	0.7	0.2	8.2	1.3	

Table 4 CaCO-2 assay results for VNFC035 and VNFC037

Conclusion: *Permeability of nucleozin (VNFC007), VNFC009, VNFC035 and VNFC037 was assessed using the Caco-2 permeability assay (Table 4). Nucleozin (VNFC007) and VNFC009 indicate an efflux ratio lower than 1. The addition of the methoxy group in compounds VNFC035 and VNFC037 has increased the efflux ratio value showing a significant improvement in the permeability of the compounds. These values indicate that the expected transport and permeability of VN035 and VN037 are active in secretion bearing a medium permeability and active in absorption bearing a low permeability respectively.*

Plasma protein binding (nucleozin (VNFC007), VNFC009, VNFC035 and VNFC037)

The binding of small molecules and peptides to serum proteins is a very important parameter for drug metabolism and pharmacokinetic studies. If a molecule displays a high affinity towards plasma proteins, the amount of drug available to diffuse into the target tissue is significantly reduced and the efficacy of the drug consequently will be poor. Determining the level of binding, therefore, is critical and will directly correlate with in vivo efficacy of the molecule.

A modified ultrafiltration assay was applied to determine the binding affinity of FLUCURE compounds (1 µM) for plasma proteins. The plasma protein binding (PPB) assay indicated that there was a high percentage of binding to the plasma protein (Table 5) for all the NP-NP inhibiting compounds VNFC007, VNFC009, VNFC035 and VNFC037. VNFC009 is a high binding compound when the mouse plasma and human filtrate indicated concentrations below the limit of quantification. VNFC035 and VNFC037 also show a high percentage of being bound (Table 6) to the plasma protein with the exception that concentration levels can now be quantified which clearly confirms a positive development in the optimization process of these compounds.

Test item	**Species**	**Filtrate**		**Retentate**		**Recovery**	**% re-maining (negative control)**
		PPB [%]	**SD**	**PPB [%]**	**SD**		
VNFC007	Mouse	95.65	0.12	96.14	5.86	0.84	114.0
	Human	97.53	0.61	97.99	4.18	0.90	97.7
VNFC009[1]	Mouse	100.00	0.00	100.06	32.47	0.72	90.2
	Human	100.00	0.00	100.47	0.29	0.78	115.7

[1] Mouse plasma and human filtrate: not found, concentration below limit of quantification: high binding compound

Table 5 Plasma protein binding (PPB) assay results for nucleozin (VNFC007) andVNFC009

		Filtrate		Retentate		Recovery	% re-maining (negative control)
Test item	**Species**	**PPB [%]**	**SD**	**PPB [%]**	**SD**		
VNFC0035	Human	97.85	0.46	98.35	5.78	0.92	89.7
VNFC0037	Human	99.49	0.11	99.50	3.23	0.92	94.4

Table 6 Plasma protein binding (PPB) assay results for VNFC035 and VNFC037

Stability of VNFC035 and VNFC037 in human plasma

The stability of test items in body and GI fluids is an important parameter, which strongly can influence the *in vivo* efficacy of a test compound. In plasma, drug candidates can undergo degradation due to enzymatic processes (proteinases, esterases) or undergo intramolecular rearrangement, or bind irreversibly (covalently) to proteins, significantly reducing bioavailability [1]. Thus the investigation of the stability in different matrices, representing models for body and gastro-intestinal fluids should be performed early in drug discovery.

In vitro assays were performed to evaluate the *in vitro* stability of selected FLUCURE compounds in body fluids and aqueous buffered solutions. A standardized test item concentration of 1 µM is applied for all assays.

The physical stability of VNFC035 and VN037 has been evaluated in the presence of human plasma and phosphate buffered saline (PBS). Almost 90% of VNFC035 was intact after 360 mins in human plasma and about the same percentage in PBS. VNFC037 indicated a higher percentage of instability when only 54% of the parent molecule remained intact after 360 mins in human plasma and 64% intact in PBS after the same period of time.

High content screening

The metabolic stability of new chemical entities is an important parameter in the lead optimization process, since the liver is the most important site of metabolism for most drugs. In vitro assays provide information on the involvement of human hepatic CYP enzymes in the metabolism rate of a test item. In the clinic a drug with low metabolic stability requires multiple daily dosing or continuous infusion to reach a concentration in the blood or target organ that is sufficient to provoke a therapeutic effect. On the other hand, a highly metabolically stable compound could accumulate in the organism possibly leading to toxic effects. Thus, metabolic stability of test items affects both efficacy and safety of therapeutics.

In vitro metabolic stability assays were performed to evaluate the metabolic stability of FLUCURE compounds in liver microsomes originating from different species. By default, metabolic stability was tested in mouse (CD-1, pooled, male) and human (pooled, mixed gender) liver microsomes. To allow a classification, the microsomal intrinsic clearance of the test items were predicted and compared to concomitantly investigated values of suitable reference compounds, i.e. and verapamil (phase I metabolism). A standardized test compound concentration of 1 µM was applied for the metabolic stability assays. In each experiment, the positive control substrate Verapamil was tested at 1 µM test concentration for phase I metabolism, respectively (0 and 60 min only).

The internal clearance i.e. the Cl_{int} values for VNFC007 in human and mouse liver microsomes were 123.5 and 356.6 [µl/min/mg] respectively. The rates for VNFC035 in human liver microsomes was 241.9 µl/min/mg. Generally, values between 50-300 µl/min/mg are an accepted rate for metabolic stability. Anything lower than 50 µl/min/mg would lead to an accumulation of the compound in the body and anything higher than 300µl/min/mg would indicate a higher dosage would be needed to

give an effective treatment. The metabolic stability of VNFC009 and VNFC037 indicate extremely high Cl_{int} µl/min/mg rates in the presence of mouse liver microsomes and an even higher rates with the human liver microsomes. This undesirable rapid metabolic rate was already well known in the Gerritz publication [1] and a solution was sought by bioisosterically replacing the isoxazole with a triazole ring. Several triazole compounds have been synthesized by partner 1, unfortunately they have not been evaluated for physico-chemical properties or preclinical *in vitro* studies.

Metabolic assays

Metabolite identification enables early identification of potential metabolic susceptibilities or issues and assists in the prediction of the metabolic pathways of potential drug candidates chosen for development. It provides a metabolism perspective that guides the synthetic route with the aim of either blocking or enhancing metabolism to optimize the pharmacokinetic and safety profiles of newly synthesized drug candidates. Information acquired from metabolite detection and characterization is useful as a guide for efforts of synthetic chemists in the structural modification of new drug candidates. In the late drug discovery and/or candidate selection phase, the metabolite identification is used to determine metabolic differences between species and to identify potential pharmacologically active or toxic metabolites.

A common method in metabolite identification includes the production of metabolites by in vitro or in vivo experiments, collection of samples at different time points and analysis of the samples by using full-scan MS. The metabolites are identified by comparison of the ion chromatograms between a blank and the other samples under consideration of the expected metabolites according to predicted gains and losses in molecular mass of the parent drug. Once molecular ions for possible metabolites are detected, they can be subjected to analysis by MS/MS and the product ion MS/MS spectra of the parent compound can subsequently compared with the corresponding fragmentation pattern of metabolite structures. The specific fragment ion that showed a shift in its m/z helps to identify the site of the modification of the molecule.

Two FLUCURE compounds were analyzed for their metabolites formed during incubations with human liver microsomes. The proposed metabolic pathways of VN035 and VN037 in hepatic microsomes is illustrated in Figure 13 and 15.

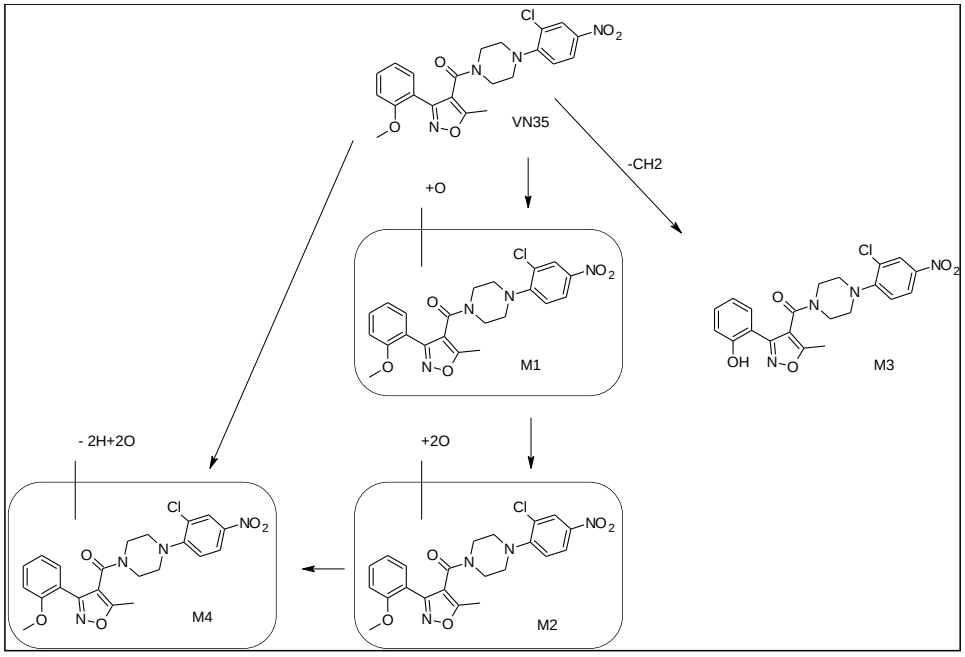

Figure 13 Proposed reaction scheme for metabolite formation from VN035 in human liver microsomal preparations

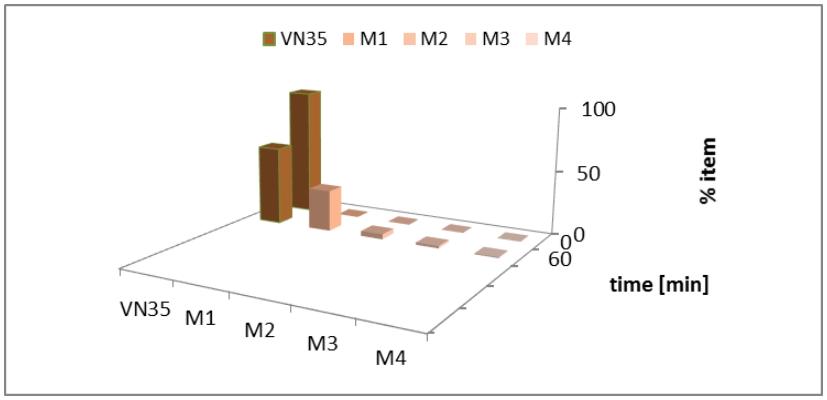

Figure 14 Production rates for metabolite formation from VN035 in human liver microsomal preparations

Almost 62% of VNFC035 remained intact after 60 min in human liver microsomal preparations (Figure 14). The major metabolite of VNFC035 in solution was M1 (Figure 13) at about 32 %, which has been identified to have an additional oxygen atom.

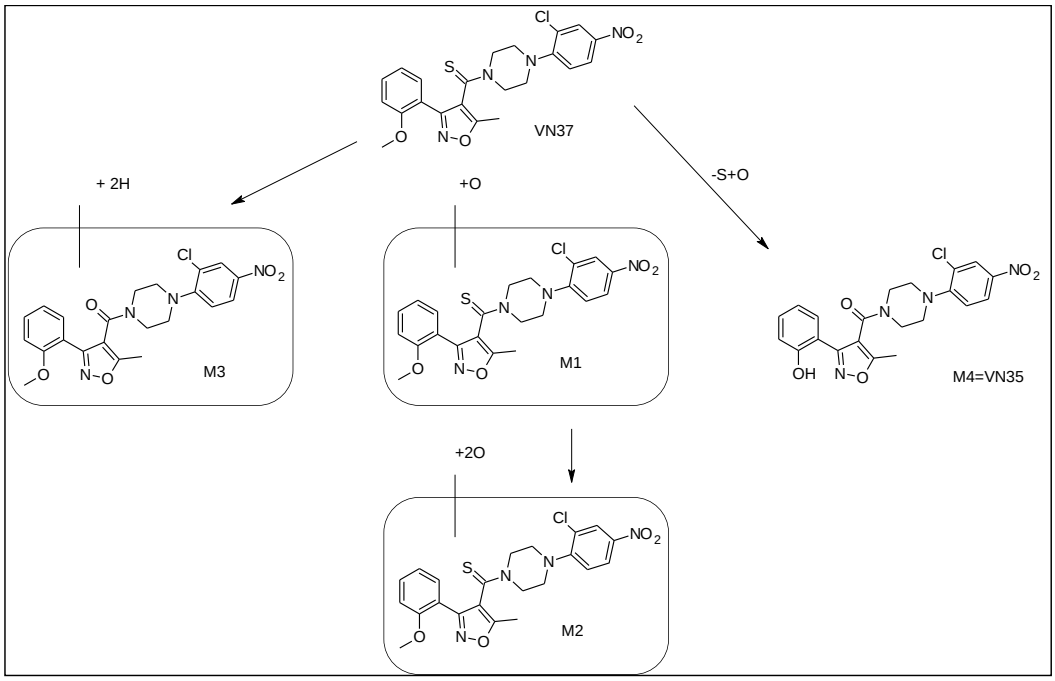

Figure 15 Proposed reaction scheme for metabolite formation from VN037 in human liver microsomal preparations

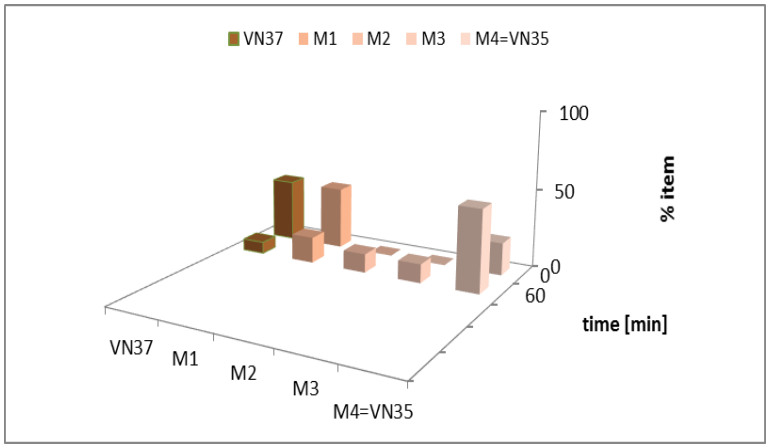

Figure 16 Production rates for metabolite formation from VN037 in human liver microsomal preparations

The major metabolite for VNFC037 formed after 60 min at 52% was VNFC035 (Figure 16). The formation of other metabolites is similar to that of VNFC035 with the addition of oxygen and hydrogen (Figure 15).

Cytotoxicity of nucleozin (VNFC007), VNFC009, VNFC035 and VNFC037

All compounds were shipped to Pharmacelsus by partners and tested with a 48 h exposure time unless otherwise stated. Prior to testing, cell lines of choice were expanded and quality controlled for sufficient viability using flow cytometry. Certain compounds were also subject to ADME and PK studies as shown below. In the toxicity assays, resazurin and ATPlite were used as toxicity indicator.

Assays were seeded into 96-well flat bottom plates and allowed to adhere overnight (for monolayer cultures). After adding several dilutions of the test compounds, viability was determined after 48 h exposure time using resazurin and ATPlite as toxicity indicator systems. The resulting data were corrected for background noise and viability was determined relative to the untreated control. Additional controls were always included to detect potential interference of the test compound with the assay resulting in artifacts (over- or underestimation of effect).

Generally the cytotoxicity results for VNFC007 and VNFC009 in THP-1 human Leukemia and 3T3 mouse cells show little or no cytotoxicity at IC_{50}. The only cytotoxicity to note for VNFC007 and VN009 is in the MDCK canine kidney cells which indicate toxicity at 5uM and 10M respectively.

Compound VNFC035 indicated a cytotoxicity with THP-1 human leukemia cells at 13.84 µM for resazurin and 12.35 µM for ATP lite. VNFC037 also showed cytotoxicity with THP-1 human leukemia cells at 29.4 µM for resazurin and 25.04 µM for ATP lite (Table 7). Results show slightly less cytotoxicity of the thioamide compounds (VNFC009 and VNFC037) compared to the amide compounds (VNFC007 and VNFC035). Other results for the MDCK canine kidney cells were difficult to determine due to problems with solubility.

Metabolic activity with Resazurin	**007**	**009**	**035**	**037**
THP human leukemia	IC50 >100uM	IC50 >100uM	IC50=13,8uM	IC50=29,4uM
3T3 mouse fibroblasts	IC50 >100uM	IC50 >100uM	IC50 >100uM	IC50 >100uM
MDCK canine kidney	IC50 >100uM	IC50 >100uM	Not determined	IC50 >100uM

Energy content of cells with ATPlite	**007**	**009**	**035**	**037**
THP human leukemia	IC50 >100uM	IC50 >100uM	IC50=12,4uM	IC50=25uM
3T3 mouse fibroblasts	IC50 >100uM	IC50 >100uM	IC50 >100uM	IC50 >100uM
MDCK canine kidney	IC50 <5uM*	IC50 <10uM*	Not determined	IC50 >100uM

Table 7 Proliferation/Viability Assays = Cytotoxicity results for VNFC007, VNFC009, VNFC035 and VNFC037

Summary for physico-chemical and ADME-T studies for nucleozin (VNC007), VNFC009, VNFC035 and VNFC037

According to Annex I of the project description, work packages 1, 2, 3, and 4 should work together on a selection of scaffolds/compound series for second half of lead optimization phase.

Previous results from physico-chemical and ADME-T studies of the compounds nucleozin (VNFC007) and VNFC009 indicated that it was necessary to improve these properties before the compounds could be considered for further advanced studies *in vivo*. It has been known in literature [1] that the addition of the methoxy group would greatly improve the solubility of the compound.

Therefore, the compounds were chemically modified with the addition of a methoxy group in the R_1 position to the VNFC isoxazoles, VNFC035 and VNFC037. These were tested for their physico-chemical and ADME-T properties i.e. solubility, permeability, physical and metabolic stability, plasma protein binding (PPB) and cytotoxicity. The results show improvements in all of these properties which confirmed that the second half of the lead optimization phase was indeed advancing forward. One of the most fundamental results that was achieved from WP4 for the VNFC series was that the identification of the major metabolite of VNFC037 was in fact VNFC035. This confirmed for us in future studies that it would be important to understand the physico-chemical and ADME-T properties of the amide derivatives to the same extent as the parent thioamide derivatives.

Several new compounds in the VNFC series have been strategically designed and synthesized on the basis of improving the low scoring physico-chemical and ADME-T assessments we received for VNFC007, VNFC009, VNFC035 and VNFC037. These compounds have been available since January 2013 by partner 1 to be assessed for their physico-chemical and ADME-T properties by partner 8. Unfortunately, no results are available due to the conflict that has developed between all the partners in the FLUCURE consortium.

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Preclinical development

In vivo pharamcokinetics

The work has been focused on planning the activities of the in vivo pharmacokinetic studies and toxicological studies. Partner 1 has been in direct contact with Dr Nicholas Meanwell, an executive director for a global leading pharmaceutical company, Bristol Myers Squibb (BMS). He has been involved with working in pharmaceutical drug development for over 40 years and has authored/co-authored over 430 scientific articles and books in his expertise area of antiviral drug development.

BMS has numerous projects in drug development for infectious diseases such as Hepatitis C virus, HIV and Influenza A. The company has been particularly successful for the development and research of several anti-influenza compounds that are similar to the compounds that have been synthesized by partner 1 in the FLUCURE project. Therefore the anti-influenza scientific articles and patents that have been published by BMS have been a valuable source of information for partner 1 in the development and research of the compounds in the FLUCURE project.

BMS has developed a number of active “triazole” derivatives against influenza. In particular “triazole 5” which is a compound equivalent to VNFC041 in the FLUCURE project. In pharmacokinetic studies they were able to establish that that the Ctrough values at 12 h for both the 10 and 50 mg/kg i.p. doses were below the 70 nM A/WSN EC50 value. Even more interestingly, the Ctrough value at 8 h for the 10 mg/kg dose was estimated to be fivefold above the EC50. This dose of 10mg/kg prompted BMS to perform studies to establish the efficacy in mice and partner 1 accordingly designed a similar study for VNFC051 (see WP3) for proof-of-concept study in a mouse model of influenza.

Partner 1 has been in contact with CROs that are specialized to perform and assess the nonclinical safety program of a candidate drug to support phase 1 clinical studies.