

Synthesis of phosphorous containing CDK9 kinase inhibitors

PhD thesis

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1. Introduction, literature background

The aim of my PhD research was to synthesise novel and effective CDK9 kinase inhibitors, as potential drug candidates against AIDS. This work was done in two laboratories. One of them was at the Rational Drugdesign Laboratory in the Cooperative Research Center of Semmelweis University. The other laboratory was in the École National Supérieur de Chimie de Montpellier in France. Although the majority of the work was done in Budapest, without the ten months long French scholarship was essential to my thesis.

1.1. Cyclin dependent kinases and the CDK9

According to the accumulated knowledge, it is clear how cell cycle is ruled by the activation and deactivation of *Cyclin Dependent Kinases* (CDK). CDKs are serine/threonine kinases. CDKs are stable and active with their essential counterparts with the cyclin subunits. These kinases are validated targets of drug development, since their role has been revealed in several proliferative disorders (e.g.: cancer) and inflammatory diseases. Several types of CDKs exist, with diverse biological roles, consequently different CDKs might be targeted in different kind of malignancies; nevertheless there are only a few molecules that inhibit only one CDK selectively.

The CDK9 is known to be involved in four diseases and it was proposed as a therapeutic target. The first and probably the most important is the HIV-1 infection and the AIDS. The second one is the cardiac hypertrophy. The CDK9 and their cyclin partners activate some anti-apoptotic signals in cancer cells, therefore these cancer cells can be killed by the inhibition of CDK9. Finally, CDK9 is involved in some inflammatory diseases.

The CDK9 forms a dimer with one of their cyclin partners (cyclin (Cyc) T1, T2a and b, K). The 80% of CDK9/CycT complexes are formed with the CycT1, as it is involved in the HIV-1 infection, as well. The most important role of CDK9/CycT1 is the phosphorylation of C-terminal domain of RNA polymerase II. As a consequence of this phosphorylation the RNA polymerase II starts the transcription of the viral genome.

1.2. The HIV virus and the AIDS

During the HIV infection and after the T-cell activation the CDK9/CycT1 is able to phosphorylate the C-terminal domain of RNA polymerase II and the polymerase will synthesise the viral mRNA.

With the exception of viral Tat protein, the other proteins are the members of the transcription machinery of the host cell. The virus hijacks the host cell signalling to turn it to its own benefit. One of the basic hypotheses of my research was that the inhibition of CDK9 could stop or, at least, slow down the HIV propagation.

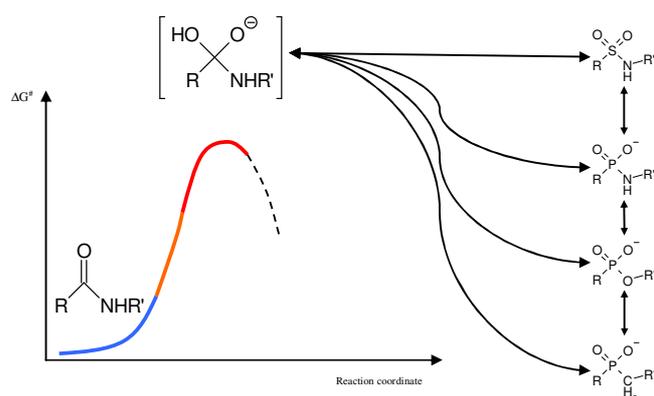
1.3. CDK9 inhibitors based on the (6-phenyl-pyrimidin-4-yl)-phenylamine core structure

As it was described above the CDK9 is a validated target in several diseases, however any specific or at least selective inhibitor had not been available at the beginning of my work. My compounds have been developed from the (6-phenyl-pyrimidine-4-yl)-phenylamine compound family which had already been known as CDK9 inhibitors. The published examples contained only a few basic substitutions on the 6-phenyl ring and on the phenylamine moiety. These molecules, according to the published biological data, have shown higher selectivity to CDK9 than the previously known compounds. We have realised that several substitution possibilities remained unexploited; therefore, I focused on these molecules during my PhD work.

1.4. The transition state analogy and its application

Theory and application of transition state analogy are a few decades old. The most important version, for my thesis, is analogues of the transition state of peptide bond hydrolysis. During the hydrolysis of peptide bond, the carbon atom of carbonyl group switches from sp^2 to sp^3 hybrid state.

This tetravalent state is instable and it stabilises by the brake up of C-N bond. According to the theory of transition state analogy peptidases can be inhibited by such molecules that chemical and electron structure is similar to the tetravalent instable state, meanwhile



they are stable in the given chemical and biological environment. Phosphoramidates have been already used as such peptidase inhibitors 30 years ago.

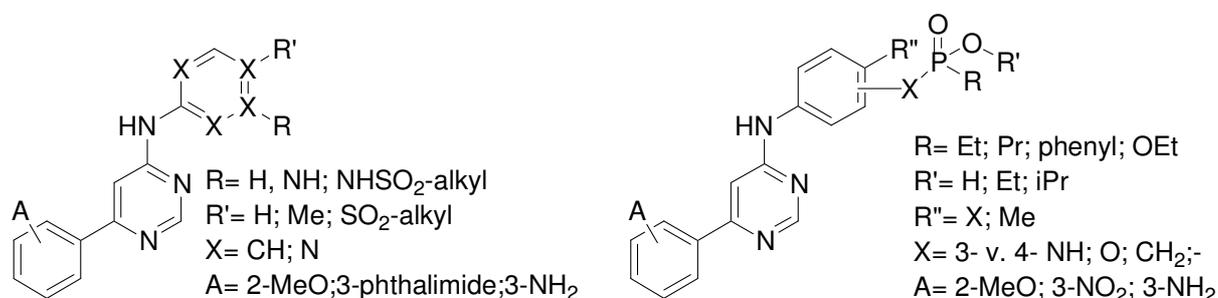
Ten years later W. E. Moree showed in a study that phosphoramidates are the most similar structures to the peptide bond transition state by its electron density. Moreover, she suggested using sulfonamides as transition state analogues. Few years later, she extended her

theory, beside the sulfonamides and phosphoramidates, to phosphonates and phosphinates. Consequently, these four structures are isosters of each other, as well.

2. Aims

My PhD research was directed to synthesise novel and effective CDK9 kinase inhibitors, as potential drug candidates against AIDS. When I joined the Rational Drugdesign Laboratory in the Cooperative Research Center of Semmelweis University my task was the development of a chemical procedure to synthesise 6-phenyl-4-chloro-pyrimidine intermediates from cheap reagents on gram scale. My task was the synthesis of some sulfonamide, sulfonyl and heterocyclic derivatives from the prepared intermediates, as well.

The most important mission of my work was the development of novel phosphorous containing compounds based on the published and prepared sulfonamide type CDK9 inhibitors, with similar biological activity. In accordance with the transition state analogy, I intended to prove the bioisosteric relationship of sulfonamides, phosphoramidates, phosphonates and phosphinates. My objective was to create an effective kinase inhibitor whose phosphorus moiety does not only improve the water solubility, but is essential to its inhibitory effect. Prepared compounds were tested in enzymatic CDK9/CycT1 assay, in first round, than some cellular assays were also performed. The best molecules were tested in cell based HIV proliferation assay.



3. Results and discussion

3.1. Preparation of 4-chloro-6-phenyl-pyrimidines

As a consequence of known biological data we focused on two substituents on the 6-phenyl ring (2-methoxy and 3-nitro) and we developed two different synthetic pathways, with the two substituents, to avoid the Suzuki-reaction.

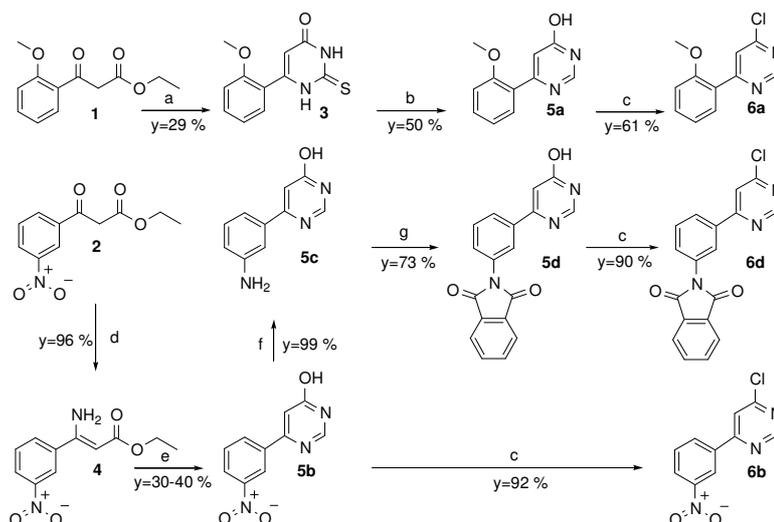


Figure 3-1 Two different pyrimidine syntheses; a = thiourea, EtOH; b = Raney-Nickel, 2 M NaOH; c = SOCl₂ or POCl₃, DMAP, toluene; d = NH₄OAc, EtOH; e = formamidinium acetate, formamide; f = H₂/Pd/C, MeOH; g = phthalic anhydride, pTsOH, DMF.

Development of two synthetic methods was necessary since the substituent of the 6-phenyl ring has a strong effect on the ring formation step. These reactions are not sensitive to the oxygen or humidity; moreover, they do not require special reagent or catalyst. There are two drawbacks of these syntheses compared to the Suzuki-reaction that the starting material determines the substituent of 6-phenyl ring, and the sequence of C-C and C-N bond (the two substituents of pyrimidine) formation is determined.

3.2. Preparation of sulfonamide, sulfonyl and heterocyclic derivatives as CDK9 inhibitors

Having the chloro-pyrimidine intermediates in hand gave me the opportunity to prepare different types of inhibitors. Among the sulfonamides two derivatives have been synthesised: a methyl and a propyl sulfonamide. In both cases the substituent of the 6-phenyl ring was a 3-amino group. The 4-methyl-3-nitro-phenylamine was reacted with the 4-chloro-6-(3-phthalimido-phenyl)-pyrimidin (**6d**). The nitro function should have been reduced to obtain the desired sulfonamides via acylation with the appropriate sulfonic acid chlorides (Fig. 3-2). Previous reduction and protection, with phthalyl moiety, of the amino group of chloropyrimidine was necessary because at this stage I would not have been able to distinguish the two nitro/amino groups from each other at the opposite sides of the molecule. Finally, the nitro group of aniline side was reduced and acylated with methyl and propylsulfonic acid chlorides.

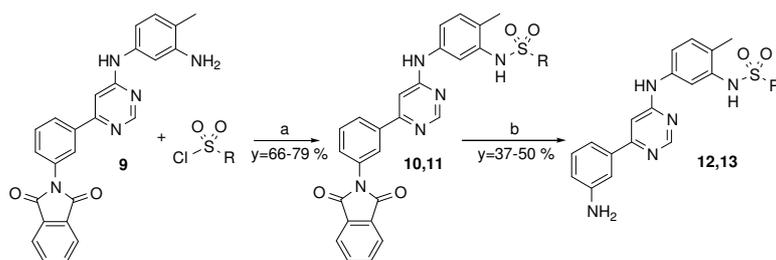


Figure 3-2 Preparation of sulfonamide derivatives; a = dry pyridine, RT; b = hydrazine hydrate, EtOH/DMF; R= methyl or propyl.

Both, the protected and the free amine form were tested against CDK9, *in vitro*. Interestingly, in case of methyl sulfonamide the protected derivative, in case of propyl sulfonamide the free amine was better kinase inhibitor.

no	R	CDK9/CycT1 IC ₅₀ (nM)
10	methyl	39
11	n-propyl	186
12	methyl	129
13	n-propyl	27

In case of sulfonyl derivatives, the strategy of synthesis had to be modified. In this case, the derivatisation of aniline with the sulfonyl moiety had to precede the coupling reaction. Two synthesised

no	R	CDK9/CycT1 IC ₅₀ (nM)
19	dimethylamine	310
20	pyrrolidine	390

anilines (**18a,b**) were coupled with the 4-chloro-6-(2-methoxyphenyl)-pyrimidine **6a** (Fig. 3-3.). The two obtained products (**19, 20**) were tested in enzymatic CDK9 assay

To obtain some heterocyclic derivatives the 2-, 3-, 4-amino-pyridine, the 2-amino-pyrimidine and the 2,4-diamino-[1,3,5]triazine were coupled with the 4-chloro-6-(2-methoxyphenyl)-pyrimidine (**6a**). Pyridines and pyrimidine derivatives were commercially available, meanwhile the triazine derivative had to be prepared in one step from 2-chloro-4,6-dinitro-[1,3,5]triazine. According to enzymatic assay results, the positions and the number of heteroatoms have influence on CDK9 inhibitory potential.

no	Heterocycle	CDK9/CycT1 IC ₅₀ (nM)
27	2-pyridyl	42
28	3-pyridyl	174
29	4-pyridyl	144
30	2-pyrimidinyl	>1 000
31	4-amino-[1,3,5]- -triazin-2-yl	355

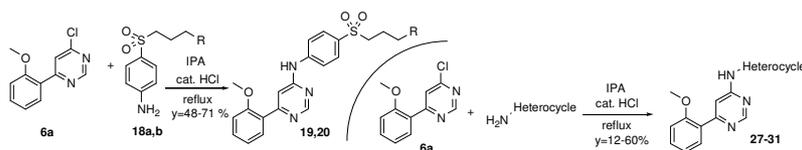


Figure 3-3 Synthesis of sulfonyl and heterocyclic derivatives, R= dimethylamine v. pyrrolidine; Heterocycle= 2-, 3-, 4-amino-pyridine; 2-amino-pyrimidine; 2,4-diamino-[1,3,5]triazine.

3.3. Synthesis of phosphonamidate containing inhibitors (Ar-N-P)

During the synthesis of sulphur containing inhibitors, a frequent problem arisen: some of the compounds had poor water solubility. This phenomenon caused difficulties during the biological tests. To overcome this problem the idea of application of different phosphorous containing functional groups emerged. As it was described in chapter 1.4, sulfonamides may have a bioisosteric relation with phosphonamidates. From the two presented sulfonamides, the propyl derivative was chosen as model compound. In case of phosphonamidate only one thing was changed to simplify the synthesis: the 3-amino substituent of the Suzuki-side was replaced with a 2-methoxy group. The propyl derivative was chosen instead of methyl because, according to own experiments and literature data, the bigger substituent is introduced to the phosphorus atom, the more stable molecule is obtained. Beside the propyl derivative synthesis of ethyl and phenyl derivatives were also planned.

In the synthesis of phosphonamidates, the same strategy can be applied as in case of sulfonamides: the entire ring system was prepared with the aniline function than it was acylated with the appropriate phosphonic acid chloride. The synthesis of the latter can be performed in two steps: first, the substituted phosphonic acid esters were prepared by the Arbuzov or Michaelis-Arbuzov reaction of ethyl or propyl-bromide and triethyl or triisopropyl-phosphite, respectively. The phenyl-phosphonate is commercially available. In the second step, a chlorination reaction had to be applied to transform specifically only one ester function into acid chloride. This reaction can be carried out with oxalyl chloride at ambient temperature.

After the acid chloride was in hand the aniline was acylated with it in dry pyridine. Despite of the very low yields, the phosphonamidates were isolated (Fig. 3-4). The low yield was predictable from literature data in view of the fact that the P-N bond of phosphonamidates can be hydrolysed very easily. The inhibitory effect of the three prepared phosphonamidates was determined. These results show that phosphonamidates are significantly weaker inhibitors of CDK9 than the sulfonamides.

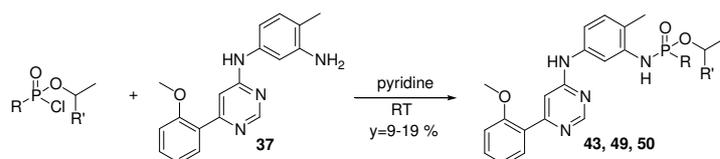


Figure 3-4 Synthesis of phosphonamidates.

no	R	R'	CDK9/CycT1 IC ₅₀ (nM)
43	propyl	Me	3 170
49	ethyl	H	1 450
50	phenyl	H	5 240

3.4. Synthesis of phosphonate containing inhibitors (Ar-O-P)

With the design of phosphonates synthesis I was in an easy situation since the reactive phosphonates intermediates were available, however the phenol derivatives of the already used anilines had to be synthesised. These phenols were easily prepared by the coupling reaction of the 5-amino-2-methyl-phenol and the chloro-pyrimidines, obtaining the desired product only (**52**, **53**).

In case of 2-methoxy substituent on the Suzuki side, the phenol can be acylated in the presence of NaH or KOtBu. In case of 3-nitro substituent only KOtBu can be applied (Fig. 3-5).

no	A	R	R'	CDK9/CycT1 IC ₅₀ (nM)
52	2-MeO	-	-	381
54a	2-MeO	ethyl	H	813
54b	2-MeO	propyl	Me	3 057
54c	2-MeO	phenyl	H	4 137
55a	3-NO ₂	ethyl	H	>10 000
55b	3-NO ₂	propyl	Me	>10 000
56a	3-NH ₂	ethyl	H	870
56b	3-NH ₂	propyl	Me	5 385

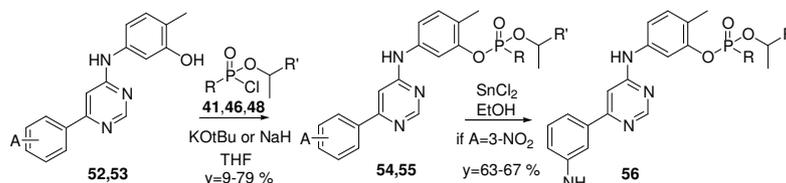


Figure 3-5 Acylation of phenols with phosphonic acid chlorides; R=ethyl (**a**), propyl (**b**), phenyl (**c**); A=2-methoxy (**52**, **54**), 3-nitro (**53**, **55**), 3-amino (**56**).

Analogue of phosphonamides described in the previous chapter had been synthesised. In addition, given that the practicability and yield of phosphonates synthesis was so much better compared to phosphonamides, some other phosphonate derivatives were prepared, as well. The phosphonate moiety was not changed, but the position of OH group was varied (Fig. 3-6).

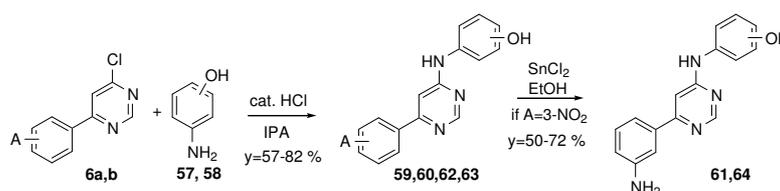


Figure 3-6 Synthesis of other phenols in meta (**57**, **59**, **60**, **61**) and para (**58**, **62**, **63**, **64**) positions; A=2-methoxy (**59**, **62**), 3-nitro (**60**, **63**), 3-amino (**61**, **64**).

The prepared phenols were acylated with phosphonic acid chlorides as it was described above (Fig. 3-7).

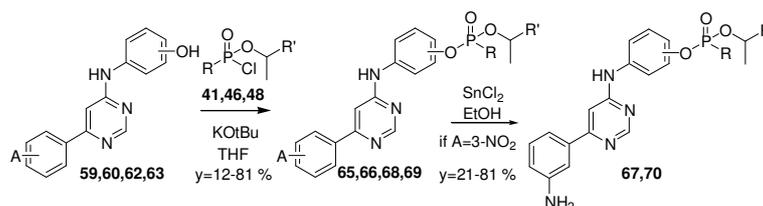


Figure 3-7 Synthesis of meta (**59**, **61**, **65**, **66**, **67**) and para (**62**, **64**, **68**, **69**, **70**) positioned phosphonates; R=ethyl (**a**), propyl (**b**), phenyl (**c**); A=2-methoxy (**59**, **62**, **65**, **68**), 3-nitro (**66**, **69**), 3-amino (**67**, **70**).

no	A	R	R'	CDK9/CycT1 IC ₅₀ (nM)	no	A	R	R'	CDK9/CycT1 IC ₅₀ (nM)
59	2-MeO	-	-	224	62	2-MeO	-	-	70
61	3-NH ₂	-	-	877	64	3-NH ₂	-	-	651
65a	2-MeO	ethyl	H	400	68a	2-MeO	ethyl	H	360
65b	2-MeO	propyl	Me	1 180	68b	2-MeO	propyl	Me	407
65c	2-MeO	phenyl	H	3 100	68c	2-MeO	phenyl	H	2 840
66a	3-NO ₂	ethyl	H	>10 000	69a	3-NO ₂	ethyl	H	>10 000
66b	3-NO ₂	propyl	Me	>10 000	69b	3-NO ₂	propyl	Me	>10 000
66c	3-NO ₂	phenyl	H	>10 000	70a	3-NH ₂	ethyl	H	3 840
67a	3-NH ₂	ethyl	H	1 800	70b	3-NH ₂	propyl	Me	6 060
67b	3-NH ₂	propyl	Me	6 430					
67c	3-NH ₂	phenyl	H	5 330					

With two examples, the specific hydrolysis of alkyl phosphonate esters was completed

(Fig. 3-8).

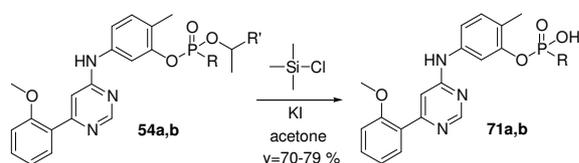


Figure 3-8 Specific hydrolysis of alkyl esters.

no	R	R'	CDK9/CycT1 IC ₅₀ (nM)
54a	ethyl	H	813
71a		-	1 490
54b	propyl	Me	3 057
71b		-	1 457

Some evident structure-activity relationships were revealed after determination of the inhibitory effects of compounds. Molecules containing nitro group are practically inactive; comparing the molecules possessing 2-methoxy or 3-amino groups the biological activity of the latter is significantly lower. The substituents of the phosphorus have an obvious effect on biological activity: the CDK9 inhibition potential is decreasing with the ethyl, propyl, phenyl order. Position of the phosphonate does not influence considerably the inhibitory effect because this is negligible beside the influence of the two factors above. Interestingly, the hydrolysis of the alkyl ester, in case of ethyl-phosphonate, decreased, in case of propyl-phosphonate, increased the biological activity, however the differences are not large.

3.5. Synthesis of phosphinate containing inhibitors (*Ar-CH₂-P*)

Besides phosphoramidates and phosphonates, the third isosteric group is the phosphinates. For their synthesis, the Pudovik reaction seemed to be appropriate, at first attempt, since many benzaldehydes are commercially available. Phosphinates isosteric with the already prepared phosphoramidates and phosphonates can be obtained with the reaction of the appropriate nitro-benzaldehydes and H-phosphinates. Given that the preparation of H-phosphinates is not easy, first, the suitability of Pudovik reaction was tested with diethyl phosphite. The Pudovik adduct was formed with 3-nitro-benzaldehyde and 4-nitro-

benzaldehyde. The resulting hydroxyl group was mesylated then the mesyloxy group was eliminated via a catalytic hydrogenation, and the nitro group was reduced in the same time. If the mesylation of hydroxyl group was skipped and the hydrogen donor was ammonium formate, an aniline was obtained which still has the α -hydroxy function group (Fig. 3-9).

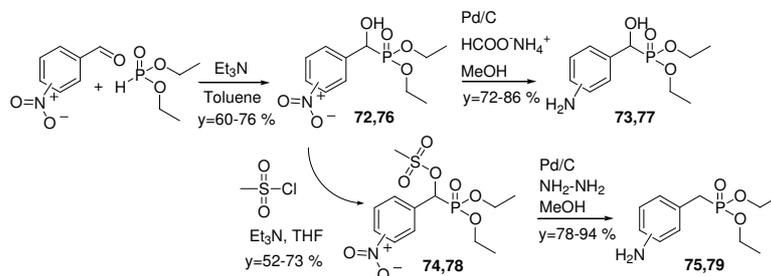


Figure 3-9 Synthesis of meta (**72,73,74,75**) and para (**76,77,78,79**) benzyl-phosphonate anilines.

Preparation of that aniline which has a methyl group in position 4 is not possible due to the lack of the aldehyde. This problem was solved by the synthesis shown on Figure 3-10.

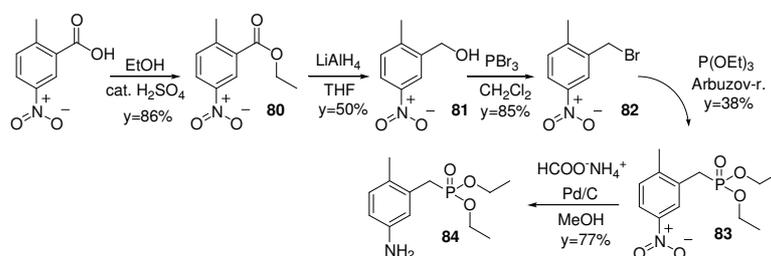


Figure 3-10 Synthesis of aniline which has a methyl group in position 4.

Biological activity of prepared compounds (Fig. 3-11 and Fig. 3-12) was determined and in some cases, very low IC_{50} s were measured. Considering that these compounds were prepared as model compounds only, to try these reactions; they have impressive biological effect.

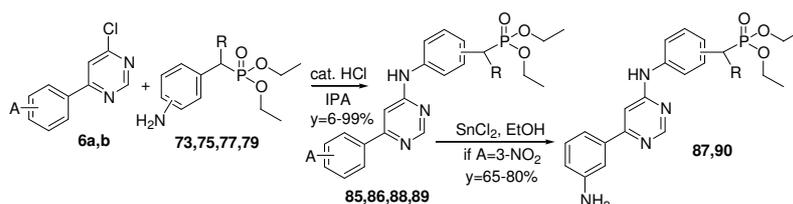


Figure 3-11 Synthesis of meta (**85, 86, 87**) and para (**88, 89, 90**) benzyl-phosphonates; R=H (**a**), OH (**b**); A=2-methoxy (**85, 88**), 3-nitro (**86,89**), 3-amino (**87, 90**).

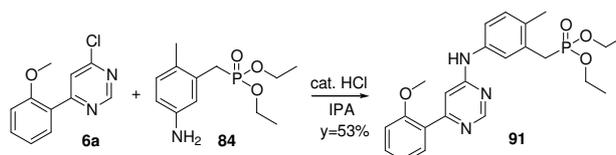


Figure 3-12 Synthesis of benzyl-phosphonate which has a methyl group in position 4.

The most important model compound, in case of phosphoramidates and phosphonates, was the derivative with the 4-methyl substituent. Its analogue from this group, compound **91**, is only a mediocre inhibitor of CDK9, which is slightly behind of our expectations.

Hydrolysis of phosphonate esters was accomplished with two examples. In this case, the situation was more simple than before, since two similar alkyl ester groups were cleaved in the same time. This hydrolysis was performed in 5 M aqueous HCl solution under reflux. The hydrochloride salts, formed in the reaction, were converted to free bases by a chemical method with propylene oxide (Fig. 3-13).

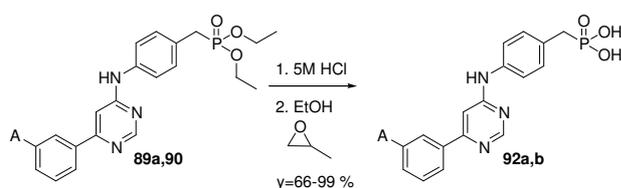


Figure 3-13 Hydrolysis of two phosphonates; A=NO₂ **89a** → **92a**; A=NH₂ **90** → **92b**.

As it was expected, biological activity of compounds was increased. In case of nitro group (**89a**→**92a**) the inactive ester became a moderate inhibitor. In case of aniline group (**90**→**92b**) the change was not so impressive, however the decrease of IC₅₀ value was still significant.

These compounds do not fit into the previously described sequence, started with phosphoramidates and phosphonates, but their phosphinate derivatives. For the preparation of these compounds H-phosphinates should be prepared (only the phenyl-H-phosphinate is commercially available) as it was described at the beginning of this chapter. Although reactions of nitro-benzaldehydes and H-phosphinates were successful, reduction of nitro group failed in each cases.

This problem was avoided similarly to the case of compound **91** (Fig. 3-10): the nitro-benzylbromide was reacted with the phosphonite intermediate of H-phosphinate synthesis in Arbuzov reaction. Reduction of nitro group was performed with thin(II)chloride in each cases (Fig. 3-14).

no	A	R	CDK9/CycT1 IC ₅₀ (nM)
85	2-MeO	H	333
86a	3-NO ₂	H	9 870
86b	3-NO ₂	OH	>14 000
87a	3-NH ₂	H	179
87b	3-NH ₂	OH	1 401
88a	2-MeO	H	490
88b	2-MeO	OH	510
89a	3-NO ₂	H	>14 000
89b	3-NO ₂	OH	4 577
90	3-NH ₂	H	3 110
91	2-MeO	-	5 220

no	A	CDK9/CycT1 IC ₅₀ (nM)
89a	NO ₂	>14 000
92a		2 100
90	NH ₂	3 110
92b		1 130

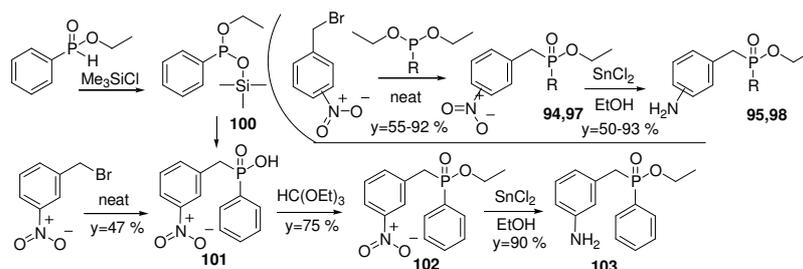


Figure 3-14 Synthesis of meta (**94,95,103**) and para (**97,98**) benzyl-phosphinate anilines, R= ethyl (a), propyl (b).

Isolated anilines were coupled with 4-chloro-6-phenyl-pyrimidines (Fig. 3-15), and then CDK9 inhibition potential of prepared compounds were determined.

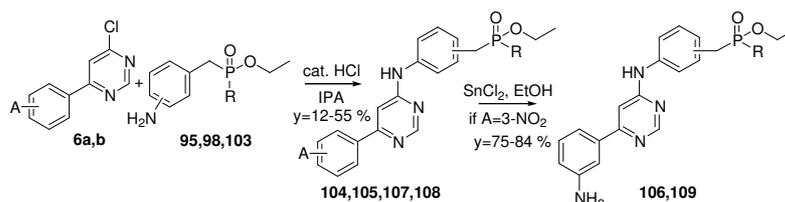


Figure 3-15 Synthesis of meta (**104, 105, 106**) and para (**107, 108, 109**) benzyl-phosphinates; R=ethyl (a), propyl (b), phenyl (c); A=2-methoxy (**104, 107**), 3-nitro (**105, 108**), 3-amino (**106, 109**).

no	A	R	CDK9/CycT1 IC ₅₀ (nM)	no	A	R	CDK9/CycT1 IC ₅₀ (nM)
104a	2-MeO	ethyl	188	107a	2-MeO	ethyl	743
104b	2-MeO	propyl	296	107b	2-MeO	propyl	652
104c	2-MeO	phenyl	517	108a	3-NO ₂	ethyl	7 774
105a	3-NO ₂	ethyl	6 432	108b	3-NO ₂	propyl	5 525
105b	3-NO ₂	propyl	8 056	109a	3-NH ₂	ethyl	7 533
105c	3-NO ₂	phenyl	>12 500	109b	3-NH ₂	propyl	7 474
106a	3-NH ₂	ethyl	327				
106b	3-NH ₂	propyl	682				
106c	3-NH ₂	phenyl	1 019				

According to *in vitro* results further substitution of the phosphorus atom, phosphinate instead of phosphonate, significantly increase the effectivity of compounds. Among the 2-methoxy-phenyl derivatives the ethyl and the propyl substitution do not make difference in the activity, both molecules has IC₅₀ lower than 300 nM; in case of phenyl substituent the measured IC₅₀ under 1 μM is also an outstanding value compared with the similar compounds. The 3-nitro-phenyl derivatives are almost inactive in accordance with our experiences. The 3-amino-phenyl derivatives are very good inhibitors compared to the already described molecules.

The initial objective was to synthesise such a molecule, which contains, at the aniline side, a methylene-phosphinate moiety in the meta position and a methyl group in the para position. Synthesis of aniline was performed similarly to compound **91** from the 2-(bromo-

methyl)-1-methyl-4-nitrobenzene (**82**) and the ethyl-phosphonite in Arbuzov reaction (Fig. 3-16). Difference was only in the longer reaction time and the lower yield (Fig. 3-17).

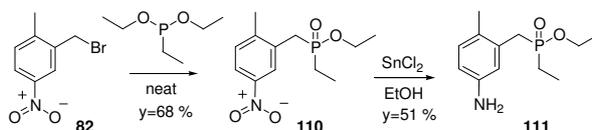


Figure 3-16 Synthesis of the benzyl-phosphinate aniline containing a 4-methyl substituent.

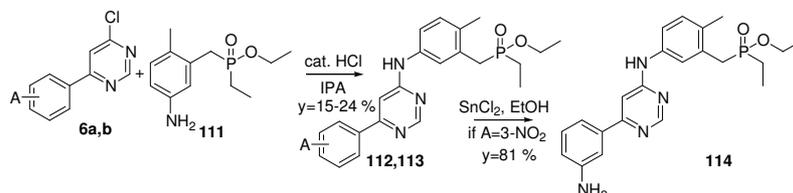


Figure 3-17 Synthesis of the benzyl-phosphinate kinase inhibitors containing a 4-methyl substituent.; A=2-methoxy (**112**), 3-nitro (**113**).

Although their synthesis is difficult, these derivatives are very good CDK9 inhibitors. A 2-methoxy (**112**) and the 3-amino (**114**) derivatives show similar biological activity which is slightly behind the effect of the best molecules.

no	A	CDK9/CycT1 IC ₅₀ (nM)
112	2-MeO	366
113	3-NO ₂	7 022
114	3-NH ₂	346

3.6. Synthesis of aryl-phosphinate containing inhibitors (Ar-P)

An idea was arisen that phosphonate and phosphinate moieties should be connected directly to the aromatic ring without the NH, the O or the methylene groups. These molecules would be restrictedly isosteric with the sulfonamides; however, they can provide key information to the structure-activity relationship.

Synthesis of these derivatives was accomplished with a palladium catalysed nucleophile substitution. To enhance the electrophilic property of aryl-halogenides (iodine or bromine) a complex was formed with palladium(0). Nucleophilic agents were diethyl-phosphite and alkyl and the phenyl H-phosphinate (Fig. 3-18).

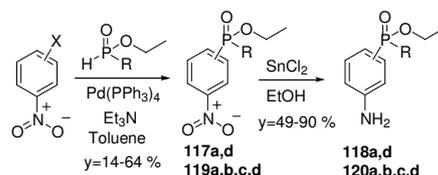


Figure 3-18 Synthesis of meta (**117**, **118**) and para (**119**, **120**) phenyl-phosphinates and phosphonates; R= ethyl (a), propyl (b), phenyl (c), ethoxy (d).

With the molecules synthesised from H-phosphinates I intended to investigate the effect of substituents of phosphorus to the biological activity, meanwhile with the molecules

synthesised from diethyl phosphite I aimed to map the difference between the phosphonic esters and acids.

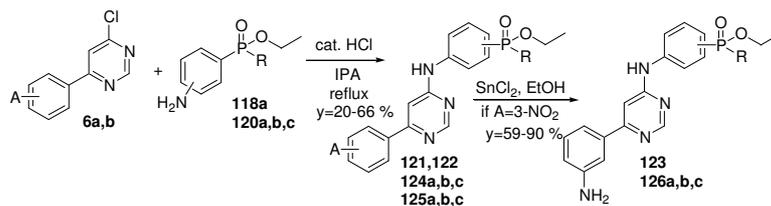


Figure 3-19 Synthesis of meta (**118**, **121**, **122**, **123**) and para (**120**, **124**, **125**, **126**) phenyl-phosphinates; R=ethyl (a), propyl (b), phenyl (c); A=2-methoxy (**121**, **124**), 3-nitro (**122**, **125**), 3-amino (**123**, **126**).

Phenyl-phosphinates did not bring resounding success in terms of biological activity. Interestingly, para derivatives were more active inhibitors than meta ones. When the R group was phenyl, only the para derivatives were prepared, the molecules were exceptional good inhibitors of CDK9. The 2-methoxy-phenyl (**124c**) derivative has lower IC_{50} than the same R= ethyl phosphinate (**124a**). In addition, the 3-amino-phenyl derivative was proven to be better inhibitor in the same context (**126c** and **126a**).

no.	A	R	CDK9/CycT1 IC_{50} (nM)
121	2-MeO	ethyl	842
122	3-NO ₂	ethyl	>12 500
123	3-NH ₂	ethyl	9 802
124a	2-MeO	ethyl	418
124b	2-MeO	propyl	1 149
124c	2-MeO	phenyl	523
125a	3-NO ₂	ethyl	>12 500
125b	3-NO ₂	propyl	>12 500
125c	3-NO ₂	phenyl	>12 500
126a	3-NH ₂	ethyl	>12 500
126b	3-NH ₂	propyl	>12 500
126c	3-NH ₂	phenyl	6 501

Diethyl ester groups were hydrolysed under acidic conditions, as it was described previously; therefore, phosphonic acids were isolated easily.

no	A		CDK9/CycT1 IC_{50} (nM)
127a	2-MeO	ester	1 040
127b	2-MeO	acid	170
128a	3-NO ₂	ester	>10 000
128b	3-NO ₂	acid	4 100
129a	2-MeO	ester	1 020
129b	2-MeO	acid	580
130	3-NO ₂	ester	>10 000
131a	3-NH ₂	ester	>10 000
131b	3-NH ₂	acid	3 320

It was revealed by *in vitro* assays that phosphonic acids have significantly lower IC_{50} s than esters. This phenomenon is the most conspicuous in case of A= 3-nitro (**128a,b**). Up to now, presented compounds with nitro group were inactive; after the hydrolysis of ester groups observable inhibitory results were obtained. The most interesting data is the IC_{50} of compound

127b, which has the lowest value among the phosphorus containing compounds.

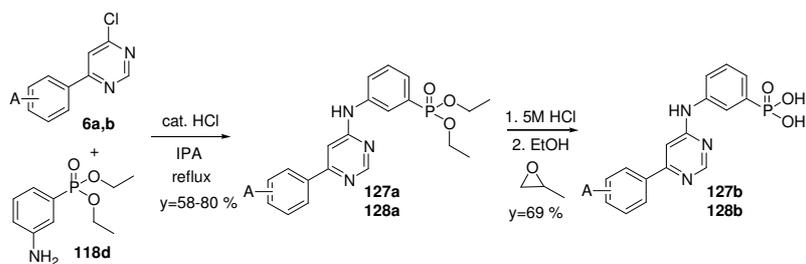


Figure 3-20 Synthesis and hydrolysis of meta positioned phenyl-phosphonates.

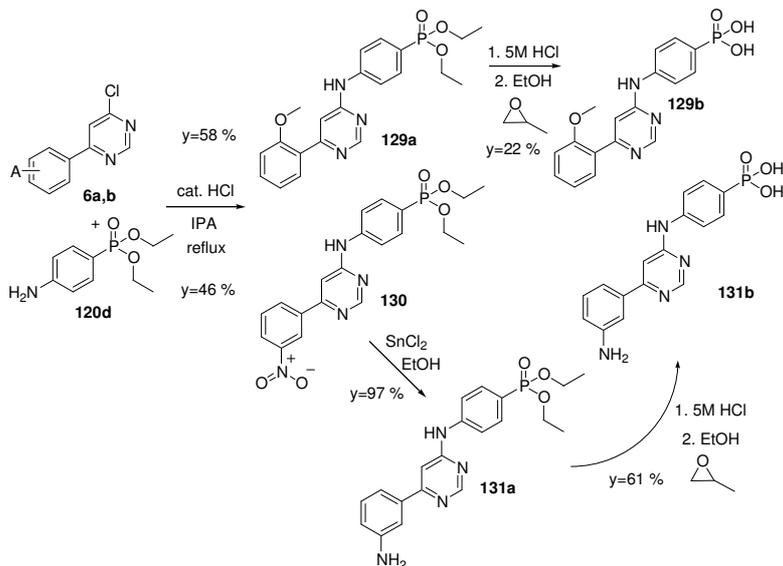


Figure 3-21 Synthesis and hydrolysis of para positioned phenyl-phosphonates.

3.7. Other biological tests

3.7.1. Determination of K_i

The already measured IC_{50} values are not universal, comparable values in various assays, but true only in the given experimental system. The universal measure is the value of K_i , which is the equilibration constant of association and dissociation of the inhibitor and the enzyme. This value depends only on the inhibitor and the enzyme; it is independent from any other parameters, therefore it provides a realistic comparison of biological activity of two molecules. The exact calculation of K_i is relatively complicated; for that reason, I do not go into the details. Meanwhile there is a simple rule for the ATP competitive inhibitors (Cheng-

Prusoff equation $K_i = \frac{IC_{50}}{1 + \frac{[ATP]}{K_m}}$): if during

no	IC_{50} (nM)	$K_i=IC_{50}/2$ (nM)	K_i (nM)
104a	188±53	94	115±17
104b	296±11	148	153±30

the IC_{50} determination the concentration of ATP is equal with the K_m , the value of K_i is half of the IC_{50} . Values calculated with the two methods show good correlation.

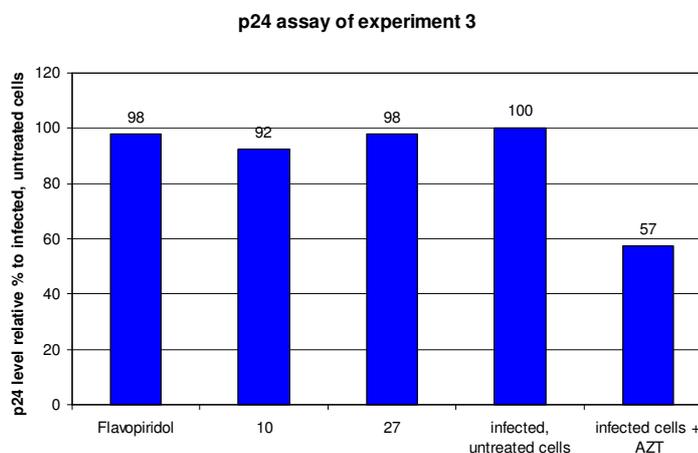
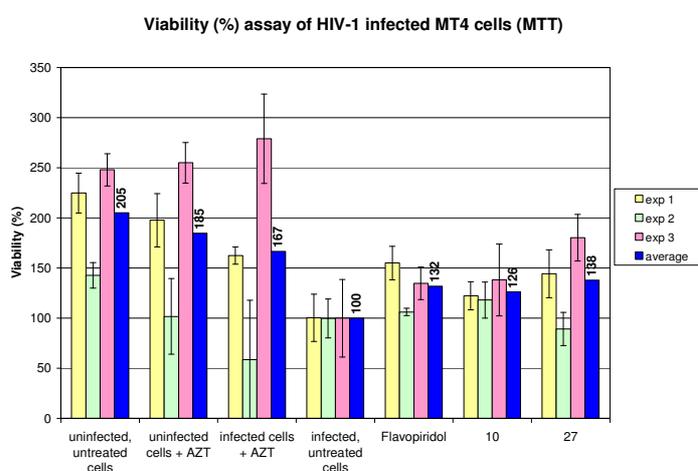
3.7.2. HIV proliferation assay

This experiment is a viability assay on HIV infected cells. If the tested compound inhibits virus proliferation, more living cells will be observed. This assay was a filter to preselect compounds that have any positive effect on infected cells. Experiments should be divided into two groups: 1. compounds without phosphorus atom (**10**, **27**); 2. compounds containing phosphorous atom (**49**, **54a**, **56a**, **71a**). Members of the second group did not show any curative effect on HIV infected cells; therefore, detailed description of results is not essential.

In case of two non-phosphorous molecules, cells were treated with 2.5 μM compound concentration. Reference compounds were flavopiridol (in 0.25 μM) and AZT (azidothymidine; in 0.025 μM). Viability assay was repeated three times, the calculated average was the basis of the comparison.

The results showed that both compounds have the same effect as flavopiridol (one of the most cited CDK9 inhibitor), and have a slightly weaker effect than the AZT. From these results, it cannot be concluded whether our compounds inhibited the virus propagation, or not. Nevertheless, it can be clearly stated that the compounds had a protective effect.

To find out whether the compounds inhibit the HIV virus propagation, the quantity of produced p24 protein should be determined. As it is known, p24 protein is a component of HIV capsid; its quantity is proportional to the amount of virus growth. Both compounds and flavopiridol reduced the quantity of p24 protein by a few percent. We hypothesise that the reason of this result was the high virus concentration. This assumption is supported by the mediocre effect of AZT.



According to the above-mentioned experiments, compound **10** and **27** are supposed to inhibit virus propagation and promote the survival of infected cells. To clarify, to what extent this effect depends on the inhibition of CDK9 or on the interaction with other human or viral biomolecules, requires further investigations.

4. Conclusions

My PhD research was focused on the synthesis of novel and effective CDK9 kinase inhibitors, as potential drug candidates against AIDS. The intermediate synthesis developed at the beginning of my work, provided me a simple tool to prepare 4-chloro-6-(substituted-phenyl)-pyrimidines in large amount. This synthesis was published in a short communication.

With the use of intermediates, some sulfonamide, sulfonyl and heterocyclic derivatives were prepared. These molecules were tested in enzymatic assays. Kinase selectivity profile and cell toxicity of the best compounds were investigated. Inhibition of HIV proliferation was also studied with two selected compounds. According to these experiments, it can be stated that the investigated molecules selectively inhibit the function of CDK9/ciklinT1 *in vitro*, this is unique in the literature. Based on the measurements these compounds are not toxic to the cell lines tested. In case of the two selected compounds, their potential to inhibit HIV-1 propagation was determined with an indirect and a direct method. Both ways showed that these compounds have curative effect on infected cells: they increased the number of surviving cells and decreased the production of viral proteins so they inhibited the virus growth.

According to the transition state analogy, I assumed that phosphonamidates, phosphonates and phosphinates would have similar biological effect to the isosteric sulfonamides, consequently they would be bioisosters. Moreover, I supposed that phosphorus containing compounds would have better pharmacokinetic properties thanks to their enhanced water solubility. Unfortunately, I did not have the opportunity to validate this last hypothesis; however, it might be proven in a later stage of drug research.

This part of the work was started with the synthesis of phosphonamidates that were prepared with a very low yield. Their biological activity is significantly weaker than that of the sulphonamides. Due to synthetic difficulties and not very promising biological results, my attention was preferably focused on synthesis of phosphonates. Synthetic feasibility of this compound family is much more favourable; therefore, 23 derivatives were prepared. Biological activity of the best molecules was near to the effect of sulfonamides. Structure-activity relationships were studied on the prepared compounds. It was concluded how the

substituents of phosphorus, the position of phosphonate moiety or the substituents on the Suzuki-side influence the *in vitro* activity. The alkyl ester of the phosphonate was selectively hydrolysed in two cases. This step, however, failed to clarify whether the ester or the acid is better inhibitor, since the activity increased in one case and it decreased in the other case .

Four compounds from the groups of phosphoramidates and phosphonates were tested in cell toxicity assay and their ability to inhibit HIV proliferation was examined in the indirect viability assay, as well. Unfortunately, they were slightly toxic on the tested MT4 cell line and they did not increase the number of surviving cells.

The third isosteric group is the group of phosphinates, which is rather ambivalent from the chemistry point of view. Some derivatives were easily synthesised with good yields, however synthesis of some others was very difficult. Besides the 18 phosphinates, 11 benzyl-phosphonates were synthesised. Several compounds from this molecular family inhibit the CDK9 in low concentration (IC_{50} = 150-300 nM). Although these values are above the effect of the best non-phosphorus compounds (IC_{50} < 50 nM), they are lower by one order of magnitude than IC_{50} of the firstly synthesised phosphoramidates (IC_{50} = 1 500-5 250 nM).

The biological activity of ester and acid type benzyl-phosphonates was investigated on two examples. It is evident that the acid is significantly better inhibitor than the ester, *in vitro*.

Synthesis of phenyl-phosphinates and phosphonates was not included among the initial goals. These molecules are only restrictedly isosteric with the sulfonamides. Synthesis of these derivatives was accomplished with a palladium catalysed nucleophile substitution. Similarly to the phosphinates: some derivatives were easily synthesised, however synthesis of some others was very difficult. Some of the phenyl-phosphinates have IC_{50} lower than 1 μ M, however, in general, these are not the most promising CDK9 inhibitors.

With the synthesis of phenyl-phosphonates, my aim was to investigate the difference between the biological activity of esters and acids. It is clear by the successful hydrolyses that phosphonic acids are always better inhibitors than phosphonic esters.

A structure-activity relationship is true for all the four molecular groups: the ethyl substituted phosphoramidate, phosphonates, phosphinates are the best inhibitors independently from the rest of the molecule. The propyl derivatives are usually a bit weaker inhibitors; the phenyl substitution exacerbates the biological effect. Examinations of variations on the Suzuki-side show that the 2-methoxy substitution is the best one. The 3-amino moiety is a bit weaker than the 2-methoxy; however there are some outstanding results. Molecules with the 3-nitro moiety are inactive apart from a few exceptions.

With two molecules, it was proven by experiments that they act in an ATP competitive manner. For these compounds, the value of K_i was determined, as well.

During the design of phosphorus containing molecules, my intention was to construct an efficient kinase inhibitor whose phosphorus moiety is crucial to its inhibitory effect, not only improves the water solubility. According to biological data obtained so far, it can be assumed, by two considerations, that it was succeeded. The first consideration is the comparison of intermediates without phosphorus moiety to the final products: it is easy in case of phosphoramidates and phosphonates. Biological activities of the corresponding anilines and phenols are better or, more or less, equal to the phosphorus containing final products. In case of phosphinates, this comparison is more difficult, since molecules similar to their hypothetical intermediates were not mentioned in the thesis. However, some examples were charted in my article published in the *Current Medicinal Chemistry*. Since the kinase inhibitory effects of phosphorus containing molecules, with some exceptions, are weaker inhibitors than the non-phosphorous intermediates, I suppose that the function of phosphorus moiety is not the improvement of water solubility as a prodrug.

The second consideration is the examination of chemical stability of phosphorus moiety. In case of phosphoramidates, it is probable that the P-N bond brakes up during the enzymatic reaction. In this case the low IC_{50} of aniline would be expected, however it is not the case. In case of phosphonates, hydrolysis of P-O bond might be occurred, however very good kinase inhibitory effects of phenols were not observed. In case of phosphinates and aryl-phosphinates and phosphonates, the possibility of hydrolysis can be excluded.

Further optimisation of pharmacological properties of these novel CDK9 inhibitors is possible by the exploitation of additional substitutions on both the Suzuki and the aniline sides. Improvement of biological activity may possible by the separation of enantiomers or by enantioselective synthesis, which topic has not been touched, yet.

5. List of own publications

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B Hegymegi-Barakonyi, R Székely, Z Varga, R Kiss, G Borbély, G Németh, P Bánhegyi, J Pató, Z Greff, Z Horváth, G Mészáros, J Marosfalvi, D Erős, C Szántai-Kis, N Breza, S Garavaglia, S Perozzi, M Rizzi, D Hafenbradl, M Ko, Y Av-Gay, BM Klebl, L Órfi, G Kéri; Signalling Inhibitors Against *Mycobacterium tuberculosis* – Early Days of a New Therapeutic Concept in Tuberculosis. *Current Medicinal Chemistry*, **15**(26), 2760-2770, (2008).

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Patent: Greff Z, Varga Z, Kéri G, Németh G, Órfi L, Szántai-Kis C; 4-Phenylamino-pyrimidine derivatives having protein kinase inhibitor activity. PCT Int. Appl. WO 2011/077171, **2011**; *Chem. Abstr.* **2011**, 155,152538.

Book chapter: Protein Kinases as Drug Targets; (ed.: Klebl B.; Gerhard M.; Michael H.); G Kéri, L Órfi, G Németh: chapter 4: Rational Drug Design of Kinase Inhibitors for Signal Transduction Therapy and chapter 5: Kinase Inhibitors in Signal Transduction Therapy; Wiley 2011, ISBN: 978-3-527-31790-5