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Efficacy of novel Syk-kinase inhibitors MT-SYK-03 and MT-SYK-322 in cellular models of autoimmunity and cancer

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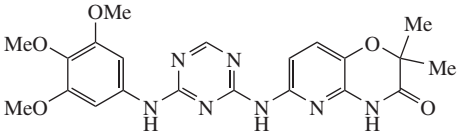
Novel rationally-designed Syk-kinase inhibitor MT-SYK-03 demonstrated equal potency with R406 (active metabolite of Fostamatinib, a phase III clinical trial candidate) in cellular models of autoimmunity and cancer with EC_{50} values in sub-micromolar range, while MT-SYK-322 was less active.

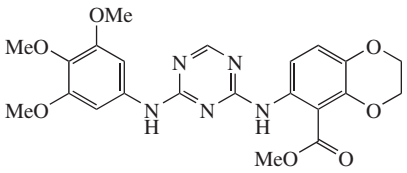
Syk-kinase is involved in different signal transduction cascades that occur in a variety of cell types.[1] It plays an important role in the immune response thus representing a promising therapeutic target for autoimmune diseases such as rheumatoid arthritis and idiopathic thrombocytopenic purpura.[2,3] Furthermore, Syk-kinase activity is required for the maturation of malignant B-cells and Syk inhibitor was shown to dose-dependently inhibit the proliferation of several Non-Hodgkin's Lymphoma (NHL) cell lines.[4]

The most promising small molecule targeting Syk-kinase, Fostamatinib (the oral methylene phosphate prodrug of the Syk-kinase inhibitor R406), now undergoes phase III clinical trials in rheumatoid arthritis[3] and phase II clinical trials in NHL.[5] Despite its impressive efficacy that proves the therapeutic potential of Syk-kinase inhibition, the use of toxic chloromethyl dialkyl phosphate precursors in the chemical synthesis of the prodrug molecule and release of formaldehyde upon conversion to the active metabolite *in vivo* fosters the search of novel Syk inhibitors.

In the present work we studied the efficacy of a novel Syk-kinase inhibitor MT-SYK-03[6] and the newly synthesized compound MT-SYK-322 (Figure 1, Scheme 1) in *in vitro* cellular assays and compared it with those of R406.[†] MT-SYK-03 and MT-SYK-322 were rationally designed using molecular docking followed by structural filtration[7,8] and are expected to possess favorable pharmacokinetics without prodrug formulation due to increased hydrophilicity (cLogP for MT-SYK-03 comprises 1.88 while cLogP for R406 is 4.32). MT-SYK-03 demonstrated significant activity in *in vitro* enzymatic assay comparable with those of R406.[6] Here the synthesis of MT-SYK-322 together with the studies of MT-SYK-03 and MT-SYK-322 in a number of additional cellular assays, which are of relevance for rheumatoid arthritis and non-Hodgkin lymphomas,[‡] are presented.

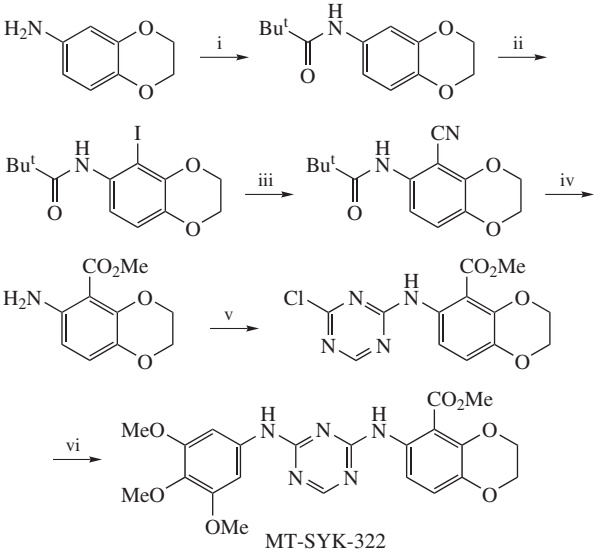


MT-SYK-03



MT-SYK-322

Figure 1 Syk-kinase inhibitors MT-SYK-03 and MT-SYK-322.



Scheme 1 *Reagents and conditions*: i, $Bu^tC(O)Cl$, Pr^i_2NEt, MeCN; ii, Bu^tLi, THF, –100 °C, then I_2; iii, $Zn(CN)_2$, $PdCl_2(dppf)$, NMP, 60 °C, 85 h; iv, aq. HCl, Δ, then MeOH/H^+; v, 2,4-dichloro[1,3,5]triazine, DMF, 60 °C, 12 h; vi, 3,4,5-$(MeO)_3C_6H_2NH_2$, DMF, 110 °C, 6 h. For detailed procedures, see Online Supplementary Materials.

The first cellular assay was used to evaluate the efficacy of compounds in blocking Syk-mediated autoimmune response. Syk-kinase is known to mediate autoimmunity partially *via* the

[†] Syntheses of R406 and MT-SYK-03 were carried out using conventional procedures described in ref. 6.

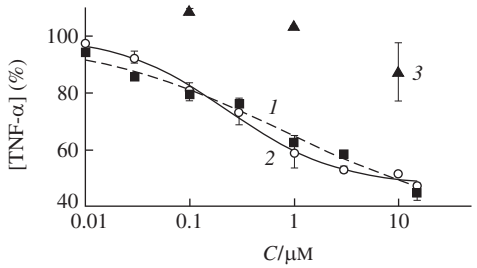


Figure 2 Inhibition of anti-IgG stimulated TNF-α release by differentiated THP-1 monocytes: (*1*) R-406, (*2*) MT-SYK-03 and (*3*) MT-SYK-322.

FCγR signaling cascade which is initiated by IgG-binding to the cell surface and finally triggers degranulation and cytokine release in various immune cells.[9] The resulting cellular response in the presence of Syk inhibitor provides an estimate of the inhibitor efficacy.[10] We used IFNγ-primed THP-1 monocytes stimulated with IgG to initiate FCγR signaling and measured the release of the tumor necrosis factor alpha (TNF-α). Both R406 and MT-SYK-03 demonstrated dose-dependent inhibition of TNF-α release with EC_{50} of 0.24 and 0.85 μM, respectively (Figure 2) while MT-SYK-322 did not show any significant inhibition. The data for R406 are in a good agreement with the previously reported EC_{50} 0.171 μM.[10]

In the second study we evaluated the anticancer properties of MT-SYK-03, MT-SYK-322 and R406. Syk-kinase inhibitors can disrupt a B-cell receptor (BCR) pathway which is essential for maturation of both normal and malignant B-cells.[9] Several subtypes of NHL have a B-cell morphology and thus are sensitive to Syk-kinase inhibitors.[4] We selected the following NHL cell lines for the present study: NAMALWA, P3H3, P3HR-1, basing on the availability from the Cell bank of the Institute of Cytology RAS (RCCC, http://www.rccc.cytspb.rssi.ru/). The effect of compounds on the cell viability is given in Table 1 and Figure 2. Both R406 and MT-SYK-03 demonstrated dose-dependent cytotoxicity with EC_{50} in sub-micromolar range in all studied cell lines. MT-SYK-322 dose-dependently inhibited proliferation of NAMALWA and P3H3 (a subline of P3HR-1) but not P3HR-1 cell line. Such difference could potentially be used to reveal additional cellular targets of MT-SYK-322 if the detailed comparison of P3H3 and P3HR-1 cell lines were available.

Table 1 EC_{50} (in μM) of Syk-kinase inhibitors in NHL cell lines.

Cell line	MT-SYK-03	MT-SYK-322	R406
NAMALWA	0.74±0.12	0.04±0.007	0.95±0.15
P3H3	0.48±0.08	0.06±0.02	0.43±0.05
P3HR-1	4.48±2.09	>10	1.61±1.21

In conclusion, novel compound MT-SYK-03 demonstrated similar potency as R406 in cellular models of autoimmunity and cancer and hence was nominated to further preclinical characterization.

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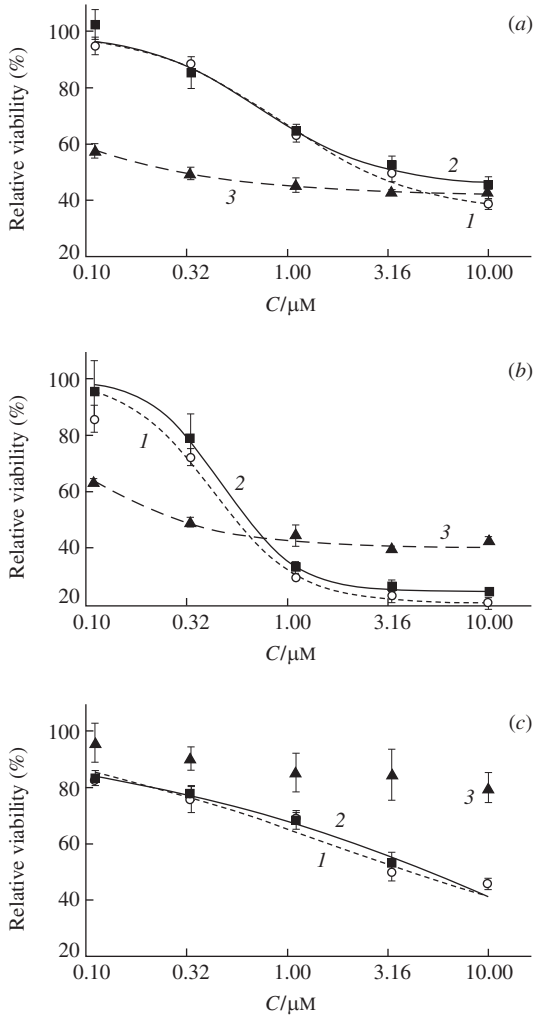

Figure 3 Cell viability of NHL cell lines (*a*) NAMALWA, (*b*) P3H3 and (*c*) P3HR-1 upon treatment with (*1*) R406 (*2*) MT-SYK-03 and (*3*) MT-SYK-322.

‡ *Cellular assays.* All human cell lines obtained from RCCC were cultured in 5% CO_2 humidified atmosphere at 37 °C in RPMI 1640 supplemented with 100 U ml^{-1} penicillin/streptomycin and 10% heat inactivated fetal bovine serum (FBS) for B-cell lymphoma cells: NAMALWA, P3H3, P3HR-1, or 2% FBS and 20 μM β-mercaptoethanol for monocytic THP-1 cells.

100× serial dilutions of the compounds were prepared in 100% DMSO, and the aliquots were stored at −20 °C. 5× working dilutions of the compounds were made in RPMI 1640 immediately prior to the experiments.

All IC_{50} and EC_{50} values in cell-based assays were determined using Origin 8.0 software. Results are presented as the mean +/– S.D.

Inhibition of THP-1 monocytes' TNF-α secretion. The effect of the compounds on IgG-induced tumor necrosis factor (TNF-α) production in macrophages was studied as described.[10] Briefly, THP-1 cells were induced to differentiate into macrophages with 1000 U ml^{-1} IFN gamma (Pharmaclon, Russia) for 6 days before stimulation. Monocyte-derived macrophages were stimulated by immobilized (plate-bound) human IgG (ICN ImmunoBiological) in 96 well plates. A negative control consisting of the F(ab') fragments was used to assess background stimulation. 20 μl of 5× working dilutions of the compounds or 5% DMSO as a control were added to the IgG-coated wells, followed by the addition of 15000 differentiated macrophages in 80 μl of media. After 20 h incubation the supernatants were collected, and secreted TNF-α levels were determined using the human TNF-α Platinum ELISA (Bender MedSystems) according to manufacturer's recommendations. The absorbance was measured in a microplate reader using 450 nm as the primary wavelength and 620 nm as the reference wavelength, the O.D. values were transformed to TNF-α concentrations using standard curve and the results were normalized to the average value of the DMSO-treated control macrophages. Experiment was carried out two times in duplicates.

NHL cells viability assay. The effect of the compounds on cell proliferation was accessed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma). Cells were plated in 96-well plates 2.0×10^4 cells per well in 100 μl media 20 h before treatment. 25 μl of 5× working dilutions of the compounds or 5% DMSO were added to assay or control wells. After 72 h of the incubation the MTT colorimetric assay was performed according to the standard protocol.[11] For data analysis, the OD value for media alone was subtracted from all wells as a background, and then all samples were normalized to the average value of the control wells. Experiment was carried out 3 times in triplicates.

Online Supplementary Materials

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.mencom.2012.11.001.

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