Novel CHK1 inhibitor MU380 exhibits significant single-agent activity in TP53-mutated chronic lymphocytic leukemia cells

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Supplementary Data

Content:

SUPPLEMENTARY METHODS ........................................................................................................ 2
SUPPLEMENTARY TABLES .......................................................................................................... 9
SUPPLEMENTARY FIGURES ...................................................................................................... 14
APPENDIX – ENANTIOSELECTIVE SYNTHESIS OF MU380 .................................................... 29
Supplementary Methods

Additional agents

Gemcitabine, fludarabine, and cytarabine were purchased from Sigma-Aldrich (St. Louis, MO, USA), ATR inhibitor VE-821 from APIs Chemical Co., Ltd (Shanghai, China), and CHK1 inhibitor CHIR-124 and STAT3 inhibitor WP1066 from Selleckchem (Munich, Germany).

All working solutions were prepared fresh by dissolving compounds in appropriate solvents. Final concentration of DMSO was maintained at less than 0.2 % in all experiments; this concentration was found non-cytotoxic in control viability measurements.

Additional cell lines and characterization of primary CLL cells

The following non-cancerous cell lines were used: RPE-1, human epithelial cells immortalized with hTERT obtained from the American Type Tissue Collection (ATCC) (CRL-4000); HS-5, human bone marrow/stromal fibroblasts transformed with a human papilloma virus 16 obtained from the ATCC (CRL-11882); primary human skin fibroblasts established “in-house” from a biopsy (“Fibroblasts normal” in Table 1); primary skin fibroblasts from a patient with ataxia-telangiectasia (cell line GM02052 obtained from Coriell Institute; NJ, USA; ATM gene mutation: c.103C>T, p.R35X) (“Fibroblasts AT” in Table 1). Additionally, three cultures of peripheral blood mononuclear cells (PBMNC) from healthy donors were used; these cultures were used either fresh or vitally frozen.

Mutation status of TP53, ATM, NOTCH1, and SF3B1 genes in CLL samples was assessed by next-generation sequencing using MiSeq (Illumina, San Diego, CA, USA); details are available upon request. Cytogenetic aberrations were detected by FISH using probes from MetaSystems (Altussheim, Germany). Complex karyotype (CK) was assessed by
conventional karyotyping by analyzing at least 15 mitoses. The CK was defined as „three or more aberrations in two or more mitoses“. Mutation status of the immunoglobulin heavy chain variable region (IGHV) was determined by PCR and sequencing; homology $\geq 98\%$ to the germ-line sequence was considered as “unmutated”.

**Antibodies for immunoblotting**

Antibodies detecting total CHK1, pS296 CHK1, pS317 CHK1, pS345 CHK1, β-actin, total H2AX, pS139 H2AX, total PARP, cleaved-PARP, total caspase-3, cleaved-caspase-3, MYC, pS15 p53, MCL1, BCL2, CDC25A, CDC25C, pS216CDC25C, CDK1, pY15 CDK1, cyclin B1 and cyclin E1 were purchased from Cell Signaling Technology (Danvers, MA, USA). Anti-NFκB antibody was from Santa Cruz Biotechnology (Dallas, TX, USA). Anti-p53 antibody (DO-1) was a gift from Dr. Borivoj Vojtesek (Masaryk Memorial Cancer Institute, Brno, Czech Republic). The secondary anti-rabbit and anti-mouse antibodies were purchased from DakoCytomation (currently Agilent Technologies, Santa Clara, CA, USA) and Cell Signaling Technology.

**Cell viability measurement**

In WST-1 assay, the cells were seeded in 96-well plates in quadruplicates using the following count per well: SU-DHL-4 cell line: $2.5 \times 10^4$ cells/well; all other leukemia and lymphoma cell lines: $5 \times 10^4$ cells/well; primary fibroblasts, the fibroblast cell lines and RPE-1 cell line $10^4$ cells/well; unstimulated primary CLL cells: $5 \times 10^5$ cells/well; healthy PBMNC: $2.5 \times 10^5$ cells/well. The cells were treated with a tested agent or DMSO (mock control) for 72 h. Final cell viability after the treatments was assessed using a WST-1 Cell Proliferation Reagent (Roche, Basel, Switzerland) according to the manufacturer’s
instructions; the spectrophotometers 1420 Multilabel Counter Victor (PerkinElmer, Waltham, MA, USA) or Sunrise™ Absorbance Reader (Tecan, Männedorf, Switzerland) were used.

In viability measurement with DiOC6 dye (Thermo Fisher Scientific, Waltham, MA, USA), cells were seeded in 96-well plates in triplicates at the density of $5 \times 10^4$ cells/well. Treatments with MU380 or DMSO (mock control) were done for 48 h. Thereafter, the cells were incubated with DiOC6 and propidium iodide (PI) for 15 min at 37°C, and cell viability was measured using flow-cytometer Accuri C6 (BD Biosciencies, San Jose, CA, USA); viable cells were DiOC6-positive and PI-negative.

**Immunoblotting and real time PCR**

Cells were seeded in six-well plates at the following density: cell lines $5 \times 10^5$/5 ml; primary CLL cells $1.25 \times 10^7$/5 ml. After respective treatments, western blotting and real time PCR procedures were performed as we previously described. In case of $\gamma$H2AX (and total H2AX) detection, histone lysates were prepared using EpiQuik™ Total Histone Extraction Kit (Epigentek, Farmingdale, NY, USA). CHK1 protein level in primary CLL cells was detected using a sensitive kit (Clarity Western ECL Substrate; Bio-Rad, Hercules, CA, USA).

Tissue samples (tumors from immunodeficient mice) were frozen in liquid nitrogen. The tissue was mechanically homogenized and lysed in 200 µl of cold RIPA buffer. Samples were agitated for 2 h at 4°C and centrifuged at 18 000 rcf for 30 min. Supernatant was collected and the procedure of western blotting was performed.

**Analysis of cell cycle**

Cells were treated in 6-well plates at the density of $1.25 \times 10^6$/5 ml. For cell cycle analysis, cells were fixed in 70% ice-cold ethanol, and DNA content was analyzed using PI staining and flow cytometer BD FACSVerse (BD Biosciencies).
Multi-parametric flow-cytometry analysis

Analysis was performed as we previously published\textsuperscript{2} with slight modifications. Cells were treated with 10 μM 5-ethynyl-2’-deoxyuridine (EdU) for 1 h, harvested, counted using CASY system and total number of $2 \times 10^6$ cells was used per one sample. Staining and washing steps were done with staining buffer (PBS containing 1% BSA) at 4°C or room temperature (RT) and in the dark. The cells were stained for viability with LIVE/DEAD Red dye (Thermo Fisher Scientific), fixed with 4% paraformaldehyde and permeabilized by 0.25% Triton-X100. Clic-iT reaction (A647, Thermo Fisher Scientific) was performed according to the manufacture protocol. Next, the cells were incubated overnight with anti-cleaved-PARP antibody (Cell Signaling Technology, 1:400) at 4°C, washed with PBS and incubated with anti-rabbit A488 antibody (Thermo Fisher Scientific, 1:1000). Finally, DNA was stained by FxCycle Violet (Thermo Fisher Scientific, 1:1000) in the presence of RNase A (Sigma-Aldrich, 100 μg/ml) for 1 h at RT. Fluorescence was analyzed using BD FACSVerse system and compensation values for multicolor analyses were calculated according to single-conjugate stained UltraComp eBeads (Thermo Fisher Scientific) assay setup within BD FACS Suite software. Cell aggregates and debris were excluded from analysis based on a dual-parameter dot plot (FSC-H vs. FSC-A). Acquired data were analyzed using FlowJo v10.4.2 software.

Analysis of mitotic cells

MEC-1 cells (1.25 × 10^5/5 ml) were cultivated for 24 h with MU380 (200 or 400 nM), VE-821 (1 or 2 μM) or combination of aforementioned lower and higher concentrations of the inhibitors. Thereafter, cells were arrested in mitosis using additional 7 h culture in the presence of 0.01% colchicine. Mitotic index was calculated from ~1 000 cells per sample, and
detected mitoses were further evaluated for the presence of chromosome damage. The procedures were as we previously described\textsuperscript{1}.

**Xenograft experiments**

The mice strain NOD-\textit{scid} $IL2R_{\gamma}^{null}$ exhibits the following immunologic defects: absent mature B-cells, mature T-cells, natural killer cells and complement, and defective macrophages and dendritic cells. Animals were housed in a pathogen-free facility, given autoclaved food, and maintained on UV-sterilized water with 12 h light/dark cycles. MEC-1 cells ($5 \times 10^6$ in 200 µl of PBS) were transplanted subcutaneously into eight- to ten-week-old mice (of both genders). After tumors became readily visible (at day +14 post-transplant), mice were randomized into treated and control groups. In experiment I, treatment consisted of seven doses of MU380 (each 20 mg/kg) administered via intraperitoneal injection between days +14 to +28 post-transplant. In experiment II, treatment involved ten doses of MU380 provided between days +14 to +25 post-transplant. The size of the tumors was measured successively using caliper, and tumor volume was calculated as “(length x width$^2$)/2”. The animals were sacrificed when the tumor volume reached 2000 mm$^3$ in the control group.

**Immunohistochemistry**

After incision of tumors, tissues were placed into 10\% neutral-buffered formalin for overnight fixation, then processed and paraffin embedded. For routine histopathological analysis, 5 µm thick paraffin sections were stained with hematoxylin and antibodies against cleaved-caspase-3 (Cell Signaling Technology), pS139 H2AX (Cell Signaling Technology), CD20 (Abcam, Cambridge, UK), and Ki-67 (DCS-diagnostics, Hamburg, Germany). Incubation with primary antibody was performed at 4 °C overnight after heat induced antigen
retrieval. Specifically bound primary antibody was visualized with a biotinylated goat antibodies followed by addition of Envision+ reagent (DakoCytomation) and 3,3-diaminobenzidine. Slides were scanned using TissueFAX system (TissueGnostics, Vienna, Austria).

**Transfection of CLL cells**

CLL cells were thawed and cultured in RPMI-1640 (20% FBS) medium without antibiotics for at least 3 hours. Then, $12.5 \times 10^6$ cells were transfected by electroporation using Neon Transfection System (ThermoFisher Scientific) (1900 V, 30 ms, 1 pulse) with specific siRNA targeting CHEK1 (200 nM; ON-TARGETplus Human CHEK1 siRNA – SMARTpool; Dharmacon Lafayette, CO, USA) or the negative control (200 nM; ON-TARGETplus Non-targeting Pool; Dharmacon). The cells were cultured in RPMI-1640 (20% FBS) medium without antibiotics for 48 h. Then, the viability was measured by flow cytometry using DiOC6 (3,3'-dihexyloxacarbocyanine iodide; Thermo Fisher Scientific) and propidium iodide (Sigma-Aldrich).

**Statistical analyses**

Synergy between gemcitabine and MU380 in individual cell lines was evaluated by Chou-Talalay test using CompuSyn software (CompuSyn, Inc., Paramus, NJ, USA). Differences between the two groups (gemcitabine alone vs. gemcitabine with MU380; leukemia vs. lymphoma cell lines; flow-cytometry and immunohistochemical analyses) were evaluated by one-way ANOVA test followed by Scheffé comparison. Differences in the sensitivity to MU380 between the genetic groups (in cell lines and primary CLL cells), *real time* PCR analyses and xenograft data were evaluated by two-way ANOVA test followed by Scheffé comparison.
Changes in the cell cycle distribution were assessed by chi-squared test. Half-maximal inhibitory concentration values (IC\textsubscript{50}) were assessed using mycurvefit.com website. Correlation between pCHK1, total CHK1, pH2AX expression and IC\textsubscript{50} values of individual cell lines was done using regression analysis. Semi-quantitative analyses of western blots were performed using ImageJ software (National Institutes of Health, Bethesda, MD, USA). The values were calculated in relation to loading control (internal standard). Quantification of immunohistochemical images was performed by ImageJ software using at least 10 representative images for each sample.

Statistical evaluation and graphs were done using Statistica 13 program (StatSoft, Tulsa, OK, USA) and GraphPad Prism (GraphPad Software, San Diego, CA, USA).

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Supplementary Tables

Supplementary Table S1: Evaluation of the combined effect of gemcitabine (Gem) with MU380 using Chou-Talalay test.

| Cell line | Gem (ng/ml) | Combination index (CI) | Effect |
|-----------|-------------|------------------------|--------|
| MEC-1     | 2.5         | 0.49                   | +++    |
|           | 5           | **0.39**               | +++    |
|           | 10          | 0.49                   | +++    |
|           | 20          | 0.77                   | ++     |
| MEC-2     | 2.5         | 1.56                   | -      |
|           | 5           | 0.58                   | +++    |
|           | 10          | **0.40**               | +++    |
|           | 20          | 0.77                   | ++     |
| BL-41     | 2.5         | 0.78                   | ++     |
|           | 5           | 0.74                   | ++     |
|           | 10          | **0.42**               | +++    |
|           | 20          | 0.66                   | +++    |
| SU-DHL-4  | 2.5         | 17.11                  | -      |
|           | 5           | 31.70                  | -      |
|           | 10          | 0.12                   | +++    |
|           | 20          | **0.06**               | ++++   |
| JEKO-1    | 0.625       | 2.50                   | -      |
|           | 1.25        | 1.54                   | -      |
|           | 2.5         | 0.36                   | +++    |
|           | 5           | **0.16**               | ++++   |
| NALM-16   | 0.625       | 0.33                   | +++    |
|           | 1.25        | **0.27**               | ++++   |
|           | 2.5         | 0.34                   | +++    |
|           | 5           | 0.48                   | +++    |
| RAJI      | 4           | 0.50                   | +++    |
|           | 8           | **0.37**               | +++    |
|           | 16          | 0.52                   | +++    |
|           | 32          | 0.92                   | -      |
| REC-1     | 2.5         | 1.35                   | -      |
|           | 5           | **0.52**               | +++    |
|           | 10          | 0.64                   | +++    |
|           | 20          | 1.15                   | -      |
| MAVER-1   | 0.4         | 2.49                   | -      |
|           | 1.6         | 0.55                   | +++    |
|           | 6.25        | **0.27**               | ++++   |
|           | 25          | 0.67                   | +++    |

| Cell line | Gem (ng/ml) | Combination index (CI) | Effect |
|-----------|-------------|------------------------|--------|
| MINO      | 1.25        | 1.77                   | -      |
|           | 2.5         | 0.74                   | ++     |
|           | 5           | **0.36**               | +++    |
|           | 10          | 0.52                   | +++    |
| OSU-CLL   | 0.625       | 4.89                   | -      |
|           | 1.25        | 1.21                   | -      |
|           | 2.5         | **0.48**               | +++    |
|           | 5           | 0.91                   | -      |
| JVM-2     | 0.625       | 0.70                   | +++    |
|           | 1.25        | **0.67**               | +++    |
|           | 2.5         | 0.75                   | ++     |
|           | 5           | 1.08                   | +      |
| JVM-3     | 0.625       | 1.65                   | -      |
|           | 1.25        | 0.53                   | +++    |
|           | 2.5         | **0.48**               | +++    |
|           | 5           | 0.89                   | +      |
| WSU-NHL   | 0.125       | 1.50                   | -      |
|           | 0.25        | 1.30                   | -      |
|           | 0.5         | 0.66                   | +++    |
|           | 1           | **0.13**               | ++++   |
| DOHH-2    | 0.625       | 1.23                   | -      |
|           | 1.25        | **0.57**               | +++    |
|           | 2.5         | 0.61                   | +++    |
|           | 5           | 1.03                   | +      |
| NALM-6    | 0.625       | 5.08                   | -      |
|           | 1.25        | 0.88                   | +      |
|           | 2.5         | 0.55                   | +++    |
|           | 5           | **0.51**               | +++    |
| Granta-519| 2.5         | 0.48                   | +++    |
|           | 5           | **0.42**               | +++    |
|           | 10          | 0.54                   | +++    |
|           | 20          | 1.06                   | +      |

CI values are the following: <0.1 very strong synergism (++++) ; 0.1-0.3 strong synergism (+++); 0.3-0.7 synergism (++); 0.7-0.85 moderate synergism (+); 0.85-0.90 slight synergism (+); 0.9-1.1 nearly additive (+); >1.20 antagonism (-).
Supplementary Table S2: CLL samples pre-treated with the pro-proliferative stimuli.

| Sample (category) | TP53 mut (%) | 17p- (%) | ATM mut (%) | 11q- (%) | Complex karyotype | ATM/TP53 mutation | Mutation analysis |
|-------------------|--------------|----------|-------------|----------|-------------------|-------------------|-------------------|
| CLL-1 (wt)        | no           | no       | no          | no       | yes               | none              | NGS               |
| CLL-56 (wt)       | no           | no       | no          | no       | ND                | none              | NGS               |
| CLL-93 (wt)       | no           | no       | no          | no       | yes               | none              | NGS               |
| CLL-34 (ATM-mut)  | no           | no       | 98          | 96       | ND                | p.H2125R          | NGS               |
| CLL-62 (ATM-mut)  | no           | no       | 96          | 99       | ND                | p.I323K           | NGS               |
| CLL-75 (ATM-mut)  | no           | no       | 99          | 97       | yes               | p.L2452R          | NGS               |
| CLL-98 (TP53-mut) | 39           | no       | ND          | no       | ND                | p.H214R           | NGS               |
| CLL-15 (TP53-mut) | 62           | no       | no          | no       | ND                | c.626_627del      | NGS               |
| CLL-16 (TP53-mut) | 99           | 95       | ND          | no       | ND                | R248Q             | FASAY             |
| CLL-99 (TP53-mut) | 20           | no       | ND          | 38       | ND                | M237I             | FASAY             |
| CLL-55 (TP53-mut) | 99           | UPD      | no          | no       | yes               | R282W             | NGS               |
| CLL-86 (TP53-mut) | 50           | 33       | ND          | no       | ND                | L265R             | FASAY             |
| CLL-88 (TP53-mut) | 99           | 87       | ND          | no       | ND                | c.741_742delinsTT | FASAY             |

NGS: next-generation sequencing; FASAY: functional analysis of separated alleles in yeast; specific details for CLL cells’ analysis can be found in Malcikova et al., Blood. 2009;114(26):5307-5314. UPD: copy-neutral loss of heterozygosity involving TP53 gene detected by Cytoscan Affymetrics Arrays; wt: samples ATM-wt/TP53-wt. NA: not applicable; ND: not determined.
## Supplementary Table S3. CLL samples used in the study.

| Sample ID | Status | c (MU380) [nM] | SF3B1 mutation | NOTCH1 mutation | IGHV status | Complex karyotype | Therapy |
|-----------|--------|----------------|----------------|-----------------|-------------|-------------------|---------|
|           |        | 100 | 200 | 300 | 400 |                    |         |
| CLL-1     | wt     | 90  | 89  | 85  | 74  | no               | no      | unmut            | yes    | no           |
| CLL-2     | wt     | 100 | 94  | 87  | 85  | no               | yes     | unmut            | no     | yes          |
| CLL-3     | ATM-mut| 88  | 76  | 63  | 56  | no               | no      | unmut            | ND     | yes          |
| CLL-4     | ATM-mut| 66  | 58  | 41  | 27  | no               | yes     | unmut            | yes    | no           |
| CLL-5     | wt     | 81  | 69  | 62  | 56  | no               | yes     | unmut            | no     | no           |
| CLL-6     | wt     | 100 | 95  | 87  | 77  | no               | no      | unmut            | no     | yes          |
| CLL-7     | TP53-mut| 49  | 48  | 38  | 34  | ND               | ND      | unmut            | ND     | no           |
| CLL-8     | TP53-mut| 93  | 79  | 71  | 68  | no               | no      | unmut            | yes    | yes          |
| CLL-9     | wt     | 89  | 82  | 73  | 63  | no               | yes     | unmut            | ND     | no           |
| CLL-10    | ATM-mut| 77  | 73  | 66  | 59  | no               | no      | unmut            | ND     | yes          |
| CLL-11    | 11q-   | 71  | 68  | 52  | 35  | no               | no      | unmut            | ND     | no           |
| CLL-12    | 11q-   | 78  | 67  | 51  | 40  | no               | no      | unmut            | ND     | no           |
| CLL-13    | wt     | 86  | 80  | 71  | 63  | yes              | yes     | unmut            | ND     | no           |
| CLL-14    | TP53-mut| 87  | 79  | 71  | 68  | yes              | no      | unmut            | ND     | no           |
| CLL-15    | TP53-mut| 75  | 66  | 65  | 52  | no               | no      | mut              | no     | no           |
| CLL-16    | TP53-mut| 79  | 56  | 38  | 32  | ND               | ND      | unmut            | yes    | no           |
| CLL-17    | 11q-   | 58  | 44  | 42  | 34  | no               | no      | unmut            | ND     | no           |
| CLL-18    | 11q-   | 68  | 65  | 62  | 57  | ND               | ND      | unmut            | ND     | yes          |
| CLL-19    | TP53-mut| 77  | 59  | 58  | 40  | no               | no      | mut              | ND     | yes          |
| CLL-20    | ATM-mut| 91  | 94  | 100 | 99  | no               | no      | unmut            | ND     | no           |
| CLL-21    | wt     | 84  | 67  | 51  | 42  | no               | no      | unmut            | no     | no           |
| CLL-22    | 11q-   | 72  | 68  | 66  | 66  | no               | no      | unmut            | ND     | no           |
| CLL-23    | 11q-   | 87  | 79  | 71  | 69  | yes              | yes     | unmut            | ND     | yes          |
| CLL-24    | 11q-   | 98  | 88  | 72  | 53  | no               | no      | unmut            | ND     | no           |
| CLL-25    | ATM-mut| 60  | 49  | 39  | 31  | no               | no      | unmut            | no     | no           |
| CLL-26    | wt     | 93  | 83  | 79  | 71  | no               | no      | mut              | ND     | yes          |
| CLL-27    | ATM-mut| 77  | 56  | 43  | 28  | no               | no      | unmut            | ND     | no           |
| CLL-28    | wt     | 81  | 70  | 60  | 52  | no               | no      | unmut            | no     | no           |
| CLL-29    | wt     | 74  | 62  | 51  | 47  | no               | no      | mut              | no     | no           |
| CLL-30    | wt     | 83  | 86  | 85  | 80  | no               | no      | mut              | ND     | yes          |
| CLL-31    | wt     | 75  | 60  | 50  | 44  | no               | no      | mut              | ND     | no           |
| CLL-32    | TP53-mut| 99  | 86  | 71  | 62  | no               | yes     | unmut            | no     | no           |
| CLL-33    | wt     | 86  | 82  | 74  | 67  | no               | no      | unmut            | yes    | yes          |
| CLL-34    | ATM-mut| 75  | 64  | 53  | 42  | no               | no      | mut              | no     | no           |
| CLL-35    | ATM-mut| 57  | 51  | 38  | 31  | no               | no      | unmut            | no     | no           |
| CLL-36    | wt     | 51  | 46  | 43  | 36  | no               | no      | mut              | ND     | yes          |
| CLL-37    | wt     | 86  | 77  | 74  | 67  | no               | no      | mut              | yes    | no           |
| CLL-38    | ATM-mut| 96  | 91  | 85  | 85  | no               | no      | unmut            | yes    | yes          |
| CLL-39    | wt     | 65  | 56  | 46  | 32  | no               | no      | mut              | ND     | no           |
| CLL-40    | wt     | 88  | 75  | 64  | 56  | no               | no      | unmut            | ND     | no           |
| CLL-41    | wt     | 62  | 57  | 47  | 39  | no               | yes     | unmut            | no     | no           |
| CLL-42    | TP53-mut| 55  | 43  | 31  | 32  | ND               | ND      | unmut            | yes    | yes          |
| CLL-43    | ATM-mut| 72  | 59  | 38  | 29  | no               | no      | unmut            | ND     | no           |
| CLL-44    | wt     | 84  | 65  | 60  | 50  | no               | no      | mut              | no     | yes          |
| CLL-45    | wt     | 81  | 67  | 54  | 45  | yes              | no      | unmut            | ND     | no           |
| CLL-46    | TP53-mut| 77  | 72  | 62  | 54  | yes              | no      | mut              | ND     | yes          |
| CLL-47    | wt     | 80  | 27  | 20  | 14  | ND               | yes     | unmut            | ND     | yes          |
| CLL-48    | wt     | 69  | 59  | 48  | 41  | ND               | ND      | unmut            | ND     | yes          |
| CLL-49    | TP53-mut| 91  | 83  | 73  | 60  | ND               | ND      | unmut            | ND     | yes          |
| CLL-50    | wt     | 74  | 60  | 51  | 51  | no               | no      | unmut            | no     | no           |
| CLL-51    | wt     | 82  | 69  | 58  | 46  | no               | yes     | unmut            | no     | yes          |
| CLL-52    | ATM-mut| 76  | 57  | 56  | 56  | yes              | no      | mut              | no     | no           |
| CLL-53    | wt     | 79  | 69  | 63  | 50  | no               | no      | mut              | no     | yes          |
| CLL-54    | TP53-mut| 47  | 30  | 16  | 10  | no               | yes     | unmut            | yes    | no           |
| Sample ID | Status |  c (MU380) [nM] | SF3B1 mutation | NOTCH1 mutation | IGHV status | Complex karyotype | Therapy |
|-----------|--------|-----------------|----------------|----------------|-------------|------------------|--------|
|           |        | 100  | 200  | 300  | 400  |               |         |
| CLL-55   | TP53-mut | 28   | 18   | 19   | 13   | no     | no    | unmut | yes | no |
| CLL-56   | wt      | 76   | 65   | 60   | 62   | yes    | yes   | unmut | no  | no |
| CLL-57   | ATM-mut | 80   | 66   | 54   | 38   | no     | no    | unmut | no  | no |
| CLL-58   | ATM-mut | 80   | 64   | 56   | 44   | yes    | no    | unmut | ND  | yes|
| CLL-59   | 11q-    | 96   | 96   | 66   | 35   | ND     | ND    | unmut | no  | no |
| CLL-60   | wt      | 58   | 43   | 28   | 25   | no     | no    | unmut | ND  | no |
| CLL-61   | 11q-    | 47   | 37   | 31   | 26   | no     | no    | unmut | no  | no |
| CLL-62   | ATM-mut | 60   | 52   | 44   | 32   | yes    | no    | unmut | no  | no |
| CLL-63   | 11q-    | 91   | 73   | 58   | 46   | no     | no    | unmut | no  | no |
| CLL-64   | ATM-mut | 93   | 81   | 74   | 60   | no     | yes   | unmut | yes | no |
| CLL-65   | wt      | 78   | 60   | 53   | 47   | no     | yes   | unmut | ND  | yes|
| CLL-66   | wt      | 94   | 86   | 79   | 67   | no     | no    | unmut | no  | no |
| CLL-67   | 11q-    | 89   | 79   | 69   | 58   | no     | no    | unmut | ND  | no |
| CLL-68   | TP53-mut | 50  | 32   | 31   | 24   | yes    | no    | unmut | ND  | yes|
| CLL-69   | TP53-mut | 82  | 76   | 67   | 64   | no     | yes   | mut   | ND  | yes|
| CLL-70   | ATM-mut | 76   | 63   | 51   | 42   | no     | no    | unmut | yes | yes|
| CLL-71   | TP53-mut | 92  | 84   | 72   | 58   | no     | no    | unmut | yes | yes|
| CLL-72   | wt      | 72   | 68   | 58   | 46   | yes    | no    | mut   | no  | no |
| CLL-73   | TP53-mut | 81  | 64   | 51   | 37   | no     | no    | unmut | yes | no |
| CLL-74   | wt      | 73   | 71   | 67   | 61   | no     | no    | mut   | ND  | no |
| CLL-75   | ATM-mut | 98   | 92   | 93   | 90   | no     | no    | unmut | yes | no |
| CLL-76   | TP53-mut | 100 | 82   | 79   | 68   | no     | no    | unmut | yes | no |
| CLL-77   | 11q-    | 84   | 74   | 64   | 54   | yes    | ND    | unmut | ND  | no |
| CLL-78   | TP53-mut | 81  | 80   | 79   | 74   | yes    | no    | unmut | yes | no |
| CLL-79   | TP53-mut | 100 | 81   | 55   | 46   | no     | no    | unmut | yes | no |
| CLL-80   | 11q-    | 47   | 46   | 26   | 15   | yes    | no    | unmut | ND  | no |
| CLL-81   | 11q-    | 86   | 70   | 63   | 53   | no     | no    | unmut | yes | no |
| CLL-82   | TP53-mut | 72  | 60   | 50   | 42   | yes    | no    | mut   | no  | yes|
| CLL-83   | wt      | 69   | 51   | 42   | 33   | no     | yes   | unmut | no  | no |
| CLL-84   | 11q-    | 80   | 54   | 48   | 42   | no     | no    | unmut | no  | no |
| CLL-85   | ATM-mut | 63   | 49   | 34   | 25   | no     | no    | unmut | ND  | yes|
| CLL-86   | TP53-mut | 73  | 51   | 37   | 28   | yes    | ND    | unmut | yes | yes|
| CLL-87   | 11q-    | 82   | 58   | 46   | 33   | no     | yes   | unmut | no  | no |
| CLL-88   | TP53-mut | 73  | 63   | 46   | 33   | ND     | ND    | unmut | yes | no |
| CLL-89   | ATM-mut | 73   | 67   | 63   | 58   | no     | no    | unmut | yes | yes|
| CLL-90   | wt      | 71   | 58   | 48   | 31   | yes    | no    | mut   | ND  | yes|
| CLL-91   | 11q-    | 81   | 71   | 63   | 49   | no     | no    | unmut | ND  | yes|
| CLL-92   | wt      | 44   | 30   | 17   | 10   | no     | no    | unmut | ND  | no |
| CLL-93   | wt      | 85   | 81   | 67   | 56   | no     | no    | unmut | yes | no |
| CLL-94   | wt      | 96   | 74   | 63   | 49   | yes    | no    | unmut | no  | no |
| CLL-95   | ATM-mut | 79   | 70   | 60   | 36   | no     | yes   | unmut | ND  | yes|
| CLL-96   | TP53-mut | 59  | 47   | 37   | 27   | ND     | ND    | unmut | yes | no |
| CLL-97   | ATM-mut | ND   |     |     |     | yes    | no    | unmut | yes | yes|
| CLL-98   | TP53-mut | ND  |     |     |     | ND     | ND    | mut   | ND  | ND |
| CLL-99   | TP53-mut | ND  |     |     |     | ND     | ND    | unmut | ND  | ND |
| CLL-100  | TP53-mut | ND  |     |     |     | ND     | ND    | unmut | yes | yes|
| CLL-101  | TP53-mut | ND  |     |     |     | ND     | ND    | unmut | yes | yes|
| CLL-102  | wt      | ND   |     |     |     | no     | no    | mut   | ND  | no |
| CLL-103  | 11q-    | ND   |     |     |     | no     | no    | unmut | ND  | no |

The samples CLL-1 to CLL-96 were used in the viability testing of non-stimulated CLL cultures; the final viability related to untreated control is listed; in all figures, tables and text of the manuscript, samples’ ID correspond to this Table S3. TP53-mut: mutation in the TP53
gene regardless of other factors; ATM-mut: mutation in the ATM gene, TP53-wt; 11q-: samples with sole deletion 11q (the other ATM-allele wt, TP53-wt); wt: samples ATM-wt/TP53-wt. Column “Therapy”, no: samples from untreated patients; yes: samples from previously treated patients. NA: not applicable; ND: not determined.
Supplementary Figures

Supplementary Figure S1. Data adopted from DepMap database (The Cancer Dependency Map Project at Broad Institute). (A) Dependency score of genes involved in essential ATR-CHK1 and nonessential ATM-p53-CHK2 pathways. (B) Dependency score of our tested cell lines. Dependency score: a lower score means that the gene is more likely to be dependent for survival in a given cell line. A score of 0 is equivalent to a gene that is not essential whereas a score of -1 corresponds to the median of all common essential genes.
Supplementary Figure S2. MU380 shows synergy with gemcitabine in lymphoid cancer cell lines. The leukemia and lymphoma cell lines were treated for 72 h with the indicated concentrations of gemcitabine (Gem) +/- 100 nM MU380. Cell viability was measured by WST-1 assay. Synergy between gemcitabine and MU380 was evaluated using Chou-Talalay test; the results are summarized in Table 1 and Supplementary Table S1.
Supplementary Figure S3. Baseline replication stress in the leukemia and lymphoma cell lines. (A) Baseline level of CHK1 isoforms and γH2AX. (B) Correlation to MU380 effects. (I) Total CHK1. (II) pS296 CHK1. (III) pS345 CHK1. (IV) pS139 H2AX. No studied factor correlated with the viability decrease observed after treatment with MU380.
Supplementary Figure S4. Effects of MU380 on viability of non-cancerous human cells. The cells were treated with indicated concentrations of the inhibitor for 72 h and viability was measured by WST-1 reagent. (A) RPE-1 cell line (immortalized epithelial cells; IC \textsubscript{50} > 10 µM). (B) HS-5 cell line (immortalized bone marrow/stromal fibroblasts; IC \textsubscript{50} = 3.7 µM). (C) Primary skin fibroblasts (IC \textsubscript{50} = 3.7 µM). (D) Primary skin fibroblasts from the patient with ataxia-telangiectasia harboring complete ATM gene inactivation (IC \textsubscript{50} > 10 µM). Origin and characterization of the cell lines is provided in Supplemental Methods.
Supplementary Figure S5. Histograms from the flow-cytometry cell cycle analysis.
Treatment time: 24 h; MU380 concentration: 400 nM.
Supplementary Figure S6. Flow-cytometry analysis of DNA synthesis rate and apoptosis induction in MEC-1 cell line. (A) Cells treated with MU380 (400 nM; 24 h) exhibited significantly reduced DNA synthesis rate compared to control untreated cells (lower EdU incorporation, $P = 0.001$) and consequently manifested extensive apoptosis as evidenced by the PARP protein cleavage ($P = 0.001$). The cell death was also confirmed using labelling with Live/Dead Red agent ($P = 0.002$).
Supplementary Figure S7. MU380 does not induce apoptosis in non-cancerous human cells. Treatment time: 48 h. (A) Human epithelial cell line RPE-1. The rise of cleaved caspase-3 (C-Caspase-3) is negligible compared to untreated control. Positive control: RPE-1 treated with doxorubicin (DOX; 1 µg/ml); NALM-6: additional positive control for western blotting (treated with 400 nM MU380). (B) Stromal/bone marrow fibroblast cell line HS-5. The rise of cleaved PARP (C-PARP) and C-Caspase-3 is negligible compared to the untreated control. The positive controls are similar to Figure A. (C) Primary skin fibroblasts. MU380 concentration: 400 nM. Positive control: MEC-1 cells treated with STAT-3 inhibitor WP1066 (5 µM; 4 h).
Supplementary Figure S8. Inhibitor VE-821 suppresses ATR-mediated signaling towards CHK1 protein in MEC-1 cell line. Cells were treated for 6 h with 400 nM MU380 (CHK1i) and indicated concentrations of VE-821 (ATRi). ATR-mediated phosphorylations pS345 and pS317 of the CHK1 protein were reduced by VE-821 in a concentration-dependent manner; the values (%) are related to the sole CHK1 inhibition when the signalling from ATR towards the CHK1 is at maximum. Densitometric analysis was done using ImageJ software.
Supplementary Figure S9. Cytogenetic analysis of mitotic chromosomes in MEC-1 cell line. (A) Control untreated cells. Two mitoses (I and II) are shown. Intact chromosomes were present in all analyzed mitoses. (B) 400 nM MU380. (C) 400 nM MU380 + 2 μM VE-821. Both types of treatments resulted in the chromosome breaks or complete chromosome pulverization (shown in the figures).
Supplementary Figure S10. Pro-proliferative stimulation of CLL cells. (A) DNA content analysis. Non-stimulated primary CLL cells (D0) (sample CLL-8) are arrested in G0/G1 phase of the cell cycle. The 10-day co-culture involving pro-proliferative stimuli (D+10) (see Methods) resulted in cell cycle entry in a proportion of the cells. (B) Real time PCR analysis of proliferation markers. Graph shows relative expression of MKi67 and BIRC5 genes (encoding proteins Ki67 and survivin, respectively) in stimulated cultures (samples withdrawn at D+7) compared to the non-stimulated counterparts (D0). MKi67 expression was stimulated more distinctively in TP53-mutated samples compared to TP53-wt ones (P = 0.063; Mann-Whitney test).
Supplementary Figure S11. Level of CDC25A and CDC25C in CLL cells. (A) Decrease of total and phosphorylated level of CDC25C in a CLL sample. (B) Variable levels of CDC25A and CDC25C in other CLL samples.
Supplementary Figure S12. Effect of MU380 on viability of non-stimulated CLL cells stratified according to additional categories. (A) *SF3B1* mutations (*P* = 0.191). (B) *NOTCH1* mutations (*P* = 0.387). (C) *IGHV* status (*P* = 0.506). (D) Complex karyotype (*P* = 0.566). (E) Therapy status (*P* = 0.145).
Supplementary Figure S13. The effect of transfection on viability of CLL cells. CLL cells were transfected with siRNA targeting CHEK1, or siRNA negative control (neg. CTR) and analysed after 48 h incubation. The percentage of viable cells was determined by gating on PI-negative and DiOC6-positive cells.
Supplementary Figure S14. Impact of structurally different CHK1 inhibitor CHIR-124 on viability of non-stimulated CLL cultures. Treatment time: 72 h; concentration range: 100-800 nM. Cell viability was measured by WST-1 reagent. CHIR-124 was tested in 10 cultures with the following genotypes: ATM-wt/TP53-wt (n = 5), TP53-mutation (n = 4) and ATM-mutation (n = 1).
Supplementary Figure S15. Effect of MU380 on the level of selected proteins.
Treatment time: 24 h; MU380 concentration: 400 nM. (A) Cleaved PARP. (B) \( \gamma \)H2AX. (C) MCL1. (D) NF-\( \kappa \)B. (E) BCL2. (F) MYC. (G) Total CHK1. Arrows mark a shift from a baseline level. The level of \( \beta \)-actin showed equal loading in all samples (data not shown). All paired samples are visualized from the same blot (image); in some cases, these samples were not immediately adjacent to each other and were therefore compiled together.
Appendix – Enantioselective Synthesis of MU380

Experimental Section

General

All chemicals were purchased from commercial suppliers and were used without further purification. The solvents were purchased as extra dry and stored over 4Å molecular sieves under nitrogen. Unless noted otherwise, the reactions were carried out in oven-dried glassware under nitrogen atmosphere.

Column chromatography was carried on silica gel (230-400 mesh). TLC plates (silica gel 60 F\textsubscript{254} or silica gel-RP18 60 F\textsubscript{254}) were visualized under UV and/or with phosphomolybdic acid, KMnO\textsubscript{4}, CAM or H\textsubscript{2}SO\textsubscript{4} in MeOH. Determination of enantiomeric excess (ee) were performed on HPLC Ultimate 3000 LC Systems (Thermo Scientific) with UV detection or Corona charged aerosol detection (CAD) using analytical columns with chiral stationary phase Chiralpak\textsuperscript{®} AD\textsuperscript{TM}, Chiralpak\textsuperscript{®} IA\textsuperscript{TM} and Chiralpak\textsuperscript{®} OJ\textsuperscript{TM}.

HRMS spectra were measured on Agilent 6224 Accurate-Mass TOF LC-MS with dual electrospray/chemical ionization mode with mass accuracy greater than 2 ppm. NMR spectra were recorded on Bruker Avance 300 MHz and 500 MHz spectrometers. Chemical shifts (\(\delta\) in ppm) of \(^1\)H NMR and \(^{13}\)C NMR were referenced to the residual signals of solvents: CDCl\textsubscript{3} [7.26 (\(^1\)H) and 77.16 (\(^{13}\)C)] and DMSO-d\textsubscript{6} [2.50 (\(^1\)H) and 39.52 (\(^{13}\)C)], chemical shifts of \(^{19}\)F to the signal of \(\alpha,\alpha,\alpha\)-trifluorotoluene [-63.72]. Data are represented as follows: chemical shift, multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet and/or multiple resonances), coupling constant (\(J\)) in Hertz, integration. IR spectra (4000-400 cm\textsuperscript{-1}) were collected on ALPHA ATR spectrometer (BRUKER); solid samples were measured neat and oily samples as films.
Reagents and conditions: a) CDI, THF, 0 °C, 1 h then HN(OCH$_3$)$_2$CH$_3$·HCl, Et$_3$N, THF, 0 °C to rt, 16 h, 99 %; b) CH$_3$CN, LiHMDS (1M in THF), THF, -78 °C, 1h then 20 min, 99 %, 99% ee.; c) 3-aminopyrazole, CH$_3$COOH, 40 °C, 16 h, 95 %, 96% ee.; d) CH$_3$CH$_2$OCH$_2$Cl, DIPEA, (CH$_2$Cl)$_2$, 80 °C, 16h, 84 %; e) NIS, CH$_3$CN, rt, 16 h, 95 %; f) 4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)-1-(trifluoromethyl)-1H-pyrazole, K$_3$PO$_4$, Pd(dppf)Cl$_2$, DME, H$_2$O, 100 °C, 3 h, 62 %; g) EtOH, aq. 3 M HCl sol., 80 °C, 3h then 7 M NH$_3$ in MeOH, rt, 2 h, 80 %. 96% ee.; h) (Boc)$_2$O, Et$_3$N, CH$_2$Cl$_2$, rt, 4 h, 97 %; i) Br$_2$, t-BuNH$_2$, CH$_2$Cl$_2$, rt, 16 h, 96 %; j) (i) CF$_3$COOH, CH$_2$Cl$_2$, rt, 2 h then 7.0 M NH$_3$ in MeOH, rt, 2 h, 97 %, 96% ee.; (ii) recrystallization from CH$_3$CN, 75 %, 99% ee.
tert-butyl (R)-3-(methoxy(methyl)carbamoyl)piperidine-1-carboxylate (2):

1, 1′-Carbonyldiimidazole (3.96 g, 24.33 mmol) was added to a solution of (R)-1-(tert-butoxycarbonyl)piperidine-3-carboxylic acid (4.65 g, 20.28 mmol) in anhydrous THF (60 mL) and the resulting mixture was stirred at 0 °C for 1 hour. Then, a suspension of N, O-dimethylhydroxylamine hydrochloride (2.334 g, 23.94 mmol) and triethylamine (3.39 mL, 24.33 mmol) in THF (60 mL) were added at 0 °C and the resulting mixture was stirred at 25 °C for 16 hours. The reaction mixture was quenched with water (300 mL), and extracted with EtOAc (3 × 600 mL). The organic extracts were washed with 0.1M aqueous HCl (300 mL), brine (300 mL), dried over MgSO₄, filtered, and concentrated under reduced pressure. The residue was purified by flash column chromatography (hexane/EtOAc = 75:25) to afford 2 (5.49 g, 99%) as a colorless oil.

\(^1\)H NMR (500 MHz, CDCl₃): \(\delta = 4.22 – 3.99\) (m, 2H), 3.72 (s, 3H), 3.17 (s, 3H), 2.91 – 2.75 (m, 2H), 2.70 (t, \(J = 12.4\) Hz, 1H), 1.91 (d, \(J = 13.5\) Hz, 1H), 1.75 – 1.60 (m, 2H), 1.53 – 1.46 (m, 1H), 1.45 (s, 9H) ppm.

\(^{13}\)C NMR (126 MHz, CDCl₃): \(\delta = 174.0, 154.8, 79.6, 61.8, 46.4, 44.3, 38.7, 32.3, 28.6, 27.5, 24.7\) ppm.

HRMS (APCI-TOF) m/z: calculated for C₁₃H₂₅N₂O₄ [M+H]: 273.1809, found 273.1809.

IR (\(\nu_{\text{max}}, \text{neat}\) = 2971, 2936, 2865, 1688, 1656, 1466, 1411, 1389, 1364, 1254, 1237, 1175, 1143, 986, 942, 886, 856, 818, 799, 768, 62, 534 cm\(^{-1}\).

tert-butyl (R)-3-(2-cyanoacetyl)piperidine-1-carboxylate (3):
CH$_3$CN (2.106 mL, 40.29 mmol) was added dropwise to a solution of LiHMDS (1.0 M in THF, 40.29 mL, 40.29 mmol) in anhydrous THF (60 mL) at -78 °C and the resulting mixture was stirred for 1 hour. Then, a solution of 2 (5.49 g, 20.16 mmol) in THF (15 mL) was added dropwise over 15 minutes at -78 °C and the resulting mixture was stirred for 5 minutes at -78 °C. The reaction mixture was quenched with saturated NH$_4$Cl (300 mL) at -78 °C, and extracted with Et$_2$O (2 × 600 mL). The organic extracts were washed with brine (300 mL), dried over MgSO$_4$, filtered, and concentrated under reduced pressure. The residue was purified by flash column chromatography (hexane/EtOAc = 60:40) to afford 3 (5.04 g, 99%) as a pale yellow oil with 99% ee.

$^1$H NMR (500 MHz, CDCl$_3$): $\delta = 3.96 - 3.82$ (m, 1H), 3.80 - 3.48 (m, 3H), 3.40 - 2.86 (m, 2H), 2.74 - 2.63 (m, 1H), 1.98 - 1.82 (m, 1H), 1.80 - 1.61 (m, 2H), 1.50 - 1.44 (m, 1H), 1.42 (s, 9H) ppm.

$^{13}$C NMR (126 MHz, CDCl$_3$): $\delta = 198.5, 154.6, 113.7, 80.3, 47.6, 44.9, 31.0, 28.4, 26.3, 23.7$ ppm.

HRMS (APCI-TOF) m/z: calculated for C$_8$H$_{13}$N$_2$O [M-Boc + H]$^+$: 153.1022, found 153.1021.

IR ($\nu_{max}$, neat) = 2975, 2935, 2863, 2257, 1722, 1677, 1468, 1420, 1365, 1324, 1293, 1263, 1240, 1142, 1066, 990, 944, 915, 858, 768, 731, 647, 533 cm$^{-1}$.

**Determination of enantiomeric excess (ee) of compound (3):**

Column : Daicel-Chiralpak-IA Analytical column (particle size 4.6 mm × 250 mm)
Injection Volume : 20 µL (a solution of 2 mg of compound 3 in 1 mL of EtOH)
Flow Rate : 1 mL/min
Mobile Phase : n-hexane/EtOH 90 : 10
Wavelength : 246 nm
Retention times : Enantiomer - A (10.95 min) + Enantiomer - B (13.00 min, required)
**tert-butyl (R)-3-(7-aminopyrazolo[1,5-a]pyrimidin-5-yl)piperidine-1-carboxylate (4):**

A mixture of 3 (4.05 g, 16.05 mmol), 3-aminopyrazole (1.332 g, 16.05 mmol) and glacial acetic acid (5.4 mL, 94.38 mmol) were stirred at 40 °C for 16 hours. The reaction mixture was concentrated under reduced pressure and the residue was purified by flash column chromatography (CH₂Cl₂/EtOAc = 50:50) to afford 4 (4.80 g, 95 %) as a white solid with 97% ee.

**¹H NMR (500 MHz, DMSO-d₆):** δ = 8.07 (d, J = 2.3 Hz, 1H), 8.01 (brs, 2H), 6.33 (d, J = 2.3 Hz, 1H), 6.05 (s, 1H), 4.08 (brs, 1H), 4.00 – 3.88 (m, 1H), 2.83 – 2.72 (m, 1H), 2.71 – 2.61 (m, 1H), 2.52 – 2.48 (m, 1H), 2.03 – 1.90 (m, 1H), 1.77 – 1.65 (m, 2H), 1.50 – 1.41 (m, 1H), 1.39 (s, 9H) ppm.

**¹³C NMR (126 MHz, DMSO-d₆):** δ = 161.4, 153.8, 148.5, 147.3, 144.1, 93.0, 86.0, 78.7, 47.8, 42.9, 29.6, 28.0, 24.6 ppm.

**HRMS (APCI-TOF) m/z:** calculated for C₁₆H₂₄N₅O₂ [M+H]⁺: 318.1925, found 318.1925.

**IR (v max, neat) = 3361, 3308, 3218, 3181, 2974, 2931, 1647, 1596, 1561, 1468, 1426, 1365, 1266, 1240, 1144, 1065, 963, 853, 769, 628 cm⁻¹.**

**m.p. = 182 °C (dec.)**

**Determination of enantiomeric excess (ee) of compound (4):**

**Column:** Daicel-Chiralpak-IA Analytical column (particle size 4.6 mm × 250 mm)

**Injection Volume:** 20 μL (a solution of 1 mg of compound 4 in 1 mL of EtOH)

**Flow Rate:** 1 mL/min

**Mobile Phase:** n-hexane/EtOH 98 : 02 + 0.5% diethylamine
Retention times: Enantiomer - A (26.38 min) + Enantiomer - B (28.87 min, required)

**tert-butyl (R)-3-(7-(bis(ethoxymethyl)amino)pyrazolo[1,5-a]pyrimidin-5-yl)piperidine-1-carboxylate (5):**

![Chemical Structure](image)

Ethoxymethyl chloride (3.99 mL, 42.99 mmol) was added dropwise to a solution of 4 (5.46 g, 17.19 mmol) and DIPEA (9.00 mL, 51.60 mmol) in 1,2-dichloroethane (120 mL) and the mixture was stirred at 70 °C for 16 hours. The reaction mixture was cooled to 25 °C, quenched with water (300 mL), and extracted with CH₂Cl₂ (3 × 600 mL). The organic extracts were dried over MgSO₄, filtered, and concentrated under reduced pressure. The residue was purified by flash column chromatography (CH₂Cl₂/EtOAc = 70:30) to afford 5 (6.264 g, 84%) as a pale yellow wax.

¹H NMR (500 MHz, CDCl₃): δ = 8.02 (d, J = 2.3 Hz, 1H), 6.53 (d, J = 2.4 Hz, 1H), 6.41 (s, 1H), 5.26 (m, 1H), 4.40 – 4.25 (m, 1H), 4.20 – 4.00 (m, 1H), 3.60 (q, J = 7.1 Hz, 4H), 3.16 – 2.94 (m, 1H), 2.88 – 2.70 (m, 1H), 2.13 – 2.05 (m, 1H), 1.90 – 1.72 (m, 2H), 1.64 – 1.54 (m, 1H), 1.47 (s, 9H), 1.20 (t, J = 7.0 Hz, 6H) ppm.

¹³C NMR (126 MHz, CDCl₃): δ = 163.2, 154.7, 150.8, 148.2, 143.7, 95.1, 94.1, 79.8, 79.4, 63.9, 48.3, 44.3, 30.3, 28.4, 25.0, 15.0 ppm.

HRMS (APCI-TOF) m/z: calculated for C₂₂H₃₆N₅O₄ [M+H]⁺: 434.2762, found 434.2762.

IR (ν max, neat) = 2974, 2931, 2897, 2867, 1687, 1604, 1542, 1460, 1415, 1390, 1363, 1300, 1289, 1265, 1254, 1238, 1171, 1147, 1065, 1005, 966, 905, 854, 823, 766, 740 cm⁻¹.
tert-butyl (R)-3-(7-(bis(ethoxymethyl)amino)-3-iodopyrazolo[1,5-a]pyrimidin-5-yl) piperidine-1-carboxylate (6):

![Chemical Structure](image)

A solution of N-iodosuccinimide (3.15 g, 14.04 mmol) acetonitrile (60 mL) was added dropwise to a solution of 5 (6.09 g, 14.04 mmol) in acetonitrile (60 mL). The reaction mixture was stirred at 25 °C for 16 hours, and then concentrated under reduced pressure. The residue was purified by flash column chromatography (hexane/EtOAc = 70:30) to afford 6 (7.44 g, 95%) as a colorless wax.

$^1$H NMR (500 MHz, CDCl$_3$): $\delta = 8.01$ (s, 1H), 6.45 (s, 1H), 5.25 – 5.19 (m, 4H), 4.45 – 4.22 (m, 1H), 4.22 – 4.00 (m, 1H), 3.57 (q, $J = 7.0$ Hz, 4H), 3.14 – 3.00 (m, 1H), 2.91 – 2.70 (m, 2H), 2.14 – 2.05 (m, 1H), 1.94 – 1.72 (m, 2H), 1.64 – 1.54 (m, 1H), 1.47 (s, 9H), 1.18 (t, $J = 7.1$ Hz, 6H), ppm.

$^{13}$C NMR (126 MHz, CDCl$_3$): $\delta = 164.9$, 155.0, 149.8, 148.6, 147.9, 94.9, 80.1, 79.6, 64.2, 48.2, 44.5, 30.5, 28.6, 25.2, 15.2 ppm.

HRMS (APCI-TOF) m/z: calculated for C$_{22}$H$_{35}$IN$_5$O$_4$ [M+H]$^+$: 560.1728, found 560.1731.

IR ($\nu_{\max}$, neat) = 2925, 2854, 2248, 1732, 1679, 1603, 1541, 1465, 1453, 1421, 1390, 1380, 1365, 1302, 1256, 1171, 1036, 1011, 727, 647 cm$^{-1}$.

1-(bromodifluoromethyl)-4-ido-1H-pyrazole (7):

![Chemical Structure](image)

500 mL (with a magnetic stirring bar) and 100 mL round-bottom flasks (both fitted with silicon septa) were evacuated and then filled with argon. Then, a solution of 4-iodopyrazole (15.00 g, 77.3 mmol) in 70 mL of anhydrous DMF (Merck) was added to the 500 mL flask.
The solution was cooled to -30 °C (ethanol/dry ice bath) and a solution of NaHMDS in THF (1M, 81.2 mL, 81.2 mmol) was added dropwise over the period of 50 min while the temperature was maintained below -20 °C. During the formation of sodium salt, the solution became green.

This solution was stirred at 0 °C for 15 minutes while 15 mL of anhydrous DMF was added to the 100 mL flask. The solvent was cooled to -30 °C (ethanol/dry ice bath) and CBr$_2$F$_2$ (8.48 mL, 92.8 mmol) was added quickly. This solution was then added to a solution of the sodium pyrazole salt dropwise at -10 °C over the period of 30 minutes.

Then, the cooling bath was removed and the mixture was stirred under argon for 18 hours. Then, the mixture was slowly poured into water (200 mL) and extracted with Et$_2$O (5 × 100 mL). The combined organic extracts were washed with water (100 mL) and brine (100 mL), dried over Na$_2$SO$_4$, and filtered. Et$_2$O ether and THF were evaporated at 30 mbar and the residue (ca. 23 g) was distilled under reduced pressure (20 mbar). The following fractions were collected:

1. 75-95 °C
2. 95-105 °C
3. > 105 °C

Then, the first and second fractions (ca. 13 g) were combined and purified by flash column chromatography (hexane/CH$_2$Cl$_2$ = 10:1 to 5:1) to afford the product 7 as a colorless oil (8.80 g, 35%).

$^1$H NMR (300 MHz, CDCl$_3$): $\delta$ = 7.90 (s, 1H), 7.78 (s, 1H) ppm.

$^{13}$C NMR (126 MHz, CDCl$_3$): $\delta$ = 148.5, 132.1, 109.5 (t, $^1$J$_{C-F}$ = 306.3 Hz), 61.3, ppm.

$^{19}$F NMR (282 MHz, CDCl$_3$): $\delta$ = -33.7 (s) ppm.

HRMS (APCI-TOF) m/z: calculated for C$_4$H$_3$BrF$_2$IN$_2$ [M+H]$^+$: 322.8487/324.8468, found 322.8485/324.8465.

4-iodo-1-(trifluoromethyl)-1H-pyrazole (8):

A mixture of compound 7 (8.80 g, 27.27 mmol) and AgF (4.50 g, 135.45 mmol) in pentane (100 mL) was stirred in a 150 mL pressure tube at 25 °C for 9 days. Then, additional AgF (1.73 g, 13.63 mmol) was added and the mixture was stirred for additional 5 days. Then,
additional AgF (0.69 g, 5.45 mmol) was added and the mixture was stirred for additional 1 day. The mixture was quickly filtered and the solvent from the filtrate was evaporated at 25 °C under reduced pressure (570 mbar). The product 8 (5.11 g, 71%) was obtained as colorless oil.

$^1$H NMR (300 MHz, CDCl$_3$): $\delta$ = 7.88 (s, 1H), 7.76 (s, 1H) ppm.

$^{13}$C NMR (126 MHz, CDCl$_3$): $\delta$ = 148.7, 132.6, 117.6 (q, $^J_{C-F}$ = 264.0 Hz), 60.9 ppm.

$^{19}$F NMR (282 MHz, CDCl$_3$): $\delta$ = -61.6 (s) ppm.

HRMS (APCI-TOF) m/z: calculated for C$_4$H$_3$F$_3$IN$_2$ [M+H]$^+$: 262.9288, found 262.9285.

**4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)-1-(trifluoromethyl)-1H-pyrazole (9):**

A mixture of compound 8 (4.50 g, 17.16 mmol), Pd(dppf)Cl$_2$ (1.257 g, 1.72 mmol), bis(pinacolato)diboron (4.59 g, 18.03 mmol) and potassium acetate (6.75 g, 68.64 mmol) in DMF (60 mL) was stirred in a pressure tube at 90 °C for 18 hours. The mixture was cooled to 25 °C, mixed with water (300 mL), and extracted with Et$_2$O (2 $\times$ 350 mL). The combined organic extracts were washed with brine (2 $\times$ 300 mL), dried over MgSO$_4$, filtered and concentrated under reduced pressure to yield the crude product 9 as a brown solid (5.40 g), which was directly used in the next step without further purification.

$^1$H NMR (500 MHz, CDCl$_3$): $\delta$ = 8.10 (s, 1H), 7.95 (s, 1H), 1.31 (s, 12H) ppm.

$^{13}$C NMR (126 MHz, CDCl$_3$): $\delta$ = 148.8, 135.3, 118.2 (q, $^J_{C-F}$ = 263.9 Hz), 110.6 (HMBC), 84.2, 25.0 ppm.

$^{11}$B NMR (160 MHz, CDCl$_3$): $\delta$ = 29.22 ppm.

$^{19}$F NMR (282 MHz, CDCl$_3$): $\delta$ = -61.25 (s) ppm.
**tert-butyl (R)-3-(7-(bis(ethoxymethyl)amino)-3-(1-(trifluoromethyl)-1H-pyrazol-4-yl)pyrazolo[1,5-a]pyrimidin-5-yl)piperidine-1-carboxylate (10):**

A solution of 6 (7.23 g, 12.90 mmol) in DME/H$_2$O (150+36 mL) was added to a mixture of 4-(4, 4, 5, 5-tetramethyl-1, 3, 2-dioxaborolan-2-yl)-1-(trifluoromethyl)-1H-pyrazole (5.07 g, 19.38 mmol), K$_3$PO$_4$ (10.98 g, 51.69 mmol) and Pd(dppf)Cl$_2$ (0.945 g, 1.29 mmol). The reaction mixture was stirred at 90 °C for 3 hours, then diluted with water (300 mL), and extracted with EtOAc (2 × 900 mL). The combined organic extracts were washed with brine (300 mL), dried over MgSO$_4$, filtered, and concentrated under reduced pressure. The residue was purified by flash column chromatography (hexane/EtOAc = 70:30) to afford 10 (4.56 g, 62 %) as a light brown solid.

$^1$H NMR (300 MHz, CDCl$_3$): $\delta = 8.37$ (s, 1H), 8.29 (s, 1H), 8.21 (s, 1H), 6.48 (s, 1H), 5.38 – 5.18 (m, 4H), 4.46 – 4.28 (m, 1H), 4.26 – 4.07 (m, 1H), 3.61 (q, $J = 7.0$ Hz, 4H), 3.19 – 2.96 (m, 1H), 2.94 – 2.72 (m, 2H), 2.21 – 2.07 (m, 1H), 1.95 – 1.75 (m, 2H), 1.70 – 1.55 (m, 1H), 1.48 (s, 9H), 1.21 (t, $J = 7.0$ Hz, 6H) ppm.

$^{13}$C NMR (126 MHz, CDCl$_3$): $\delta = 164.0$, 155.0, 148.5, 147.2, 141.7, 140.8, 123.7, 119.5 (q, $J_{C-F} = 262.0$ Hz), 116.7, 99.8, 94.9, 80.1, 79.8, 64.3, 48.7, 44.3, 30.6, 28.6, 25.2, 15.2 ppm.

$^{19}$F NMR (282 MHz, CDCl$_3$): $\delta = -61.14$ (s) ppm.

HRMS (APCI-TOF) m/z: calculated for [M+H]$^+$: 568.2854, found 568.2851.

IR ($\nu_{\text{max}}$, neat) = 3142, 3095, 2972, 2937, 2889, 2859, 1681, 1590, 1542, 1488, 1424, 1410, 1382, 1365, 1308, 1283, 1270, 1221, 1195, 1165, 1142, 1106, 1088, 1074, 1034, 1000, 969, 908, 868, 663, 628, 466 cm$^{-1}$.

m.p. = 122 – 124 °C
(R)-5-(piperidin-3-yl)-3-(1-(trifluoromethyl)-1H-pyrazol-4-yl)pyrazolo[1,5-a]pyrimidin-7-amine (11):

Aqueous 3 M HCl (90 mL) was added to a solution of 10 (4.56 g, 3.00 mmol) in ethanol (90 mL) and the reaction mixture was stirred at 80 °C for 3 hours. The solvents were evaporated, the residue was dissolved in 7 M NH₃ in MeOH (90 mL) and the mixture was stirred at 25 °C for 2 hours. The solvents were evaporated under reduced pressure and the residue was purified by flash column chromatography (CH₂Cl₂/7 M NH₃ in MeOH = 90:10) to afford 11 (2.25 g, 80 %) as a white solid with 96% ee.

¹H NMR (500 MHz, DMSO-d₆): δ = 8.70 (s, 1H), 8.55 (s, 1H), 8.48 (s, 1H), 7.74 (s, 2H), 6.07 (s, 1H), 3.24 – 3.15 (m, 1H), 3.05 – 2.96 (m, 1H), 2.83 – 2.72 (m, 2H), 2.63 – 2.55 (m, 1H), 2.07 – 1.97 (m, 1H), 1.81 – 1.67 (m, 2H), 1.61 – 1.49 (m, 1H) ppm.

¹³C NMR (126 MHz, DMSO-d₆): δ = 164.9, 148.0, 145.3, 141.9, 141.2, 123.4, 119.2 (q, J_C-F = 261.0 Hz), 117.4, 97.2, 86.4, 51.7, 46.1, 45.1, 30.3, 26.1 ppm.

¹⁹F NMR (282 MHz, DMSO-d₆): δ = -61.71 (s) ppm.

HRMS (APCI-TOF) m/z: calculated for C₁₅H₁₇F₃N₇ [M+H]⁺: 352.1492, found 352.1492.

IR (ν_max, neat) = 3336, 3305, 3162, 2957, 2937, 2854, 2827, 1654, 1567, 1483, 1421, 1397, 1316, 1292, 1260, 1209, 1161, 1133, 981, 941, 915, 800, 763, 640, 627, 620, 501 cm⁻¹.

m.p. = 221 – 224 °C
Determination of enantiomeric excess (ee) of compound (11):

Column : Daicel-Chiralpak-AD Analytical column (particle size 4.6 mm×250 mm)
Injection Volume : 20 µL (a solution of 1 mg of compound 11 in 1 mL of IPA)
Flow Rate : 1 mL/min
Mobile Phase : n-hexane/IPA 90 :10+ 0.5% diethylamine
Retention times : Enantiomer - A (16.58 min) + Enantiomer - B (21.90 min, required)

tert-butyl (R)-3-(7-amino-3-(1-(trifluoromethyl)-1H-pyrazol-4-yl)pyrazolo[1,5-a]pyrimidin-5-yl)piperidine-1-carboxylate (12):

Di-tert-butyl dicarbonate (1.518 g, 6.96 mmol) was added to a mixture of 11 (2.220 g, 6.33 mmol) and triethylamine (4.41 mL, 31.59 mmol) in CH₂Cl₂ (90 mL) and the mixture was stirred at 25 °C for 4 hours. The resulting solution was quenched with aqueous saturated NaHCO₃ (150 mL) and the mixture was extracted with CH₂Cl₂ (3 × 600 mL). The organic extracts were dried over MgSO₄, filtered, and concentrated under reduced pressure. The residue was purified by flash column chromatography (CH₂Cl₂/MeOH = 95:05) to afford 12 (2.760 g, 97 %) as a pale yellow solid.

¹H NMR (300 MHz, CDCl₃): δ = 8.36 (s, 1H), 8.27 (s, 1H), 8.19 (s, 1H), 6.07 (s, 1H), 5.67 (s, 2H), 4.41 – 4.26 (m, 1H), 4.22 – 3.96 (m, 1H), 3.18 – 2.99 (m, 1H), 2.92 – 2.67 (m, 2H), 2.20 – 2.05 (m, 1H), 1.96 – 1.74 (m, 2H), 1.70 – 1.62 (m, 1H), 1.48 (s, 9H) ppm.

¹³C NMR (126 MHz, CDCl₃): δ = 164.2, 155.1, 146.7, 145.6, 141.6, 141.3, 123.6, 119.5 (q, J_C-F = 262.1 Hz), 116.8, 99.7, 87.8, 79.8, 48.7, 44.4, 30.4, 28.6, 25.2 ppm.

¹⁹F NMR (282 MHz, CDCl₃): δ = -61.13 (s) ppm.
HRMS (APCI-TOF) m/z: calculated for C_{20}H_{25}F_{3}N_{7}O_{2} \ [M+H]^+: 452.2016, found 452.2015.

IR (v_{\text{max}}, \text{neat}) = 3453, 3312, 3179, 2977, 2934, 2859, 1668, 1645, 1624, 1591, 1563, 1526, 1477, 1417, 1366, 1319, 1288, 1170, 917, 883, 803, 733, 634 cm\(^{-1}\)

m.p. = 124 – 127 °C

tert-butyl (R)-3-(7-amino-6-bromo-3-(1-(trifluoromethyl)-1H-pyrazol-4-yl)pyrazolo[1,5-a] pyrimidin-5-yl)piperidine-1-carboxylate (13):

To a solution of 12 (2.760 g, 6.09 mmol) in CH\(_2\)Cl\(_2\) (60 mL) was added \(t\)-BuNH\(_2\) (45 mL). Then, a solution of Br\(_2\) (0.315 mL, 6.09 mmol) in CH\(_2\)Cl\(_2\) (15 mL) was added dropwise and the resulting mixture was stirred at 25 °C for 16 hours. The reaction mixture was concentrated under reduced pressure and the residue was purified by flash column chromatography (CH\(_2\)Cl\(_2\)/MeOH = 97:03) to afford 13 (3.105 g, 96 %) as a white solid.

\(^1\)H NMR (300 MHz, CDCl\(_3\)): \(\delta = 8.30\) (s, 1H), 8.28 (s, 1H), 8.16 (s, 1H), 6.11 (s, 2H), 4.47 – 4.27 (m, 1H), 4.25 – 4.05 (m, 1H), 3.34 – 3.18 (m, 1H), 3.17 – 3.00 (m, 1H), 2.94 – 2.71 (m, 1H), 2.18 – 2.07 (m, 1H), 1.94 – 1.76 (m, 2H), 1.74 – 1.63 (m, 1H), 1.48 (s, 9H) ppm.

\(^13\)C NMR (126 MHz, CDCl\(_3\)): \(\delta = 160.6, 154.9, 144.5, 143.5, 141.6, 141.6, 123.7, 119.5\) (q, \(J_{C-F} = 263.0\) Hz), 116.3, 100.4, 85.5, 79.7, 48.1, 44.3, 42.8, 29.8, 28.7, 25.2 ppm.

\(^19\)F NMR (282 MHz, CDCl\(_3\)): \(\delta = -61.16\) (s) ppm.

HRMS (APCI-TOF) m/z: calculated for C\(_{20}\)H\(_{24}\)BrF\(_3\)N\(_7\)O\(_2\) \ [M+H]^+: 530.1121, found: 530.1119.
IR (ν_{max}, neat) = 3435, 3297, 3235, 3198, 2976, 2928, 2854, 1676, 1642, 1618, 1588, 1540, 1468, 1417, 1397, 1365, 1317, 1288, 1245, 1174, 1150, 1065, 941, 802, 626 cm\(^{-1}\).

m.p. = 110 – 113 °C

(R)-6-bromo-5-(piperidin-3-yl)-3-(1-(trifluoromethyl)-1H-pyrazol-4-yl)pyrazolo[1,5-a]pyrimidin-7-amine (MU380):

Trifluoroacetic acid (17.85 mL, 233.16 mmol) was slowly added to a solution of 10 (3.09 g, 5.82 mmol) in CH\(_2\)Cl\(_2\) (75 mL). The reaction mixture was stirred at 25 °C for 2 hours, then the solvents were evaporated. The residue was dissolved in 7 M NH\(_3\) in MeOH (45 mL) and the solution was stirred at 25 °C for 2 hours. The reaction mixture was concentrated under reduced pressure and the residue was purified by flash column chromatography (CH\(_2\)Cl\(_2\)/7 M NH\(_3\) in MeOH = 95:05) to afford MU380 (2.430 g, 97 %) as a white solid with 96% ee.

**Determination of enantiomeric excess (ee) of MU380:**

Column : Daicel-Chiralpak-OJ Analytical column (particle size 4.6 mm×250 mm)

Injection Volume : 20 \(\mu\)L (a solution of 1 mg of compound MU380 in 1 mL of EtOH)

Flow Rate : 1 mL/min

Mobile Phase : n-hexane/EtOH = 95:05+ 0.5% diethylamine

Retention times : Enantiomer - A (30.07 min, required) + Enantiomer - B (35.76 min)

**Recrystallization of MU380:**

MU380 (2.430 g, 96% ee) in acetonitrile (33 mL) was refluxed for 1 hour, then the solution was allowed to cool to 25 °C and stirred for 1 hour. The precipitated white solid was filtered, washed with acetonitrile (6 mL) and dried under vacuum to afford enantiomerically pure MU380 (1.815 g, 75%) with >99% ee.
Determination of enantiomeric excess (ee) of MU380:

Column: Daicel-Chiralpak-OJ Analytical column (particle size 4.6 mm x 250 mm)

Injection Volume: 20 µL (a solution of 1 mg of compound MU380 in 1 mL of EtOH)

Flow Rate: 1 mL/min

Mobile Phase: n-hexane/EtOH = 95:05 + 0.5% diethylamine

Retention times: Enantiomer - A (29.82 min, required)

$^1$H NMR (500 MHz, DMSO-d$_6$): $\delta = 8.72$ (s, 1H), 8.54 (s, 1H), 8.51 (s, 1H), 3.23 – 3.15 (m, 2H), 3.14 – 3.09 (m, 1H), 2.99 – 2.93 (m, 1H), 2.80 – 2.72 (m, 1H), 2.58 – 2.52 (m, 1H), 2.04 – 1.94 (m, 1H), 1.82 – 1.68 (m, 2H), 1.57 – 1.46 (m, 1H) ppm.

$^{13}$C NMR (126 MHz, DMSO-d$_6$): $\delta = 161.2$, 145.5, 143.2, 141.9, 141.7, 123.8, 119.1 (q, $^{1}J_{C\text{-F}} = 261.1$ Hz), 116.8, 98.2, 83.9, 50.4, 45.9, 43.4, 29.5, 26.0 ppm.

$^{19}$F NMR (282 MHz, DMSO-d$_6$): $\delta = -61.76$ (s) ppm.

HRMS (APCI-TOF) m/z: calculated for C$_{15}$H$_{16}$BrF$_3$N$_7$ [M+H]$^+$: 430.0597, found 430.0595.

IR ($\nu_{\text{max, neat}}$) = 3292, 3201, 3153, 2935, 2855, 1642, 1628, 1591, 1541, 1419, 1397, 1316, 1289, 1263, 1207, 1169, 1133, 1104, 940, 921, 868, 802, 747, 664, 622, 560 cm$^{-1}$.

m.p. = 147 – 148 °C
$^1$H NMR (500MHz) spectrum of compound 2 in CDCl$_3$

$^{13}$C NMR (126 MHz) spectrum of compound 2 in CDCl$_3$
HRMS of compound 2

calculated mass: \([\text{M-H}]^+ = 273.1809\)
observed: \([\text{M-H}]^+ = 273.1809\) mass accuracy < 0.1 ppm

IR spectrum of compound 2
$^{1}$H NMR (500MHz) spectrum of compound 3 in CDCl$_3$

$^{13}$C NMR (126 MHz) spectrum of compound 3 in CDCl$_3$
C_{13}H_{20}N_2O_3

HRMS of compound 3

IR spectrum of compound 3
HPLC chromatogram of compound 3 (racemic)
HPLC chromatogram of compound 3 (non-racemic)
**H NMR (500MHz) spectrum of compound 4 in DMSO-\textit{d6}**

**\textsuperscript{13}C NMR (126 MHz) spectrum of compound 4 in DMSO-\textit{d6}**
HRMS of compound 4

IR spectrum of compound 4

C_{14}H_{23}N_{5}O_{2}  \quad \text{mono } m/z \ 317.1852

APCI (+) (MMI)
nitrogen flow 5 L/min, gas temperature 325°C, nebulizer 45 psi, skimmer 65 V,
vaporizer 250°C, fragmentor 16 V, dissolved in methanol

observed: [M+H]^+ \sim 318.1925 \quad \text{mass accuracy} < 0.1 \text{ ppm}
HPLC chromatogram of compound 4 (racemic)
HPLC chromatogram of compound 4 (non-racemic)
$^{1}\text{H NMR (500MHz) spectrum of compound 5 in CDCl}_3$

$^{13}\text{C NMR (126 MHz) spectrum of compound 5 in CDCl}_3$
HRMS of compound 5

Calculated mass: [M+H]^+ = 434.2762
Observed: [M+H]^+ = 434.2762
Mass accuracy < 0.1 ppm

IR spectrum of compound 5
$^1$H NMR (500MHz) spectrum of compound 6 in CDCl$_3$

$^{13}$C NMR (126 MHz) spectrum of compound 6 in CDCl$_3$
HRMS of compound 6

IR spectrum of compound 6
$^1$H NMR (300MHz) spectrum of compound 7 in CDCl$_3$

$^{13}$C NMR (126 MHz) spectrum of compound 7 in CDCl$_3$
$^{19}$F NMR (282 MHz) spectrum of compound 7 in CDCl₃
$^1$H NMR (300MHz) spectrum of compound 8 in CDCl$_3$

$^{13}$C NMR (126 MHz) spectrum of compound 8 in CDCl$_3$
$^{19}$F NMR (282 MHz) spectrum of compound 8 in CDCl$_3$

$^1$H NMR (300MHz) spectrum of compound 9 in CDCl$_3$
$^{13}$C NMR (126 MHz) spectrum of compound 9 in CDCl$_3$

$^{19}$F NMR (282 MHz) spectrum of compound 9 in CDCl$_3$
$^1$H NMR (300 MHz) spectrum of compound 10 in CDCl$_3$.

$^{13}$C NMR (126 MHz) spectrum of compound 10 in CDCl$_3$.
$^{19}\text{F NMR (282MHz) spectrum of compound 10 in CDCl}_3$

**HRMS of compound 7**

- **APCI + (MMI)**
  - Nitrogen flow 5 L/min, gas temperature 300°C, nebulizer 45 psi, skimmer 65 V, vaporizer 200°C, fragmentor 20 V, dissolved in methanol

- **C$_{26}$H$_{38}$F$_3$N$_3$O$_4$**
  - Mono m/z = 567.2781
  - Calculated mass: [M+H]$_+$ = 568.2854
  - Observed: [M+H]$_+$ = 568.2851
  - Max. mass error = 0.5 ppm
IR spectrum of compound 10

$^1$H NMR (500MHz) spectrum of compound 11 in DMSO-$d_6$
\(^{13}\)C NMR (126 MHz) spectrum of compound \(\text{11}\) in DMSO-\(d_6\)

\(^{19}\)F NMR (282MHz) spectrum of compound \(\text{11}\) in DMSO-\(d_6\)
HRMS of compound 11

IR spectrum of compound 11
HPLC chromatogram of compound 11 (racemic)
HPLC chromatogram of compound 11 (non-racemic)
HPLC chromatogram of compound 11
H NMR (300MHz) spectrum of compound 12 in CDCl₃

¹³C NMR (126 MHz) spectrum of compound 12 in CDCl₃
$^{19}$F NMR (282MHz) spectrum of compound 12 in CDCl$_3$

HRMS of compound 12
IR spectrum of compound 12

$^1$H NMR (300MHz) spectrum of compound 13 in CDCl$_3$
$^{13}$C NMR (126 MHz) spectrum of compound 13 in CDCl$_3$

$^{19}$F NMR (282 MHz) spectrum of compound 13 in CDCl$_3$
HRMS of compound 13

IR spectrum of compound 13
$^1$H NMR (500MHz) spectrum of MU380 in DMSO-$d_6$ 

$^{13}$C NMR (126 MHz) spectrum of MU380 in DMSO-$d_6$
$^{19}$F NMR (282MHz) spectrum of MU380 in DMSO-$d_6$

$C_{15}H_{15}BrF_3N_7$  
mono m/z = 429.0524

ACPI + (MMI)  
nitrogen flow 5 L/min, gas temperature 300°C, nebulizer 45 psi, skimmer 65 V, vaporizer 200°C, fragmentor 20 V, dissolved in methanol

X10^2 +ACPI Scan (0.095-0.478 min, 24 scans) Frag=20.0V PK-03-03_APCIpos_fr20_0001.d Subtract (2)

430.0595

Calculated mass: [M+H]$^+$ = 430.0597  
Observed: [M+H]$^+$ = 430.0595  
Max. mass error ~0.4 ppm

HRMS of MU380
IR spectrum of MU380
HPLC chromatogram of compound **MU380** (96% ee, before recrystallization)
HPLC chromatogram of compound **MU380** (after recrystallization)