**SUPPORTING INFORMATION**

# Synthetic Studies on Centromere-Associated Protein-E (CENP-E) Inhibitors: 2. Application of Electrostatic Potential Map (EPM) and Structure-Based Modeling to Imidazo[1,2-*a*]pyridine Derivatives as Anti-Tumor Agents

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**Computational 3D model of CENP-E protein with lead compound 1a**

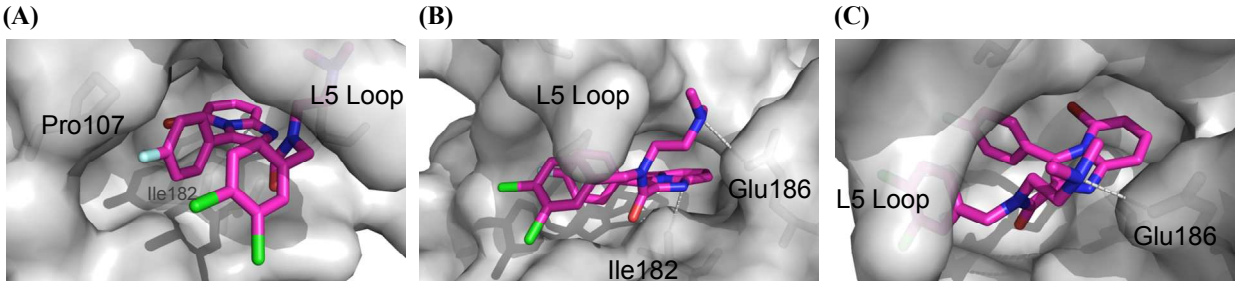


**Figure S1.** (A) hydrophobic pocket including Pro107, (B) hydrophobic pocket containing Ile182, and (C) hydrophobic L5 loop.

**CENP-E Enzyme Assay**

ATPase assay for determining human kinesin activities was performed in 384 well plates using 62.5 ng/mL CENP-E motor domain (Cytoskeleton), 22 µg/mL Microtubule (Cytoskeleton) and 25 µM ATP. Reactions were performed in 6 µL of reaction buffer (20 mM PIPES-KOH, pH 6.8, 3.0 mM $MgCl_2$, 3.0 mM KCl, 1.0 mM EGTA, 1.0 mM dithiothreitol, 0.01% w/v Brij35, and 0.2% w/v BSA) for 60 min at room temperature. The amount of ADP produced during the ATPase reaction was determined with ADP-Glo (Promega). The luminescence was measured with Envision (PerkinElmer Inc., MA).

To evaluate the inhibitory activity of test compounds at high ATP concentration, compounds were pre-incubated with CENP-E motor domain and Microtubule for 60 min and the ATPase reactions were initiated by adding ATP sufficient to yield a final concentration of 500 µM. After incubation for 20 min at room temperature, the amount of ADP was determined with ADP-Glo. The luminescence was measured with Envision.

**Site-directed Point Mutagenesis Experiments**

Human CENP-E motor domain mutant proteins, I182L and T183A with a GST tag at the N-terminus were prepared by a wheat germ cell-free protein synthesis system using WEPRO7240 extract (CellFree Sciences) according to the manufacturer's protocol. Mutation was introduced by overlap-extension PCR method. Expressed mutants were purified by Glutathione Sepharose 4B resin (GE Healthcare). The ATPase activity for the CENP-E mutant proteins was measured by ADP-Glo assay. The amino acid alignment between human and mouse CENP-E is described in Table S1.

**Table S1. Amino Acid Alignment between human and mouse**

| | 61 | 62 | 63 | 64 | 65 | 66 | 67 | 68 | 69 | 70 | 71 | 72 | 73 | 74 | 75 | 76 | 77 | 78 | 79 | 80 | 81 | 82 | 83 | 84 | 85 | 86 | 87 | 88 | 89 | 90 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Human | N | V | Y | E | E | I | A | A | P | I | I | D | S | A | I | Q | G | Y | N | G | T | I | F | A | Y | G | Q | T | A | S |
| Mouse | N | V | Y | E | E | I | A | V | P | I | I | S | S | A | I | Q | G | Y | N | G | T | I | F | A | Y | G | Q | T | A | S |
| | 91 | 92 | 93 | 94 | 95 | 96 | 97 | 98 | 99 | 100 | 101 | 102 | 103 | 104 | 105 | 106 | 107 | 108 | 109 | 110 | 111 | 112 | 113 | 114 | 115 | 116 | 117 | 118 | 119 | 120 |
| Human | G | K | T | Y | T | M | M | G | S | E | D | H | L | G | V | I | P | R | A | I | H | D | I | F | Q | K | I | K | K | F |
| Mouse | G | K | T | H | T | M | M | G | S | E | D | C | L | G | V | I | P | R | A | I | H | D | I | F | Q | R | I | K | K | F |
| | 121 | 122 | 123 | 124 | 125 | 126 | 127 | 128 | 129 | 130 | 131 | 132 | 133 | 134 | 135 | 136 | 137 | 138 | 139 | 140 | 141 | 142 | 143 | 144 | 145 | 146 | 147 | 148 | 149 | 150 |
| Human | P | D | R | E | F | L | L | R | V | S | Y | M | E | I | Y | N | E | T | I | T | D | L | L | C | G | T | Q | K | M | K |
| Mouse | P | E | R | E | F | L | L | R | V | S | Y | M | E | I | Y | N | E | T | I | T | D | L | L | C | N | A | Q | K | M | K |
| | 151 | 152 | 153 | 154 | 155 | 156 | 157 | 158 | 159 | 160 | 161 | 162 | 163 | 164 | 165 | 166 | 167 | 168 | 169 | 170 | 171 | 172 | 173 | 174 | 175 | 176 | 177 | 178 | 179 | 180 |
| Human | P | L | I | I | R | E | D | V | N | R | N | V | Y | V | A | D | L | T | E | E | V | V | Y | T | S | E | M | A | L | K |
| Mouse | P | L | I | I | R | E | D | T | N | R | T | V | Y | V | S | D | L | T | E | E | V | V | Y | T | A | E | M | A | L | K |
| | 181 | 182 | 183 | 184 | 185 | 186 | 187 | 188 | 189 | 190 | 191 | 192 | 193 | 194 | 195 | 196 | 197 | 198 | 199 | 200 | 201 | 202 | 203 | 204 | 205 | 206 | 207 | 208 | 209 | 210 |
| Human | W | I | T | K | G | E | K | S | R | H | Y | G | E | T | K | M | N | Q | R | S | S | R | S | H | T | I | F | R | M | I |
| Mouse | W | L | A | T | G | E | K | N | R | H | Y | G | I | T | K | M | N | Q | R | S | S | R | S | H | T | I | F | R | M | I |

**Kinesin Selectivity Assay**

ATPase assays for Eg5 motor domain (Cytoskeleton) and KHC motor domain (Cytoskeleton) were performed in reaction buffer (20 mM PIPES-KOH, pH 6.8, 3.0 mM $MgCl_2$, 3.0 mM KCl, 1.0 mM EGTA, 1.0 mM dithiothreitol, 0.01% w/v Brij35, and 0.2% w/v BSA)

using Microtubule and 100 μM ATP. The amount of ADP was determined with ADP-Glo. The luminescence was measured with Envision.

**Kinase Enzyme Assay**

**Table S2.** List of Kinases Evaluated for **(+)-(*S*)-12**

| | | | |
|---|---|---|---|
| AKT1 | c-Kit | IKKβ | PDGFRβ |
| ASK1 | c-Met | INSR | PI3Kα |
| Aurora-B | CSK | JAK1 | PKA |
| BRAF | EGFR | JNK1 | PLK1 |
| CDC7 | EPHA5 | MAPKAPK2 | ROCK1 |
| CDK1 | ERK1 | MEK1 | SRC |
| CDK2 | FAK | MEKK1 | SRPK1 |
| CHK1 | FGFR1 | NEK2 | Tie2 |
| CK1δ | GSK3β | p38α | VEGFR2 |

**Cellular Assay**

**Cell Cycle Synchronization**

The synchronous cells were used to monitor the cell cycle-dependent effects of the compounds in detail. We mainly used HeLa cells, a human cervix adenocarcinoma cell line, in the experiments because of their technical availability for various assays. HeLa cellswere plated at a concentration of 100,000 cells/well in 6 well plates with 2 mL of Dulbeccos's Modified Eagle medium (DMEM; GIBCO) containing 10% fetal bovine serum (FBS) until the cells adhered to the plates, and then the cells were treated with 2 mM of thymidine (Sigma Aldrich, USA). ($1^{st}$ block). Sixteen hours after the first treatment, thymidine was removed followed by 8 h incubation with DMEM containing 10% FBS and then treated again with 2 mM of thymidine for

16 h ($2^{nd}$ block). Seven hours after release from the second block, cells thus synchronized at the G2 phase were treated with test compounds at the indicated concentration for 4 h. The cells treated with test compounds were collected by trypsinization, and were fixed with 70% EtOH for cell cycle analysis.

**FACS Analysis**

FACS analysis was performed as described previously[S1], Briefly, the synchronous HeLa cells at G2 phase were treated with the indicated concentration of test compound for 4 h, and the cells then collected by trypsinization followed by fixation with 70% of EtOH overnight at −20 °C. After washing the fixed cells with FACS buffer (Dulbecco's Phosphase Buffered Saline (PBS; GIBCO) containing 4% FBS) twice, the cells were incubated in 100 μL of FACS buffer in presense of AlexaFluor 488-conjugated anti-phosho Histone H3 antibody (1:20 dilution; cell signaling, USA) and RNase (0.2 mg/ml; Invitrogen) for 30 min at room temperature in dark. The stained cells were washed with FACS buffer for three times, and suspended with FACS buffer containing 10 mg/mL of propidium iodide (PI; Sigma Aldrich, USA). Then thousand cells were analyzed by FACScalibur (Becton, Fickson and Company, NJ, USA) to detect pHH3 positive cells. Percentage of pHH3 positive cells was obtained by the following formula; % pHH3 positive cells = numbers of pHH3 positive cells/(10,000 – cell debris) × 100.

**Growth Inhibition Assay**

HeLa cells were plated in 96-well plates at a concentration of 2000 cells/well with 100 μM of DMEM, followed by being cultured at 37 °C with 5% $CO_2$ for 8 hr or more until the cells adhered to the culture plates. Cells were then treated with the indicated concentration of test compound. Three days after drug treatment, 50 μL of CellTiter-Glo Mixture (CellTiter-GloTM

Luminescent cell Viability Assay, Promega) were added into each well of 96-well plates, and chemical luminescence was measured by microplate vender according to the manufacturer's protocol (PerkinElmer). Proliferative inhibition rate was obtained by the following formula: proliferative inhibition rate (%) = (1- luminescence of test compound/luminescence of control) × 100.

**Immunofluorescence**

Immunofluorescence measurements were performed as described previously.[S1] The following antibodies were used: anti-CENP-B (sc22788; Santa Cruz Biotechnology), anti-BubR1 (612503), anti-α-tubulin (T9026; Sigma-Aldrich), anti-p53 (sc126; Santa Cruz Biotechnology), anti-53BP1 (sc22760; Santa Cruz Biotechnology), and HEC1 (ab3613; Abcam). Images were captured with a Plan-APOCHROMAT 100× oil lens on an Axiovert 200M microscope (Carl Zeiss).

**In vivo PD Assay**

Colo205 cells were selected for the in vivo study because, in addition to their technical availability, the Colo205 cells were sensitive to CENP-E inhibition. Colo205 cells (a human colon adenocarcinoma cell line) were xenografted into 5-week old nude mice by subcutaneous injection (5 x $10^6$ cells/mouse). Test compounds dissolved in 0.1 mol/L of citric acid with with 10% DMSO, 9% cremophor EL and 18% PEG 400, were intraperitoneally administered into xenografted mice with 150–400 $mm^3$ of tumors at the indicated dose, followed by a second intraperitoneal administration. At 24 h after the first administration, tumors were collected and homogenized in radioimmunoprecipitation assay (RIPA) buffer containing protease and

phosphatase inhibitors.The cell lysates were suspended in 2 × SDS sample buffer. After 5 min boiling, the samples were kept at −20 °C until Western blotting analysis.

**Western Blotting Analysis**

Western blotting was performed as described previously[S2]. Briefly, SDS-PAGE was carried out followed by transfer of proteins onto PVDF (polyvinylidene difluoride) membrane. The membranes were blocked with Block-Ace solution (Snow Brand Milk Product Co., Ltd.), and then incubated with primary antibodies in TBS buffer containing 0.05% Tween 20. As primary antibodies, anti-phospho-histone H3 (#9701, cell signaling) and anti-GAPDH (MAB374, Millipore) antibodies were used at a concentration of 0.1 to 1 μg/mL. Following the primary antibody treatment, the membranes were incubated with HRP-conjugated anti-mouse IgG (NA9301V, GE Healthcare) or HRP-conjugated anti-Rabbit + IgG (NA9301V, GE Healthcare). Immunoblotted proteins were visualized by chemiluminescence (Supersignal West Femto Maximum Sensitivity Substrate, # 34096, Thermo Scientific), and visualized images were captured by luminoimage analyzer LAS-1000 (Fuji Film).

**In vivo Biological Activity Testing**

Colo205 cells were xenografted into nude mice as described in the above in vivo PD assay section. Mice bearing tumors (100–250 $mm^3$) were selected and randomized into control and tested groups. The xenografted mice were treated with test compounds by intraperitoneal administration at the indicated doses twice a day × 2 every 5 days for 2 cycles. Each group included five animals. Tumor dimensions were measured regularly by calipers during the experiments and tumor volume was calculated. Tumor growth inhibition (% *T/C*) was calculated

by the following formula: tumor growth inhibition (% *T/C*) = {(tumor volume tested − tumor volume tested on day 0)/(tumor volume control − tumor volume control on day 0)} × 100

All in vivo procedures were performed in accordance with protocols approved by the Institutional Animal Care and Use Committee (IACUC) at Takeda Pharmaceutical Company Ltd (Experimental Protocol Number: 00004407).

**Electrostatic Potential Map (EPM) Analysis**

Electrostatic potential maps for each compound were calculated for the simplified dimethyl amide analogs (Figure S2). The partial charges, local energy minimum conformations and molecular surfaces for each compound were computed employing MOE (version 2011.10). During each procedure, the MMFF94x force field was set for the molecular mechanic calculation. The molecular surfaces computed are shown as Connolly surfaces in solid display style. The surfaces were colored by screened electrostatic potentials (transparency of front: 64; electrostatic color map ranges: from -15 (red) through 0 (white) to +15 (blue)).

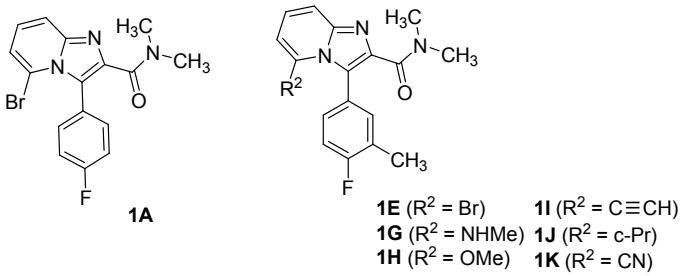


**Figure S2.** Simplified dimethyl amide analogs for EPM analysis.

**X-ray Structure Analysis**

*Crystal data for* **(+)-(*S*)-14**: $C_{32}H_{33}Cl_2FN_4O_5$, *MW* = 643.54; crystal size, 0.17 x 0.05 x 0.04 mm; colorless, prism; triclinic, space group *P*1, *a* = 10.5392(2) Å, *b* = 11.6005(2) Å, *c* = 12.8463(2) Å, $\alpha$ = 85.8900(10)°, $\beta$ = 88.4340(11)°, $\gamma$ = 89.6050(11)°, *V* = 1565.95(5) Å$^3$, *Z* = 2, *D*x = 1.365 g/cm$^3$, *T* = 100 K, $\mu$ = 2.3114 mm$^{-1}$, $\lambda$ = 1.5419 Å, $R_1$ = 0.064, $wR_2$ = 0.139, GOF = 1.034.

The absolute configuration of **(+)-(*S*)-14** was determined as *S* based on the Flack parameter,[S3] 0.05(2). All measurements were made on a Rigaku R-AXIS RAPID diffractometer using graphite monochromated Cu-K$\alpha$ radiation. The structure was solved by direct methods with SHELXS-97[S4] and was refined using full-matrix least-squares on $F^2$ with SHELXL-97.[S4] All non-H atoms were refined with anisotropic displacement parameters. CCDC 1037306 for compound **(+)-(*S*)-14** contains the supplementary crystallographic data for this paper. These data can be obtained free of charge from The Cambridge Crystallographic Data Centre via www.ccdc.cam.ac.uk/data_request/cif.

*Crystal data for* **(−)-(*R*)-14**: $C_{32}H_{33}Cl_2FN_4O_5$, *MW* = 643.54; crystal size, 0.20 x 0.19 x 0.05 mm; colorless, platelet; triclinic, space group *P*1, *a* = 10.5301(2) Å, *b* = 11.5817(2) Å, *c* = 12.8513(2) Å, $\alpha$ = 85.8039(8)°, $\beta$ = 88.4421(8)°, $\gamma$ = 89.6201(8)°, *V* = 1562.50(5) Å$^3$, *Z* = 2, *D*x = 1.368 g/cm$^3$, *T* = 100 K, $\mu$ = 2.3165 mm$^{-1}$, $\lambda$ = 1.5419 Å, $R_1$ = 0.063, $wR_2$ = 0.160, GOF = 1.035.

The absolute configuration of **(−)-(*R*)-14** was determined as *R* based on the Flack parameter,[S3] 0.04(2). All measurements were made on a Rigaku R-AXIS RAPID diffractometer using graphite monochromated Cu-K$\alpha$ radiation. The structure was solved by direct methods with SHELXS-97[S4] and was refined using full-matrix least-squares on $F^2$ with SHELXL-97.[S4]

All non-H atoms were refined with anisotropic displacement parameters. CCDC 1037305 for compound **(−)-(*R*)-14** contains the supplementary crystallographic data for this paper. These data can be obtained free of charge from The Cambridge Crystallographic Data Centre via www.ccdc.cam.ac.uk/data_request/cif.

**ORTEP drawing of (+)-(*S*)-14 and (−)-(*R*)-14**

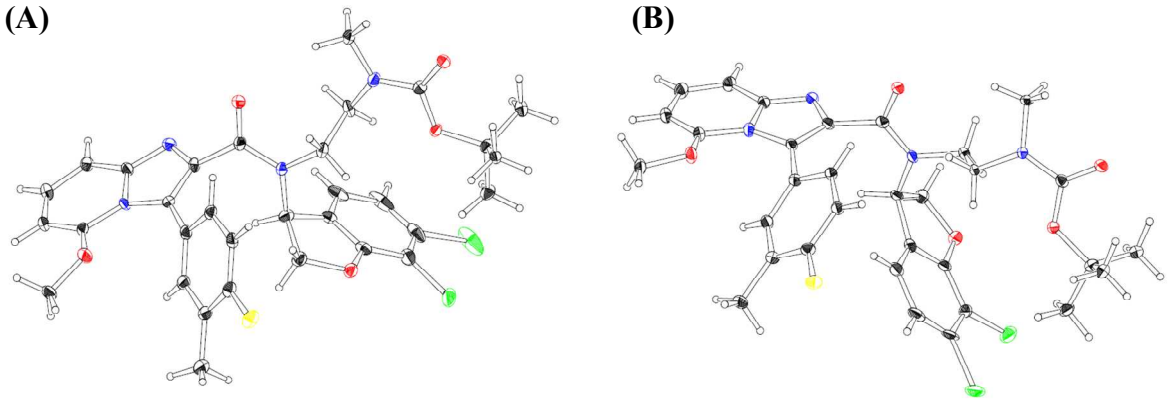


**Figure S3.** (A) ORTEP drawing of **(+)-(*S*)-14**. (B) ORTEP drawing of **(−)-(*R*)-14**. Displacement ellipsoids are drawn at the 20% probability level.

**References**

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