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Article

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Ethyl 2-((4-chlorophenyl)amino)thiazole-4-carboxylate and derivatives are potent inducers of Oct3/4

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ABSTRACT: The Octamer-binding transcription factor 4 (Oct3/4) is a master gene in the transcriptional regulatory network of pluripotent cells. Repression of Oct3/4 in embryonic stem cells (ESCs) is associated with cell differentiation and loss of pluripotency, whereas forced overexpression in cooperation with other transcriptional factors, such as Nanog, Sox2 and Lin28, can reprogram somatic cells back into pluripotent cells, termed induced pluripotent stem cells (iPSCs). However, random integration and potential tumorigenic transformation caused by viral transduction limit the clinical application of iPSCs. Performing a cell-based High Throughput Screening (HTS) campaign, we identified several potential small molecules as inducers of Oct3/4 expression. Here we report a lead structure ethyl 2-((4-chlorophenyl)amino)-thiazole-4-carboxylate, termed O4I2, showing high activity in enforcing Oct3/4 expression. Based on chemical expansion we further identified derivatives having increased activities towards Oct3/4 induction. Thus O4I2 and its derivatives should provide a new class of small molecules suitable for iPSC generation.

Introduction

Oct3/4 (Octamer-binding transcription factor 4), also referred to POU5F1, Oct4A, Oct3 or Oct4, is a member of the POU family of transcription factors[1] expressed specifically in pluripotent cells, including embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs).[2] Evidence is given that loss-of-function mutation of Oct3/4 leads to differentiation of ESCs into trophoblast,[3] while gain-of-function or over-expression leads to differentiation into primitive endoderm and mesoderm,[4] supporting the important role of Oct3/4 in regulation of the cellular pluripotency core network. It had been shown by the groups of Yamanaka and Thomson that terminally differentiated cells can be de-differentiated back to pluripotency by viral transduction with a set of defined factors, Oct3/4, Sox2, KLF4 and c-Myc,[5, 6] or alternatively Oct3/4, Sox2, Nanog and Lin28.[7] However, the low efficiency and risks associated with the genetic transformation handicap the wide use of iPSCs in the clinic,[8] where iPSCs could be an infinite source for patient-specific cells and tissue. The desire for improved protocols for the generation of iPSCs boosts researches into reprogramming technology to increase efficacy and address safety concerns.[9, 10] Of particular interest are small molecules that can replace defined factors and enhance reprogramming efficiency.[11-13] Recently, an azaindole derivative was reported to enhance Oct3/4 activation and thereby promote reprogramming efficiency of mouse embryonic fibroblast transfected with four defined factors.[14] Moreover, scientists successfully generated mouse iPSCs from mouse embryonic fibroblasts (MEFs) cells by using a combination of seven small molecules, indicating that small molecule driven induction of defined factors can be sufficient in reprogramming.[15] However, human fibroblasts still seem to be more refractory, and so far researchers had to over-express at least one transcription factor, namely Oct3/4, to

achieve generation of human iPSCs, in combination with different small molecules. Thus, Oct3/4 seems to be a non-redundant defined factor in human cell reprogramming.[16, 17]

Results and discussion

Initially, we performed a High Throughput Screening (HTS) campaign using HEK293 cells stably expressing luciferase reporter under the control of a larger human Oct3/4-promoter region (HEK-Oct3/4) and identified hundreds of compounds from a pool of 250,000 small molecules able to elevate luminescent intensity in the threshold of 150% effect relative to cells treated with DMSO alone. Interestingly, analysis of the hit compounds using Instant JChem (ChemAxon, Hungary) revealed a cluster of dozens of compounds containing a thiazole-ring. Among them, ethyl 2-(4-chlorophenylamino)thiazole-4-carboxylate (Fig. 1A), referred to as **O**ct**4** **i**nducing compound **2** (O4I2) to distinguish from O4I1 we reported recently,[18] and others with 2-(phenylamino)thiazole-4-carboxylate moiety exhibited approximate 2-fold higher activity at 20 μM. Although in general HTS is an efficient approach to identify desired small molecule(s) using a well designed assay, it suffers from classes of small molecules, that appear as frequent hitters in divers assays in a drug-unlike fashion, called pan-assay interference compounds (PAINS).[19, 20] Very recently, 2-aminothiazoles (in concentrations above 200 μM) have been identified as one of the PAINS in biological protein binding assays.[21] Nevertheless the authors emphasized that 2-aminothiazoles at low concentrations (like 20 μM) could still show specific activities in HTSs.[21] Considering that our results were obtained from a cell-based HTS at a recommended concentration[21] and numerous approved drugs, such as Talipexole and Pramipexole, are based on 2-aminothiazole as pharmacophore,[22] we were curious to investigate O4I2 in detail.

To independently verify the results obtained from HTS, we followed Hantzsch thiazole synthesis[23] and successfully synthesized O4I2 in a reaction of 1-(4-chlorophenyl)thiourea with ethyl 3-bromo-2-oxopropanoate in ethanol in a good yield of 89%. The detailed reaction protocol as well as compound characterization is described in the experimental section.

Using the independently newly synthesized, purified O4I2, we analyzed the response to O4I2 on the level of luminescence and found the positive correlation to concentrations at 72 hrs of treatment, showing the highest activation in 3.5-fold at 40 μM, while we could not detect clear difference within the signals after 48 hrs treatment at the tested concentrations, suggesting that a long-term exposure might be required to achieve a higher activation (Fig. 1B). Previous reports illustrated the importance of the Oct3/4 protein in completely accomplishing conversion from somatic cells into iPSCs by binding to the corresponding promoters of other pluripotency related genes and thereby facilitating their expressions.[24] Hence, we immunoblotted non-infected HEK293 cells after 72 hrs treatment to determine the protein level of Oct3/4. The results demonstrated a clear accumulation of Oct3/4 in a concentration-dependent manner (Fig. 1C). Moreover, the level of Nanog protein was increased (Fig. 1C). Analysis by qRT-PCR revealed a significant induction of stemness-associated genes after stimulation with O4I2, including Oct3/4, Nanog and Sox2 in HEK293 cells, while the level of Lin28 remained unaffected and Rex1 was even reduced (Fig. 1D).

Our results showed that O4I2 might not only promote Oct3/4 expression by activating its promoter site, but also stabilize the Oct3/4 protein in HEK293 cells. Next, we directly assayed the transcriptional activity of Oct3/4 using a luciferase reporter driven by an Oct3/4 transcriptional response element in embryonal carcinoma NCCIT cells (NCCIT-Oct3/4) and found an up to 4-fold increased luminescence intensity after 72 hrs treatment (Fig. 2A). In good

agreement, the results obtained from an online luciferase assay, in which the signal of luminescence was constantly recorded in NCCIT-Oct3/4 cells treated with increasing concentrations of O4I2, also interestingly showed transcriptional activation induced not only by Oct3/4 but also Nanog over the tested period (SI. 1 and 2). Moreover, we accordingly observed that O4I2 markedly stimulated Oct3/4 even at 5 μM after 72 hrs treatment on the translational level (Fig. 2B). Of note, the accumulation of Oct3/4 in NCCIT occurred already 2 hrs after incubation with 20 μM O4I2 (Fig. 2C), while the detectable Sox2 increase appeared much later, implicating compound-mediated activation of Oct3/4 might be different between embryonic kidney HEK293 cells and embryonal carcinoma NCCIT cells. Next, we examined the inducible ability of O4I2 in HeLa cells, which do not express Oct3/4.[25] The results clearly showed the concentration-dependent activation of Oct3/4, as well as its downstream target Nanog, thus implying that O4I2-mediated induction was independent of the cellular intrinsic endogenous Oct3/4 level (Fig. 2D).

Human fibroblasts are terminally differentiated, accessible cells, which have been shown to be amenable to reprogramming with relatively high efficiency and thus have emerged as a favourable donor source in iPSCs generation. To test if our newly discovered small molecule could induce pluripotent marker activation in fibroblasts, we investigated the expression of several reprogramming-associated markers measured by qRT-PCR after 48 hrs treatment with O4I2 in human primary skin fibroblasts (HFs) and detected a marked increase of Oct3/4 expression (15-fold), as shown in Fig. 3A. Moreover, the induction of Lin28 and Rex1 was even higher than Oct3/4, showing 20- and 30-fold increase in expression, respectively. We further detected a moderate activation of Nanog (2-fold). Interestingly, a mesenchymal marker N-Cadherin was notably repressed. We next examined the status of stemness markers at the protein

level in HFs, which were treated with O4I2 for 72 hrs. Immunoblot analysis revealed a considerable accumulation of Lin28 and Nanog (Fig. 3B). We performed immunocytochemistry and successfully detected co-expression of Oct3/4 and Sox2 (Fig. 3C; upper), as well as Nanog and Klf4 (Fig. 3C; lower) in HF cells treated with O4I2 for 5 days. However the level of Oct3/4 induced by small molecule was still much lower than that in non-treated embryonal NCCIT cells analyzed by immunoblotting (Fig 3D). It is well-known that levels of alkaline phosphatase (ALP) activity are elevated in ESCs and iPSCs.[4, 5, 7, 9, 11, 12, 15, 24] Therefore, we examined activity of ALP in O4I2-treated fibroblasts after 5 days, and included propidium iodide (PI) staining to visualize dead cells. In comparison to DMSO (mock) treatment, a slight but clear increase in the ALP-FITC signal could be detected indicating that an alternation of endogenous cellular properties occurred (SI. 3).

To further elucidate the activity, we next studied the influence of various substituents on the amino-group of O4I2 through chemical modification illustrated in SI. 4. The derivatives are listed in Tab. 1 and grouped as three sub-families, namely variant in 4-position of *N*-phenyl (indicated in pink), halogen- (indicated in violet) and disubstituted-variants (indicated in orange), whose respective activities were measured in HEK-Oct3/4 reporter cells and illustrated in Fig. 4. Regarding the para-variants, we found that the depletion of chlorophenyl-group (**2**), or replacement with butyl- (**3**), allyl- (**4**), acetyl- (**5**), benzyl-group (**7**) or rigid, bulk substituents (**8**) resulted in a significant reduction (**3**, **4**, **7**) or a complete loss (**2**, **5**, **8**) of activity, whereas removal of chloride (**6**) led to a slight functional enhancement, suggesting that the phenyl-group may be preferable in this position. Therefore, we developed another series of O4I2 derivatives based on 2-(phenylamino)-thiazole-4-carboxylate to investigate the influence of various halogens at different substituent position on the activity. As shown in Fig. 4, highlighted in violet, the

results demonstrated that compounds with chloro- and fluoro-groups in particular at *m*-position (compounds **9** and **15**) showed more activity in the luciferase assay. Moreover, we synthesized various dichloro-substituted derivatives and found that only *m,p*- or *o,p*-disubstituted could hold (**17**) or slightly enhance (**18**) luciferase activity. Finally, we examined if the aqueous solubility played a role in the activity. Thus we converted 4 compounds (**O4I2, 9, 18** and **20**) to their corresponding carboxylic acids (**22-25**) by heating with 4 N HCl, indicated as acid-variant in Fig. 4. To our surprise, no activity was observed with any of these four compounds. Further conversion of **22** with *N,N*-dimethylpropane-1,3-diamine to its corresponding amide (**26**) under dicyclohexylcarbodiimid (DCC) and *N*-hydroxybenzotriazol (HOBt) in dried CH_2Cl_2 also did not result in activity enhancement in HEK-Oct3/4 (SI. 4). These results might indicate a requirement of the short hydrophobic group in the thiazole moiety for sufficient activation, and further substantiate that the 2-aminothiazole derivatives do not act as PAINS in our cell-based luciferase reporter assay. For a more detailed analysis, other carboxamides should be synthesized and studied in the near future.

Conclusion

Oct3/4 is an essential element in the maintenance and generation of pluripotent cells and small molecule to activate Oct3/4 is still scant. In this work, we present a class of compounds carrying 2-aminothiazoles as potential Oct3/4 inducers identified from a cell-based HTS. Given that in very recent papers, 2-aminothiazole based compounds had been described to be pan-active in biological assays and therefore had been termed "PAINS",[19-21, 26] we carefully validated their specific activity. Using a chemical approach, we structurally verified the lead compound O4I2 and showed that (i) O4I2 activates Oct3/4 in HEK293 and embryonal NCCIT cells, and also in Oct-deficient HeLa and terminally-differentiated human fibroblasts, and (ii) that O4I2 activates

also other pluripotency-associated genes such as Lin28 and Nanog in a drug-like manner. Studying the structure-activity relationship of O4I2 based on 25 derivatives revealed an indispensable role of the phenyl-group and that its chloro- or fluoro-m-substituted derivatives further enhanced activity, while conversion to carboxylic acids led to a completed loss of activity Thus, the results obtained in our study provide clear evidence that a small molecule, namely O4I2, acts as a potent Oct3/4 inducer in various human cell lines including human fibroblasts. Importantly, the basic 2-aminothiazole structure itself, as well as its carboxylic acid derivatives, showed no activity. We completely agree that this basic 2-aminothiazole structure should have the potential to interact with many biological target molecules, based on its ability to form hydrogen bonds and aromatic interactions. Considering the complexity of the control of Oct3/4 expression, this could be even beneficial for the desired biological effect. The careful validation of our results using re-synthesis, chemical expansion of the basic structural motif and highly purified compounds for activity evaluation confirm the highly specific activity of the compounds described here. Interestingly, the very low concentration used in our initial screening, has been in the concentration range recommended by Devine et al.,[21] to avoid false positive results. Taken together, our results provide evidence, that this class of compounds could be a useful tool for chemical-mediated induction of pluripotency in somatic cells.

Experimental section

Cell culture

HEK293 and HeLa cells were cultured in DMEM (Gibco, Germany) containing 10% FBS (PAA, Germany) and 1% Penicillin/Streptomycin (Pen/Strep, Gibco, Germany) under 5% CO_2 at 37 °C in a humidified atmosphere. HF and HFF cells were under the same conditions, but with 20% FBS and 15% FBS, respectively. Oct3/4 stable reporter HEK293 cells were maintained in DMEM medium (10% FCS, 1% 1 M Hepes – Buffer, 0.5% Pen/Strep and 0.5% 200 mM glutamine with fresh addition of 100 µg/mL Hygromycin before use). NCCIT cells were maintained in RPMI1640 (Gibco, 10% FBS and 1% PS). NCCIT cells were infected Cignal Lenti Oct3/4 (SA Bioscience, Qiagen, Germany) according to the manufacturer's instructions and cultured in RPMI 1640 containing 10% FBS and 1% Pen/Strep with fresh addition of 400 ng/mL Puromycin (Santa Cruz, Germany).

Isolation and culture of primary human fibroblasts

Experiments with primary human fibroblasts were conducted with patients' informed consent and were approved by the Medical Ethics Committee of the Medical Faculty Mannheim, Heidelberg University (2009-350N-MA). The procedure was published previously.[18] Briefly, freshly retained human skin samples from redundant operation material containing the dermis and adipose tissue were cut into small pieces. In the presence of 0.25% trypsin in PBS the tissue was then cut with scalpels in order to dissociate the tissue until reaching a pasty consistency. These tissue fragments were then resuspended in fibroblast cell culture medium (DMEM containing 10% FBS, 1x non-essential amino acids, 1% penicillin/streptomycin and 50 µM ß-mercaptoethanol), plated on tissue culture dishes and kept at 37°C in a humidified atmosphere at

5% CO2 until fibroblasts grew out. These fibroblasts were further expanded and used for experiments.

Luciferase reporter assay

Beetle juice kit (PJK, Germany) was used to determine the luciferase activity as previously reported.[18] Briefly, HEK293-Oct3/4 or NCCIT-Oct3/4 reporter cells were seeded into 24-well tissue culture plates at a density of 100,000 per well for 24h. After treatment with compounds as indicated in the text, the cells were harvested with 80 µL Beetle lysis-juice at 37°C for 10 min. The protein concentration was determined by Bradford assay (Sigma, Germany). 100 µL reaction mixture containing luciferin and ATP was added to 20 µL cell lysis in a white plate (Gibco, Germany), incubated for 3 min and measured by a luminometer plate-reader. The activity was determined as percent luminous intensity of treated cells over control cells from at least five-independent experiments.

qRT-PCR

Total RNA was isolated by using GenElute™ Mammalian Total RNA Miniprep Kit (Sigmal, Germany) to reverse-transcriptional synthesize cDNA by using random primers. Quantitative reverse-transcription real-time-PCR was performed using a Light Cycler 480 (Roche, Germany) following a manufacturer's protocol using the following primer pairs (MWG, Eurofins, Germany): (Oct3/4) 5s: GAAGTTGGAGAAGGTGGAAC; 3as: GGTGATCCTCTTCTGCTTCAG; (Nanog) 5s: GAACTGTGTTCTCTTCCACC; 3as: CACCTGTTTGTAGCTGAGGTTC; (Sox2): 5s: CAAGACGCTCATGAAGAAGG; 3as: CATGTGCGCGTAACTGTCCATG; (Rex1) 5s: GATCTTCAACGAGTCCACCAG; 3as: GAAAGGTGGGAGATCCTCCTCTTC; (Lin28A) 5s: GTGGATGTCTTTGTGCACCAG; 3as:

GACACGGATGGATTCCAGAC. ß-Actin 5s: CTGACTACCTCATGAAGATCCTC; 3as: CATTGCCAATGGTGATGACCTG was used as an endogenous control. Data were normalized to the value of DMSO-treated cells showing the mean ± SD of quadruplicates and are representative of two independent experiments.

Immunoblotting

The immunoblotting was performed as previously described.[18, 27] Briefly, the cells were incubated with the compounds as indicated in the text and lysed using urea-lysis buffer (1 mM EDTA, 0.5% Triton X-100, 5 mM NaF, 6 M Urea, 1 mM Na_3VO_4, 10 µg/mL Pepstatin, 100 µM PMSF and 3 µg/mL Aprotinin in PBS). Enhanced chemiluminescence (ECL) immunoblot analysis was performed. 40µg of total protein was resolved on 10% SDS-PAGE gels and immunoblotted with Oct3/4 (Cell Signaling, NEB, Germany), Nanog (Abcam, USA), Lin28 (Epitomics, Biomol, Germany), Vinculin (Cell Signaling, NEB, Germany) or ß-Actin antibodies (Sigma Aldrich, Germany). Primary antibodies were incubated at a 1:1000 dilution in TBS (pH 7.5) with 0.1% Tween-20 and 5% BSA/milk with gentle agitation overnight at 4°C. Secondary antibodies (Cell Signaling) were incubated in TBS (pH 7.5) with 5% BSA/Milk and 0.1% Tween-20 at a 1:10,000 dilution for 1 hr at room temperature.

Immunofluorescence

As we reported,[18] human fibroblasts were seeded in a 96-well plate coated with Geltrex (Invitrogen, Germany) at a density of 10,000 cells/well. After 24 hrs the cells were incubated with O4I2 for 5 days in DMEM/F12 Glutamax containing 20% knockout serum replacement, 10 ng/mL bFGF and 1 mM ß-mercaptoethanol. Afterwards, the cells were fixed with 4% PFA at RT for 15 min, and blocked with blocking buffer (5% goat serum and 0.3% Triton X-100 in PBS)

for 1 hr. Blocking solution was aspirated and incubated with Oct3/4 (1:400, Cell Signaling), Nanog (1:1000, Cell Signaling), Sox2 (1:100, Cell Signaling) and Klf4 (1:100, Biomol) in antibody dilution buffer (1% BSA and 0.2% Triton X-100 in PBS) at 4°C overnight. The secondary antibody (Goat anti-mouse Alexa Flour® 594 or 488, Dianova, Germany) was added and incubated for 1 hr. Hoechst 33258 (1 μg/mL in PBS) was used to visualize nuclei.

Statistics

Statistical and graphical data analyses were performed using Microsoft Excel 2007 software. The statistical significance of compared measurements was performed by using the Student's one-tailed t-test.

Chemistry

Reagents

Solvents and reagents obtained from commercial suppliers were at least of reagent grade and were distilled or dried according to prevailing methods prior to use, if necessary. For monitoring the reactions, Merck silica gel plastic plates 60 F254 (detection by UV) for TLC (thin-layer chromatography) were used. Chromatography was accomplished on silica (0.060−0.200 mm, 60 Å). HPLC analysis confirmed the purity of each compound was at least >96%.

Analytical Methods

^{1}H and ^{13}C NMR spectra were recorded on a Varian 300 MHz NMR system (^{1}H: 300 MHz, ^{13}C: 75 MHz). Chemical shifts are reported in ppm from tetramethylsilane with solvent as the internal standard (^{1}H DMSO-d_6: δ 2.50; ^{13}C DMSO-d_6: δ 39.5). The following abbreviations were used to explain the multiplicities in NMR spectra: s =singlet, d = doublet, t = triplet, q =

quartet, m = multiplet. High-resolution mass spectra (HRMS) were recorded on a Bruker ApexQe hybrid 9.4 T FT-ICR (ESI). The purity of the compounds was determined by HPLC (Agilent 1200 HPLC system, detection by UV at 277 nm) on the column Poroshell 120 SB-C18 (4.6×100mm 2.7 Micron) and ≥95% unless otherwise indicated. Elemental analyses were performed on an Element Analyzer Perkin Elmer EA 240 or 2400 CHN.

General procedure for the syntheses

Method: A solution of ethyl bromopyruvate (1.1 mmol) and thiourea (1.0 mmol) in absolute ethanol 20 mL was stirred under reflux monitored by TLC. Afterwards the mixture was diluted in sodium bicarbonate (saturated) and extracted with ethyl acetate and dried over the sodium sulfate. The combined organic phase was evaporated and the residue was purified by chromatography if required to get the product in high yield (>70%). The carboxylic acids were obtained from conversion of corresponding ester by incubation with 4 N HCl under reflux.

Ethyl 2-((4-chlorophenyl)amino)thiazole-4-carboxylate (O4I2, 1) 89%

^{1}H-NMR (300 MHz; DMSO-d_6): 1.29 (t, ^{3}J=6.9, 3H), 4.27 (q, ^{3}J=7.2, 2H), 7.35-7.40 (m, 2H), 7.65-7.70 (m, 2H), 7.79(s, 1H), 10.52 (s, 1H). ^{13}C--NMR (DMSO-d_6): 14.2, 60.4, 118.4, 118.9, 124.9, 128.8, 139.6, 142.3, 160.7, 162.8. HRMS (ESI) Calculated m/z: 283.0303; found *m/z:* 283.0303 $[M+H]^+$. Anal. Calcd for $C_{12}H_{11}ClN_2O_2S$: C, 50.98; H, 3.92; N, 9.91. Found: C, 50,84; H, 4.22; N, 9.83. IR (cm^{-1}): 2495, 2161, 2022, 1729, 1543, 1436, 1199, 1086, 1017, 792, 706.

Ethyl 2-aminothiazole-4-carboxylate (2) 75%

^{1}H-NMR (300 MHz; DMSO-d_6): 1.25 (t, ^{3}J=6.9, 3H), 4.19 (q, ^{3}J=7.2, 2H), 7.21 (s, 2H), 7.45 (s, 1H). ^{13}C-{^{1}H}-NMR (DMSO-d_6): 14.2, 60.1, 116.9, 142.3, 161.0, 168.1. HRMS (ESI)

Calculated m/z: 195.0199; found *m/z:*195.0199 $[M+Na]^+$. Anal. Calcd for $C_6H_8N_2O_2S \cdot 0.25H_2O$: C, 40.78; H, 4.85; N, 15.85. Found: C, 41.01; H, 4.79; N, 15.85. IR (cm^{-1}): 3437, 3257, 3126, 2981, 2512, 2159, 2031, 1976, 1687, 1615, 1535, 1515, 1367, 1335, 1234, 1117, 1074, 1020, 961, 875, 784, 746, 665

Ethyl 2-(butylamino)thiazole-4-carboxylate (3) 79%

^{1}H-NMR (300 MHz; DMSO-d_6): 0.89 (t, ^{3}J=7.5, 3H), 1.23-1.38 (m, 5H), 1.49-1.55 (m, 2H), 3.19 (q, ^{3}J=6.9, 2H), 4.20 (q, ^{3}J=7.2, 2H), 7.47(s, 1H), 7.77 (s, 1H). ^{13}C-{^{1}H}-NMR (DMSO-d_6): 13.6, 14.2, 19.5, 30.6, 44.2, 60.1, 116.1, 142.5, 161.0, 168.3. HRMS (ESI) Calculated m/z: 251.0825; found *m/z:*251.0825 $[M+Na]^+$. Anal. Calcd for $C_{10}H_{16}N_2O_2S$: C, 52.61; H, 7.06; N, 12.27. Found: C, 52.44.; H, 7.01; N, 12.20. IR (cm^{-1}): 3190, 3128, 2956, 2930, 2516, 2159, 2030, 1976, 1722, 1590, 1227, 1203, 1092, 713

Ethyl 2-(allylamino)thiazole-4-carboxylate (4) 91%

^{1}H-NMR (300 MHz; DMSO-d_6): 1.26 (t, ^{3}J=6.9, 3H), 3.86-3.90 (m, 2H), 4.21 (q, ^{3}J=7.2, 2H), 5.10-5.14 (m, 1H), 5.20-5.27 (m, 1H), 5.83-5.95 (m, 1H), 7.51 (s, 1H), 7.96 (t, ^{3}J=5.7, 1H). ^{13}C-{^{1}H}-NMR (DMSO-d_6): 14.2, 46.5, 60.1, 115.9, 116.7, 134.6, 142.3, 161.0, 168.2. HRMS (ESI) Calculated m/z: 213.0692; found *m/z:* 213.0692 $[M+H]^+$. Anal. Calcd for $C_8H_{10}N_2O_2S$: C, 48.57; H, 5.08; N, 14.13. Found: C, 48.87; H, 5.31; N, 14.28. IR (cm^{-1}): 3179, 3123, 2985, 2520, 2159, 2030, 1976, 1724, 1577, 1415, 1200, 1141, 1089, 1028, 1001, 916, 729, 700

Ethyl 2-acetamidothiazole-4-carboxylate (5)

^{1}H-NMR (300 MHz; DMSO-d_6): 1.28 (t, ^{3}J=6.9, 3H), 2.13 (s, 3H), 4.26 (q, ^{3}J=6.9, 2H), 8.01 (d, , ^{5}J=0.9, 1H), 12.49 (s, 1H). ^{13}C-{^{1}H}-NMR (DMSO-d_6): 14.2, 22.4, 60.5, 122.4, 140.8, 158.0,

161.0, 169.0. HRMS (ESI) Calculated m/z: 237.0304; found *m/z:*237.0304 $[M+Na]^+$. Anal. Calcd for $C_8H_{10}N_2O_3S$: C,44.85; H,4.71; N,13.08. Found: C, 44.65; H, 4.47; N, 13.21. IR (cm^{-1}): 3223, 2991, 2513, 2159, 2031, 1976, 1726, 1700, 1613, 1552, 1293, 1203, 1013, 754, 734, 687

Ethyl 2-(phenylamino)thiazole-4-carboxylate (6) 81%

^{1}H-NMR (300 MHz; DMSO-d_6): 1.29 (t, ^{3}J=6.9 Hz, 3H), 4.27 (q, ^{3}J=6.9 Hz, 2H), 6.98 (t, ^{3}J=7.5, 1H), 7.33 (t, ^{3}J=7.8 Hz, 2H), 7.61-7.63 (m, 2H), 7.76 (s, 1H), 10.34 (s, 1H). ^{13}C-{^{1}H}-NMR (DMSO-d_6): 14.2, 60.4, 117.0, 118.4, 121.7, 129.0, 140.7, 142.4, 160.8, 163.2. HRMS (ESI) Calculated m/z: 271.0512; found *m/z:*271.0512 $[M+Na]^+$. Anal. Calcd for $C_{12}H_{12}N_2O_2S{\cdot}0.5\ H_2O$: C, 56.01; H, 5.09; N, 10.89. Found: C, 56.41; H, 5.03; N, 10.65. IR (cm^{-1}): 3281, 3058, 1714, 1685, 1597, 1527, 1454, 1377, 1319, 1206, 1089, 1018, 966, 889, 843, 752, 692, 667.

Ethyl 2-(benzylamino)thiazole-4-carboxylate (7) 75%

^{1}H-NMR (300 MHz; DMSO-d_6): 1.25 (t, ^{3}J=7.2, 3H), 4.20 (q, ^{3}J=7.2, 2H), 4.45 (d, ^{3}J=6, 2H), 7.26-7.27 (m, 5H), 7.51 (s, 1H), 8.30 (t, ^{3}J=6, 1H). ^{13}C-{^{1}H}-NMR (DMSO-d_6): 14.2, 47.7, 60.1, 116.8, 127.0, 127.4, 128.3, 138.7, 142.3, 161.0, 168.3. HRMS (ESI) Calculated m/z: 263.0849; found *m/z:* 263.0849 $[M+H]^+$. Anal. Calcd for $C_{13}H_{14}N_2O_2S{\cdot}0.5H_2O$: C, 57.55; H, 5.57; N, 10.32. Found: C, 57.66; H, 5.26; N, 10.09. IR (cm^{-1}): 3178, 3112, 2982, 2886, 1516, 2159, 1031, 1976, 1726, 1588, 1576, 1423, 1195, 1090, 1067, 1018, 972, 760, 726, 695

Diethyl 2,2'-(1,4-phenylenebis(azanediyl))dithiazole-4-carboxylate (8) 75%

^{1}H-NMR (300 MHz; DMSO-d_6): 1.29 (t, ^{3}J=7.2, 6H), 4.26 (q, ^{3}J=6.9, 4H), 7.59 (s, 4H), 7.71 (s, 2H), 10.37 (bs, 2H). ^{13}C-{^{1}H}-NMR (DMSO-d_6): 14.3, 60.4, 118.0, 118.3, 135.4, 142.1, 160.8, 163.7. HRMS (ESI) Calculated m/z: 419.0842; found *m/z:* 419.0842 $[M+H]^+$. Anal. Calcd for

$C_{18}H_{18}N_4O_4S_2$: C, 51.66; H, 4.34; N, 13.39. Found: C, 51.41; H, 4.27; N, 13.13. IR (cm^{-1}): 3056, 2519, 2159, 2023, 1976, 1817, 1591, 1517, 1494, 1254, 1196, 1088, 1016, 757, 736, 669

Ethyl 2-((3-chlorophenyl)amino)thiazole-4-carboxylate (9) 81%

^{1}H-NMR (300 MHz; DMSO-d_6): 1.30 (t, ^{3}J=7.2, 3H), 4.27 (q, ^{3}J=6.9, 2H), 7.01 (dd, ^{3}J=7.8, ^{4}J=1.2, 1H), 7.34 (t, ^{3}J=7.8, 1H), 7.45 (dd, ^{3}J=8.1, ^{4}J=0.9, 1H), 7.83 (s, 1H), 7.93 (t, ^{4}J=2.1, 1H), 10.60 (s, 1H). ^{13}C-{^{1}H}-NMR (DMSO-d_6): 14.2, 60.5, 115.3, 116.3, 119.1, 121.1, 130.6, 133.4, 142.0, 142.3, 160.6, 162.5. HRMS (ESI) Calculated m/z: 283.0303; found *m/z:* 283.0303 $[M+H]^+$. Anal. Calcd for $C_{12}H_{11}ClN_2O_2S$: C, 50.98; H, 3.92; N, 9.91. Found: C, 50.59; H, 4.29; N, 10.26. IR (cm^{-1}): 3267, 3097, 2986, 2517, 2163, 2024, 1702, 1593, 1538, 1475, 1448, 1380, 1335, 1211, 1091, 1023, 864, 762, 731, 682.

Ethyl 2-((2-cholorphenyl)amino)thiazole-4-carboxylate (10) 72%

^{1}H-NMR (300 MHz; DMSO-d_6): 1.28 (t, ^{3}J=6.9, 3H), 4.25 (q, ^{3}J=7.2, 2H), 7.06-7.12 (m, 1H), 7.33-7.38 (m, 1H), 7.47-7.50 (m, 1H), 7.80 (s, 1H), 8.27-8.30 (m, 1H), 9.84 (s, 1H).^{13}C-{^{1}H}-NMR (DMSO-d_6): 14.2, 60.4, 119.8, 121.7, 123.1, 124.1, 127.8, 129.7, 137.2, 141.9, 160.7, 164.0. HRMS (ESI) Calculated m/z: 301.0122; found *m/z:* 305.0122 $[M+Na]^+$. Anal. Calcd for $C_{12}H_{11}ClN_2O_2S \cdot 0.25\ H_2O$: C, 50.18; H, 4.04; N, 9.75. Found: C, 50.32; H, 4.02; N, 9.73. IR (cm^{-1}): 3135, 3066, 2935, 2833, 1704, 1549, 1469, 1436, 1375, 1327, 1297, 1205, 1083, 1067, 1007, 949, 869, 806, 734, 658.

Ethyl 2-((4-bromophenyl)amino)thiazole-4-carboxylate (11) 80%

^{1}H-NMR (300 MHz; DMSO-d_6): 1.29 (t, ^{3}J=6.9 Hz, 3H), 4.27 (q, ^{3}J=7.2 Hz, 2H), 7.48-7.52 (m, 2H), 7.59-7.64 (m, 2H), 7.80(s, 1H), 10.52 (s, 1H). ^{13}C-{^{1}H}-NMR (DMSO-d_6): 14.2, 60.4,

112.8, 118.8, 118.9, 131.7, 140.0, 142.3, 160.6, 162.7. HRMS (ESI) Calculated m/z: 326.9797; found *m/z:* 326.797 $[M+H]^+$. Anal. Calcd for $C_{12}H_{11}BrN_2O_2S$: C, 44.05; H, 3.39; N, 8.56. Found: C, 43.87; H, 3.71; N, 8.27. IR (cm^{-1}): 3641, 3265, 2979, 2514, 2162, 2023, 1729, 1590, 1544, 1484, 1435, 1199, 1084, 1015, 792, 703.

Ethyl 2-(3-bromophenylamino)thiazole-4-carboxylate (12) 77%

^{1}H-NMR (300 MHz; DMSO-d_6): 1.30 (t, ^{3}J=7.2, 3H), 4.27 (q, ^{3}J=6.9, 2H), 7.13-7.16 (m, 1H), 7.25-7.31 (m, 1H), 7.48-7.52 (m, 1H), 7.82 (s, 1H), 8.06-8.07 (m, 1H), 10.57 (s, 1H). ^{13}C-{^{1}H}-NMR (DMSO-d_6): 14.6, 60.5, 115.7, 119.1, 121.9, 124.0, 130.9, 142.2, 160.6, 162.5. HRMS (ESI) Calculated m/z: 326.9797; found *m/z:* 326.9797 $[M+H]^+$. Anal. Calcd for $C_{12}H_{11}BrN_2O_2S{\cdot}0.5H_2O$: C, 42.87; H, 3.60; N, 8.33. Found: C, 43.18; H, 3.34; N, 8.17. IR (cm^{-1}): 3337, 3112, 2979, 2514, 2159, 2030, 1976, 1699, 1581, 1547, 1481, 1451, 1370, 1220, 1158, 1117, 1094, 1075, 1026, 989, 972, 862, 799, 771, 754, 715, 682, 652

Ethyl 2-(2-bromophenylamino)thiazole-4-carboxylate (13) 87%

^{1}H-NMR (300 MHz; DMSO-d_6): 1.27 (t, ^{3}J=6.9, 3H), 4.24 (q, ^{3}J=6.9, 2H), 7.03-7.09 (m,1H), 7.34-7.42 (m, 1H), 7.64-7.68 (m, 1H), 7.78 (s, 1H), 8.05-8.08 (m,1H), 9.71 (s, 1H). ^{13}C-{^{1}H}-NMR (DMSO-d_6): 14.2, 60.4, 114.9, 119.5, 123.3, 125.3, 128.5, 133.1, 138.6, 142.0, 160.7, 164.7. HRMS (ESI) Calculated m/z: 326.9797; found *m/z:* 326.9797 $[M+H]^+$. Anal. Calcd for $C_{12}H_{11}BrN_2O_2S{\cdot}0.25\ H_2O$: C, 43.05; H, 3.49; N, 8.45. Found: C, 43.34; H, 3.54; N, 8.06. IR (cm^{-1}): 3642, 3143, 2934, 2515, 2162, 2024, 1975, 1702, 1548, 1327, 1297, 1204, 1081, 1038, 1007, 867, 729.

Ethyl 2-((4-fluorophenyl)amino)thiazole-4-carboxylate (H14) 99%

^{1}H-NMR (300 MHz; DMSO-d_6): 1.29 (t, ^{3}J=6.9, 3H), 4.26 (q, ^{3}J=7.2, 2H), 7.14-7.20 (m, 2H), 7.63-7-67 (m, 2H), 7.75 (s, 1H), 10.40(s, 1H). ^{13}C-{^{1}H}-NMR (DMSO-d_6): 14.2, 60.4, 115.5 (^{2}J=22 Hz), 118.4, 118.6 (^{3}J=7.7 Hz), 137.2 (^{4}J=2.3 Hz), 142.3, 165.6 (^{1}J=237 Hz), 160.7, 163.3. HRMS (ESI) Calculated m/z: 267.0598; found *m/z:* 267.0598 $[M+H]^{+}$. Anal. Calcd for $C_{12}H_{11}FN_2O_2S{\cdot}0.67H_2O$: C, 51.79; H, 4.47; N, 10.07. Found: C, 51.33; H, 4.28; N, 9.74. IR (cm^{-1}): 3102, 2517, 2159, 2030, 1976, 1712, 1623, 1565, 1527, 1502, 1445, 1411, 1368, 1294, 1251, 1207, 1172, 1156, 1092, 1015, 871, 859, 838, 797, 772, 760

Ethyl 2-((3-fluorophenyl)amino)thiazole-4-carboxylate (15) 76%

^{1}H-NMR (300 MHz; DMSO-d_6): 1.30 (t, ^{3}J=7.2 Hz, 3H), 4.27 (q, ^{3}J=7.2 Hz, 2H), 6.75-6.81 (m, 1H), 7.24-7.38 (m, 2H), 7.73-7.78 (m, 1H), 7.82(s, 1H), 10.62 (s, 1H). ^{13}C-{^{1}H}-NMR (DMSO-d_6): 14.2, 60.5, 103.7 (d, ^{2}J = 26.7 Hz), 107.8 (d, ^{2}J = 21.2 Hz), 112.8 (d, ^{4}J = 2.5 Hz), 119.1, 130.5 (d, ^{3}J = 9.8 Hz), 102.3, 142.3 (d, ^{3}J = 11.4 Hz), 160.7, 162.5 (d, ^{1}J = 240.0 Hz), 162.6. HRMS (ESI) Calculated m/z: 289.0417; found *m/z:* 289.0417 $[M+Na]^{+}$. Anal. Calcd for $C_{12}H_{11}FN_2O_2S{\cdot}0.25\ H_2O$: C, 53.23; H, 4.28; N, 10.35. Found: C, 53.42; H, 4.56; N, 10.02. IR (cm^{-1}): 3263, 3102, 2988, 2517, 2163, 2024, 1976, 1696, 1609, 1541, 1486, 1458, 1378, 1333, 1210, 1146, 1091, 1018, 954, 850, 756, 681.

Ethyl 2-((2-fluorophenyl)amino)thiazole-4-carboxylate (16) 41%

^{1}H-NMR (300 MHz; DMSO-d_6): 1.29 (t, ^{3}J=7.2, 3H), 4.26 (q, ^{3}J=7.2, 2H), 7.02-7.04 (m, 1H), 7.17-7.29 (m, 2H), 7.79 (S, 1H), 8.39-8.45 (m, 1H), 10.17 (s, 1H). ^{13}C-{^{1}H}-NMR (DMSO-d_6): 14.2, 60.4, 115.2 (^{2}J=18.6 Hz), 119.6, 120.1 (^{4}J=1.5 Hz), 122.7 (^{3}J=7.4 Hz), 124.6 (^{3}J=3.5 Hz), 128.6 (^{2}J=10.7 Hz), 142.0, 151.8 (^{1}J=242.2 Hz), 160.7, 163.3. HRMS (ESI) *m/z*: $[M+H]^{+}$. HRMS (ESI) Calculated m/z: 267.0598; found *m/z:* 267.0598 $[M+H]^{+}$. Anal. Calcd for

$C_{12}H_{11}FN_2O_2S_2 \cdot 0.5H_2O$: C, 52.35; H, 4.39; N, 10.18. Found: C, 52.68; H, 3.98; N, 9.98. IR (cm^{-1}): 3249, 3119, 2978, 1516, 2031, 1976, 1719, 1704, 1620, 1530, 1503, 1488, 1458, 1326, 1312, 1255, 1217, 1187, 1173, 1087, 1173, 1087, 1022, 959, 771, 751, 732, 683

Ethyl 2-((3,4-dichlorophenyl)amino)thiazole-4-carboxylate (17) 84%

^{1}H-NMR (300 MHz; DMSO-d_6): 1.30 (t, ^{3}J=7.2, 3H), 4.27 (q, ^{3}J=7.2 Hz, 2H), 7.47 (dd, ^{3}J=9 Hz, ^{4}J=2.4 Hz, 1H), 7.56 (d, ^{3}J=8.7 Hz, 1H), 7.85 (s, 1H), 8.15 (d, ^{4}J=2.4 Hz, 1H), 10.70(s, 1H). ^{13}C-{^{1}H}-NMR (DMSO-d_6): 14.2, 60.5, 117.0, 117.9, 119.5, 122.6, 130.7, 131.2, 140.6, 142.3, 160.6, 162.3. HRMS (ESI) Calculated m/z: 316.9913; found *m/z:* 316.9913 $[M+H]^+$. Anal. Calcd for $C_{12}H_{10}Cl_2N_2O_2S \cdot 0.2\ H_2O$: C, 44.93; H, 3.27; N, 8.73. Found: C, 45.29; H, 3.61; N, 8.68. IR (cm^{-1}): 2159, 2024, 1976, 1719, 1698, 1590, 1538, 1473, 1442, 1216, 1132, 1216, 1132, 1092, 1023, 813, 791.

Ethyl 2-((2,4-dichlorophenyl)amino)thiazole-4-carboxylate (18) 82%

^{1}H-NMR (300 MHz; DMSO-d_6): 1.28 (t, ^{3}J=7.8, 3H), 4.24 (q, ^{3}J=6.9, 2H), 7.42-7.46 (m, 1H), 7.62-7.63 (m, 1H), 7.84 (s, 1H), 8.39-8.42 (m, 1H), 9.94 (s, 1H). ^{13}C-{^{1}H}-NMR (DMSO-d_6): 14.2, 60.4, 120.4, 122.0, 122.0, 126.4, 127.8, 128.9, 136.4, 141.8, 160.6, 163.4. HRMS (ESI) Calculated m/z: 316.9913; found *m/z:* 316.9913 $[M+H]^+$. Anal. Calcd for $C_{12}H_{10}Cl_2N_2O_2S \cdot 0.2\ H_2O$: C, 44.93; H, 3.27; N, 8.73. Found: C, 45.11; H, 3.62; N, 8.45. IR (cm^{-1}): 3347, 3117, 2981, 2921, 1704, 1587, 1520, 1469, 1381, 1302, 1202, 1084, 1025, 976, 849, 818, 785, 737.

Ethyl 2-((2,6-dicholorphenyl)amino)thiazole-4-carboxylate (19) 82%

^{1}H-NMR (300 MHz; DMSO-d_6): 1.25 (t, ^{3}J=7.2, 3H), 4.20 (q, ^{3}J=6.9, 2H), 7.36-7-41 (m, 2H), 7.59-7.62 (m, 1H), 7.68 (s, 1H), 9.95 (s, 1H). ^{13}C-{^{1}H}-NMR (DMSO-d_6): 14.2, 60.3, 118.8,

118.8, 118.8, 129.2, 129.3, 133.7, 160.7, 166.1. HRMS (ESI) Calculated m/z: 338.9722; found *m/z:* 338.9722 $[M+Na]^+$. Anal. Calcd for $C_{12}H_{10}Cl_2N_2O_2S \cdot 0.2\ H_2O$: C, 44.93; H, 3.27; N, 8.73. Found: C, 45.11; H, 3.63; N, 8.46. IR (cm^{-1}): 2974, 2827, 1697, 1552, 1434, 1377, 1328, 1282, 1210, 1082, 1011, 953, 876, 782, 716.

Ethyl 2-((2,5-dichlorophenyl)amino)thiazole-4-carboxylate (20) 84%

^{1}H-NMR (300 MHz; DMSO-d_6): 1.30 (t, ^{3}J=7.2, 3H), 4.27 (q, ^{3}J=7.2, 2H), 7.08-7.11 (m, 1H), 7.48-7.51 (m, 1H), 7.87 (s, 1H), 8.70-8.71(m, 1H), 10.03 (s, 1H). ^{13}C-{^{1}H}-NMR (DMSO-d_6): 14.1, 60.5, 119.5, 120.0, 120.8, 122.6, 130.8, 132.0, 138.2, 141.6, 160.6, 162.7. HRMS (ESI) Calculated m/z: 338.9722; found *m/z:* 338.9722 $[M+Na]^+$. Anal. Calcd for $C_{12}H_{10}Cl_2N_2O_2S \cdot 0.2\ H_2O$: C, 44.93; H, 3.27; N, 8.73. Found: C, 45.18; H, 3.50; N, 8.60. IR (cm^{-1}): 3319, 3108, 2500, 2163, 2024, 1975, 1695, 1586, 1528, 1404, 1372, 1331, 1251,1166, 1094, 1033, 870, 787, 717.

Ethyl 2-(3,5-dichlorophenylamino)thiazole-4-carboxylate (21) 83%

^{1}H-NMR (300 MHz; DMSO-d_6): 1.30 (t, ^{3}J=7.2,3H), 4.27 (q, ^{3}J=6.9, 2H), 7.14 (t,^{4}J=1.8, 1H), 7.73 (d, ^{4}J=1.8, 1H), 7.88 (s, 1H), 10.77 (s, 1H). ^{13}C-{^{1}H}-NMR (DMSO-d_6): 14.1, 60.5, 114.9, 119.8, 120.4, 134.3, 142.2, 142.7, 160.5, 162.0. HRMS (ESI) Calculated m/z: 338.9722; found *m/z:* 338.9722 $[M+Na]^+$. Anal. Calcd for $C_{12}H_{10}Cl_2N_2O_2S \cdot 0.2\ H_2O$: C, 44.93; H, 3.27; N, 8.73. Found: C, 45.23; H, 3.34; N, 8.82. IR (cm^{-1}): 3347, 3117, 2981, 2921, 1704, 1587, 1520, 1469, 1381, 1302, 1202, 1084, 1025, 976, 849, 818, 785, 737.

2-(4-Chlorophenylamino)thiazole-4-carboxylic acid (22)

^{1}H-NMR (300 MHz; DMSO-d_6): 7.35-7.38 (m, 2H), 7.69-7.74 (m, 3H), 10.48 (s, 1H). ^{13}C-{^{1}H}-NMR (DMSO-d_6): 118.4, 118.6, 124.8, 128.8, 139.7, 143.4, 162.1, 162.5. HRMS (ESI)

Calculated m/z: 276.9809; found *m/z*: 276.9809 $[M+Na]^+$. Anal. Calcd for $C_{10}H_7ClN_2O_2S$: C, 47.16; H, 2.77; N, 11.00. Found: C, 47.33; H, 2.70; N, 10.87. IR (cm^{-1}): 2501, 2172, 2032, 1732, 1539, 1432, 1197, 1085, 1011, 793, 711.

2-(3-Chlorophenylamino)thiazole-4-carboxylic acid (23) 67%

^{1}H-NMR (300 MHz; DMSO-d_6): 6.99-7.02 (m, 1H), 7.34 (t, ^{3}J=8.1, 1H), 7.46-7.50 (m, 1H), 7.77 (s, 1H), 7.92 (t, ^{3}J=2.1, 1H), 10.54 (s, 1H). ^{13}C-{^{1}H}-NMR (DMSO-d_6): 115.3, 116.2, 118.9, 121.0, 130.5, 133.4, 142.1, 143.3, 162.0, 162.3. HRMS (ESI) Calculated m/z: 276.9809; found *m/z*: 276.9809 $[M+Na]^+$. Anal. Calcd for $C_{10}H_7ClN_2O_2S$: C, 47.16; H, 2.77; N, 11.00. Found: C, 47.23; H, 2.72; N, 10.62. IR (cm^{-1}): 2522, 2160, 2027, 1700, 1536, 1478, 1450, 1377, 1334, 1210, 1093, 866, 767, 729, 683.

2-(2,4-Dichlorophenylamino)thiazole-4-carboxylic acid (24) 56%

^{1}H-NMR (300 MHz; DMSO-d_6): 7.43 (dd, ^{3}J=9.0, ^{4}J=2.7, 1H), 7.62 (d, ^{3}J=2.4, 1H), 7.79 (s, 1H), 8.47 (d, ^{3}J=9.0, 1H), 9.89 (s, 1H). ^{13}C-{^{1}H}-NMR (DMSO-d_6): 120.5, 122.3, 123.3, 126.6, 128.2, 129.8, 136.9, 143.3, 162.5, 163.5. HRMS (ESI) Calculated m/z: 310.9419; found *m/z*: 310.9419 $[M+Na]^+$. Anal. Calcd for $C_{10}H_6Cl_2N_2O_2S$: C, 41.54; H, 2.09; N, 9.69. Found: C, 41.88; H, 2.02; N, 9.54. IR (cm^{-1}): 3350, 3114, 2980, 2917, 1703, 1585, 1522, 1471, 1380, 1300, 1198, 1083, 1027, 974, 845, 813.

2-(2,5-Dichlorophenylamino)thiazole-4-carboxylic acid (25) 59%

^{1}H-NMR (300 MHz; DMSO-d_6): 7.08 (dd, ^{3}J=8.7, ^{4}J=2.7, 1H), 7.49 (d, ^{3}J=8.4, 1H), 7.83 (s, 1H), 8.69 (d, ^{4}J=2.4, 1H), 9.98 (s, 1H), 12.84(s,1H). ^{13}C-{^{1}H}-NMR (DMSO-d_6): 119.8, 120.3, 121.0, 123.0, 131.2, 132.5, 138.8, 143.2, 162.5, 163.0. HRMS (ESI) Calculated m/z: 310.9419; found

m/z: 310.9419 $[M+Na]^+$. Anal. Calcd for $C_{10}H_6Cl_2N_2O_2S$: C, 41.54; H, 2.09; N, 9.69. Found: C, 41.89; H, 1.98; N, 9.66. IR (cm^{-1}): 3300, 3121, 2504, 2163, 1695, 1587, 1523, 1377, 1336, 1259, 1169, 1090, 1031, 870, 719.

Synthesis of 2-((4-Chlorophenyl)amino)-N-(3-(dimethylamino)propyl)thiazole-4-carboxamide (26)

To a solution of **22** (1 mmol) in dried CH_2Cl_2 (20 mL) DCC (1.1 mmol) and HOBt (1.1 mmol) were added and stirred under N_2 for 2 hrs. After adding *N,N*-dimethylpropane-1,3-diamine (4 mmol) the mixture was further stirred for 3 days. The mixture was diluted with 200 mL CH_2Cl_2 and extracted with saturated Na_2CO3 aqueous solution. The organic phase was dried over Na_2SO_4. After removal of solvent, the residue was purified by column chromatography (ethyl acetate : cyclohexan = 1:1) to obtain pure compound. ^{1}H-NMR (300 MHz; DMSO-d_6): 10.52 (s, 1H), 10.01 (s, 1H), 8.12 (d, ^{3}J=8.5, 1H), 7.82 (d, ^{3}J=8.4, 1H), 7,69 (m, 1H), 7.52 (m, 1H), 6.56 (s, 1H), 3.18 (m, 2H), 1.81 (m, 10H). HRMS (ESI) Calculated m/z: 339.1041; found *m/z*: 339.1041 $[M+H]^+$.

FIGURES.

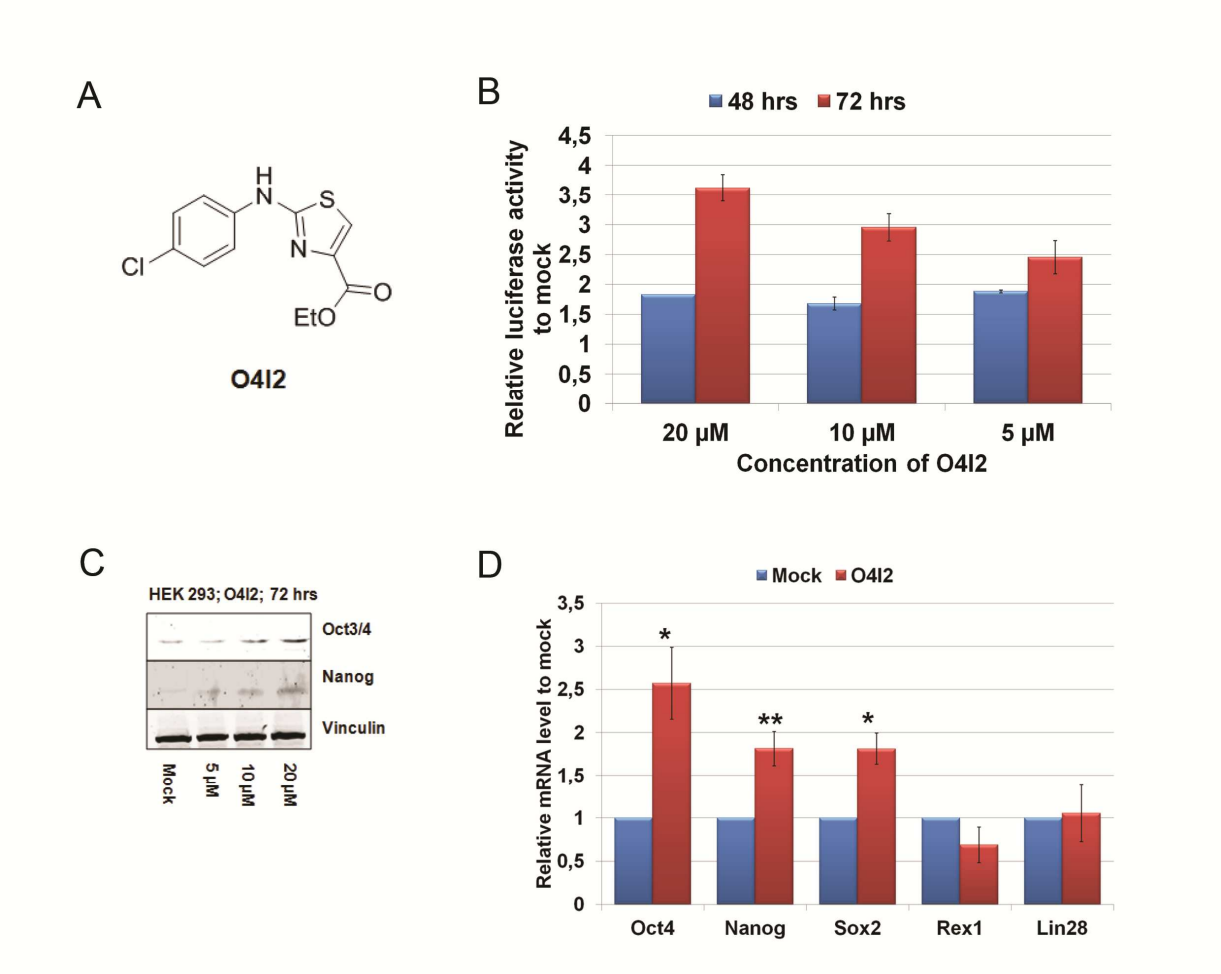


Figure 1. O4I2 activates Oct3/4 in HEK293 cells. A) Structure of O4I2. B) Concentration dependent activation of Oct3/4 detected in HEK-Oct3/4 reporter cells. C) Accumulation of Oct3/4 and Nanog in HEK293 cells detected by immunoblot (72 hrs treatment). D) Increased expression of pluripotency related genes in HEK293 cells treated with O4I2 for 72 hrs quantified by qRT-PCR. Bars indicate standard deviation (*: $p<0.05$; **: $p<0.01$)

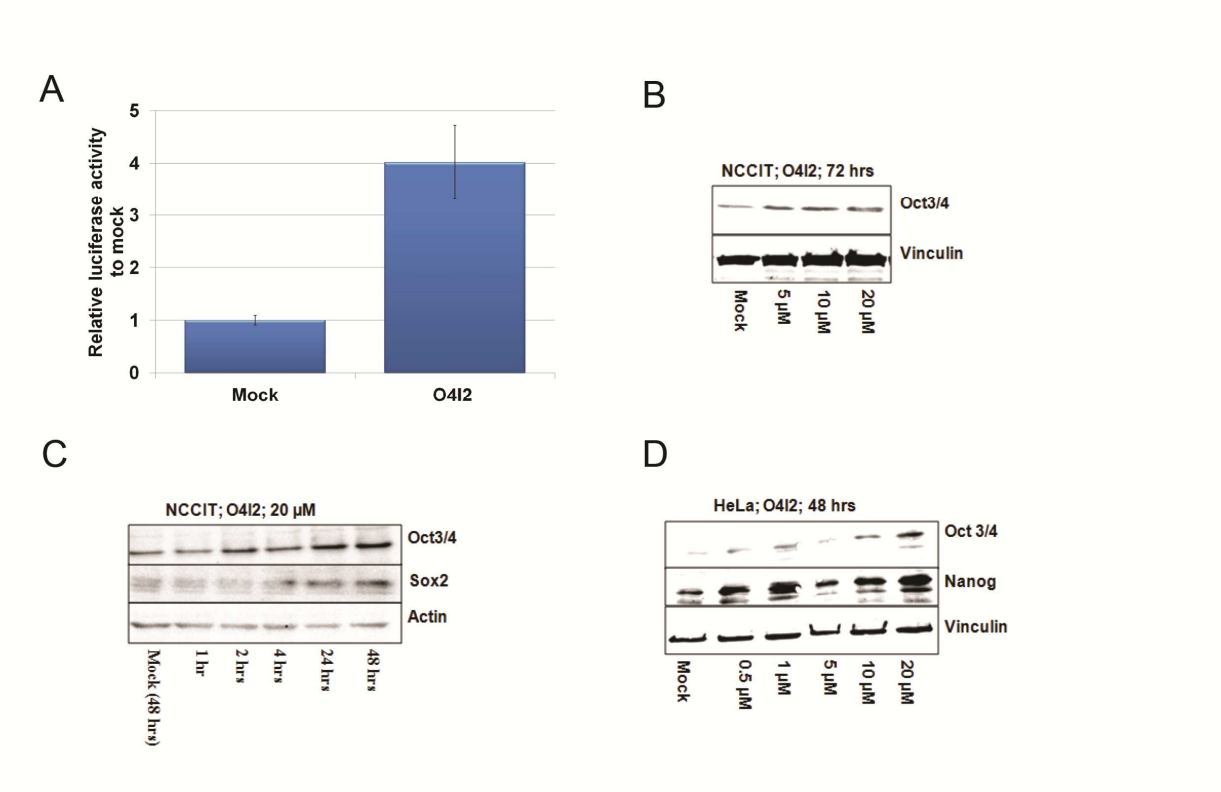


Figure 2. O4I2 activates Oct3/4 in NCCIT and HeLa cells. A) O4I2 induced transcriptional activity of luciferase reporter gene in NCCIT-Oct3/4 reporter cells after 72 hrs treatment. B) The level of Oct3/4 was stabilized in a concentration-dependent study (72 hrs), as well as C) in a time-dependent study. D) Expression of Oct3/4-protein in HeLa cells after 48 hrs treatment.

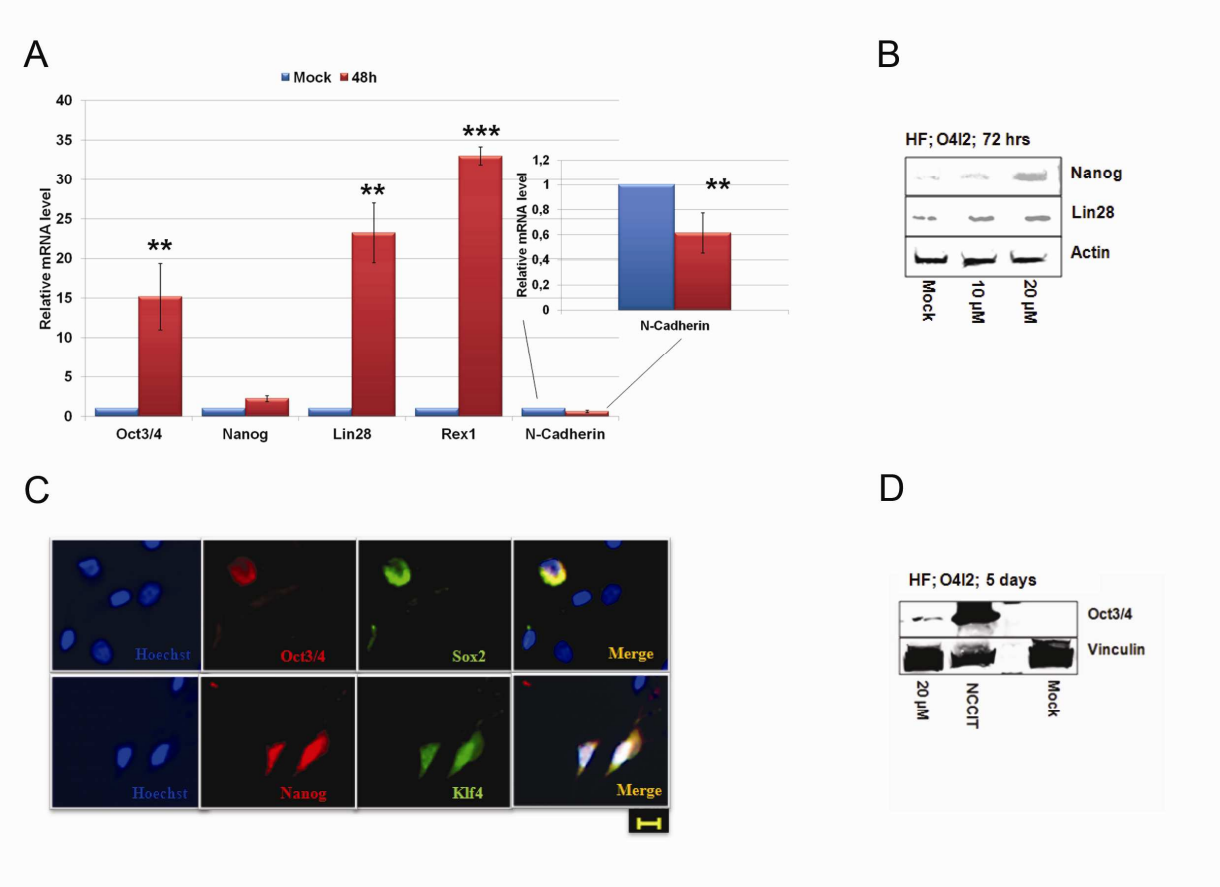


Figure 3. O4I2 activates Oct3/4 on the transcriptional and translational level in human fibroblasts. A) O4I2 promoted the expression of pluripotency-associated genes as analyzed by qRT-PCR in human neonatal foreskin fibroblasts (HFFs) after 48 hrs. B) Nanog and Lin28 were stimulated in response to O4I2 in primary human fibroblasts (HFs) after 72 hrs treatment. C) The co-expression of Oct3/4 (red) and Sox2 (green), as well as Nanog (red) and KLF4 (green) in HFF after 5 days incubation detected by the respective antibodies and nuclei were stained by Hoechst 33258. Bar 20 µm. D) Induction of Oct3/4 in the HFF cells by immunoblot; NCCIT was used as positive control. (**: $p<0.01$; ***: $p<0.001$)

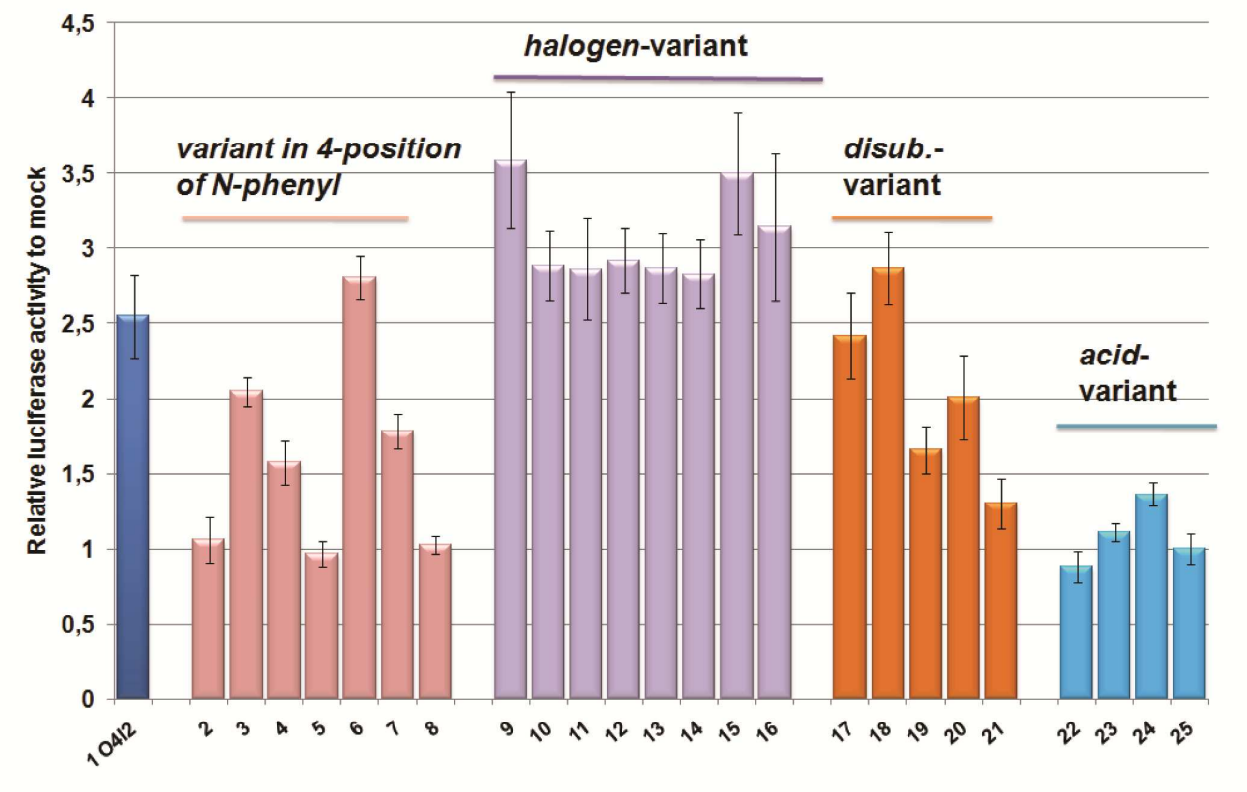
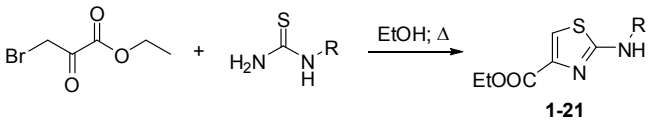


Figure 4: The effect of O4I2 derivatives on Oct3/4 expression in HEK-Oct3/4 cells.

TABLES.

Table 1. Structures of O4I2 derivatives.[a]



1-21

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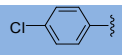
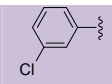
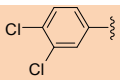
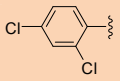
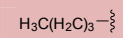
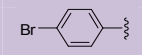
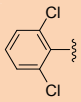
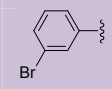
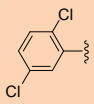
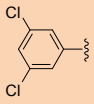
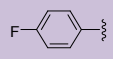
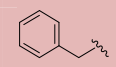
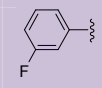
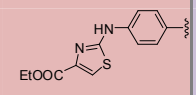
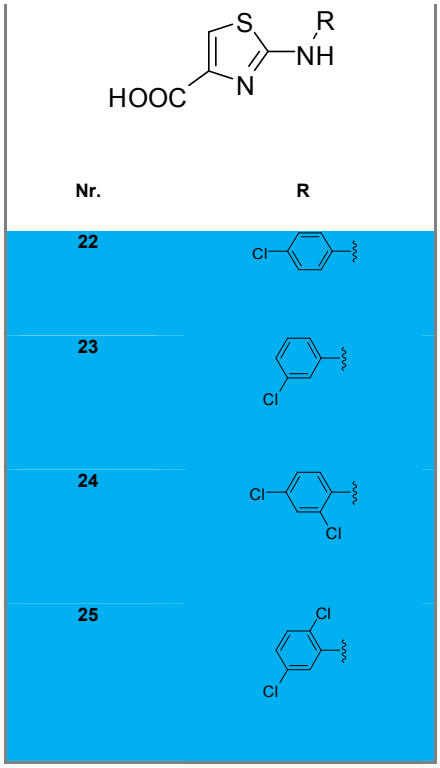
1 O4I2		9		17	
2		10		18	
3		11		19	
4		12		20	
5		13		21	
6		14			
7		15			
8		16			

[a] Compound O4I2 is highlighted in blue; variants are para- (pink); halogen- (violet) and disubstituted-variants (orange).

Table 2. Carboxylic acid derivatives



ASSOCIATED CONTENT

Supporting Information.

Five figures supplied as Supporting Information. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

X.C. designed experiments, analyzed data, synthesized and characterized compounds, performed biological assays and wrote manuscript. H.Y. and S.S. synthesized and performed luciferase assay. D.R. performed biological assays. F.W. and A.G. generated stable cell lines for screening. S.R. performed online luciferase reporter assay. H.A. and H.F prepared and characterized cell lines. N.M. performed analysis of screening data. M.A.A. and J.A. S.G. participated in screening and experiments design. J.U. isolated human fibroblasts. R.M. designed experiments and analyzed data, designed and organized screening and screening assays, and coordinated project. S.W. designed experiments, designed screening, analyzed data and wrote manuscript.

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Conflict of Interest

The authors declare no competing financial interest

Abbreviation

ALP: Alkaline Phosphatase; **c-Myc**: v-myc avian myelocytomatosis viral oncogene homolog; **E-Cadherin**: Epithelial Cadherin; **ESCs**: Embryonic Stem Cells; **HEK293**: a human embryonic kidney cell line; **HEK-Oct3/4**: Oct3/4 promoter-driven luciferase reporter HEK293 cells; **HeLa**: a human cervical cancer cell line; **HF**: Human adult primary Fibroblasts; **iPSCs**: Induced-Pluripotent Stem Cells; **Klf4**: Kruppel-like factor 4; **Lin28**: Lin-28 homolog A; **mCiPSCs**: mouse Chemically induced-Pluripotent Stem Cells; **MEF**: Mouse Embryonic Fibroblasts; **MET**: Mesenchymal–Epithelial Transition; **Nanog**: Nanog homeobox; **N-Cadherin**: Neural Cadherin; **NCCIT**: a human embryonal carcinoma cell line; **Oct3/4**: Octamer-binding transcription factor 3/4; **PAINS**: Pan-Assay Interference compounds; **PI**: Propidium Iodide; **qRT-PCR**: quantitative Real-time Polymerase Chain Reaction; **Rex1**: RNA exonuclease 1 homolog; **Sox2**: Sex determining region Y-box 2.

REFERENCES

1. Yamanaka, S. Pluripotency and nuclear reprogramming. *Philos. Trans.: Biol. Sci.* **2008,** 363, 2079-2087.

2. Wang, X.; Dai, J. W. Concise Review: Isoforms of OCT4 Contribute to the Confusing Diversity in Stem Cell Biology. *Stem Cells* **2010,** 28, 885-893.

3. Zaehres, H.; Lensch, M. W.; Daheron, L.; Stewart, S. A.; Itskovitz-Eldor, J.; Daley, G. Q. High-efficiency RNA interference in human embryonic stem cells. *Stem Cells* **2005,** 23, 299-305.

4. Niwa, H.; Miyazaki, J.; Smith, A. G. Quantitative expression of Oct-3/4 defines differentiation, dedifferentiation or self-renewal of ES cells. *Nat. Genet.* **2000,** 24, 372-376.

5. Takahashi, K.; Yamanaka, S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell* **2006,** 126, 663-676.

6. Yu, J. Y.; Vodyanik, M. A.; Smuga-Otto, K.; Antosiewicz-Bourget, J.; Frane, J. L.; Tian, S.; Nie, J.; Jonsdottir, G. A.; Ruotti, V.; Stewart, R.; Slukvin, I. I.; Thomson, J. A. Induced pluripotent stem cell lines derived from human somatic cells. *Science* **2007,** 318, 1917-1920.

7. Takahashi, K.; Tanabe, K.; Ohnuki, M.; Narita, M.; Ichisaka, T.; Tomoda, K.; Yamanaka, S. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell* **2007,** 131, 861-872.

8. Mummery, C. Induced Pluripotent Stem Cells - A Cautionary Note. *N. Engl. J. Med.* **2011,** 364, 2160-2162.

9. Stadtfeld, M.; Hochedlinger, K. Induced pluripotency: history, mechanisms, and applications. *Gene. Dev.* **2010,** 24, 2239-2263.

10. Zhu, S. Y.; Wei, W. G.; Ding, S. Chemical Strategies for Stem Cell Biology and Regenerative Medicine. *Annu. Rev. Biomed. Eng.* **2011,** 13, 73-90.

11. Huangfu, D. W.; Maehr, R.; Guo, W. J.; Eijkelenboom, A.; Snitow, M.; Chen, A. E.; Melton, D. A. Induction of pluripotent stem cells by defined factors is greatly improved by small-molecule compounds. *Nat. Biotechnol.* **2008,** 26, 795-797.

12. Huangfu, D. W.; Osafune, K.; Maehr, R.; Guo, W.; Eijkelenboom, A.; Chen, S.; Muhlestein, W.; Melton, D. A. Induction of pluripotent stem cells from primary human fibroblasts with only Oct4 and Sox2. *Nat. Biotechnol.* **2008,** 26, 1269-1275.

13. Pandian, G. N.; Sato, S.; Anandhakumar, C.; Taniguchi, J.; Takashima, K.; Syed, J.; Han, L.; Saha, A.; Bando, T.; Nagase, H.; Sugiyama, H. Identification of a small molecule that turns ON the pluripotency gene circuitry in human fibroblasts. *ACS Chem Biol* **2014,** 9, 2729-36.

14. Li, W.; Tian, E.; Chen, Z. X.; Sun, G.; Ye, P.; Yang, S.; Lu, D.; Xie, J.; Ho, T. V.; Tsark, W. M.; Wang, C.; Horne, D. A.; Riggs, A. D.; Yip, M. L. R.; Shi, Y. Identification of Oct4-activating compounds that enhance reprogramming efficiency. *Proc. Natl. Acad. Sci. U. S. A.* **2012,** 109, 20853-20858.

15. Hou, P. P.; Li, Y. Q.; Zhang, X.; Liu, C.; Guan, J. Y.; Li, H. G.; Zhao, T.; Ye, J. Q.; Yang, W. F.; Liu, K.; Ge, J.; Xu, J.; Zhang, Q.; Zhao, Y.; Deng, H. K. Pluripotent Stem Cells Induced from Mouse Somatic Cells by Small-Molecule Compounds. *Science* **2013,** 341, 651-654.

16. Zhu, S. Y.; Li, W. L.; Zhou, H. Y.; Wei, W. G.; Ambasudhan, R.; Lin, T. X.; Kim, J.; Zhang, K.; Ding, S. Reprogramming of Human Primary Somatic Cells by OCT4 and Chemical Compounds. *Cell Stem Cell* **2010,** 7, 651-655.

17. Yuan, X.; Wan, H. F.; Zhao, X. Y.; Zhu, S. Y.; Zhou, Q.; Ding, S. Brief Report: Combined Chemical Treatment Enables Oct4-Induced Reprogramming from Mouse Embryonic Fibroblasts. *Stem Cells* **2011,** 29, 549-553.

18. Cheng, X.; Dimou, E.; Alborzinia, H.; Wenke, F.; Gohring, A.; Reuter, S.; Mah, N.; Fuchs, H.; Andrade-Navarro, M. A.; Adjaye, J.; Gul, S.; Harms, C.; Utikal, J.; Klipp, E.; Mrowka, R.; Wolfl, S. Identification of 2-[4-[(4-Methoxyphenyl)methoxy]-phenyl]acetonitrile and Derivatives as Potent Oct3/4 Inducers. *J Med Chem* **2015,** 58, 4976-83.

19. Baell, J.; Walters, M. A. Chemical con artists foil drug discovery. *Nature* **2014,** 513, 481-483.

20. Baell, J. B. Observations on screening-based research and some concerning trends in the literature. *Future Med. Chem.* **2010,** 2, 1529-1546.

21. Devine, S. M.; Mulcair, M. D.; Debono, C. O.; Leung, E. W.; Nissink, J. W.; Lim, S. S.; Chandrashekaran, I. R.; Vazirani, M.; Mohanty, B.; Simpson, J. S.; Baell, J. B.; Scammells, P. J.; Norton, R. S.; Scanlon, M. J. Promiscuous 2-Aminothiazoles (PrATs): A Frequent Hitting Scaffold. *J Med Chem* **2015,** 58, 1205-14.

22. Bennett, J. P., Jr.; Piercey, M. F. Pramipexole--a new dopamine agonist for the treatment of Parkinson's disease. *J Neurol Sci* **1999,** 163, 25-31.

23. R. H. Wiley, D. C. E., L. L. Behr. *Org. Reactions,* **1951,** 6, 367.

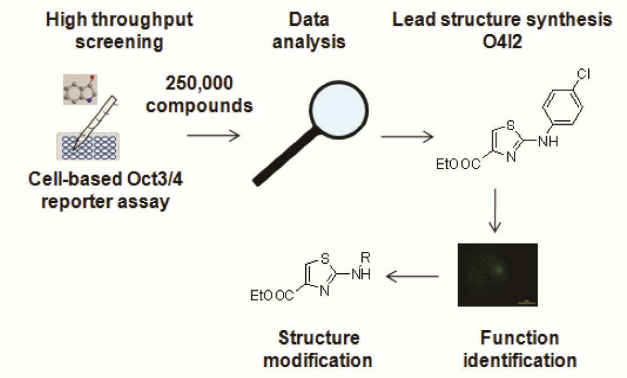
24. Polo, J. M.; Anderssen, E.; Walsh, R. M.; Schwarz, B. A.; Nefzger, C. M.; Lim, S. M.; Borkent, M.; Apostolou, E.; Alaei, S.; Cloutier, J.; Bar-Nur, O.; Cheloufi, S.; Stadtfeld, M.; Figueroa, M. E.; Robinton, D.; Natesan, S.; Melnick, A.; Zhu, J. F.; Ramaswamy, S.; Hochedlinger, K. A Molecular Roadmap of Reprogramming Somatic Cells into iPS Cells. *Cell* **2012,** 151, 1617-1632.

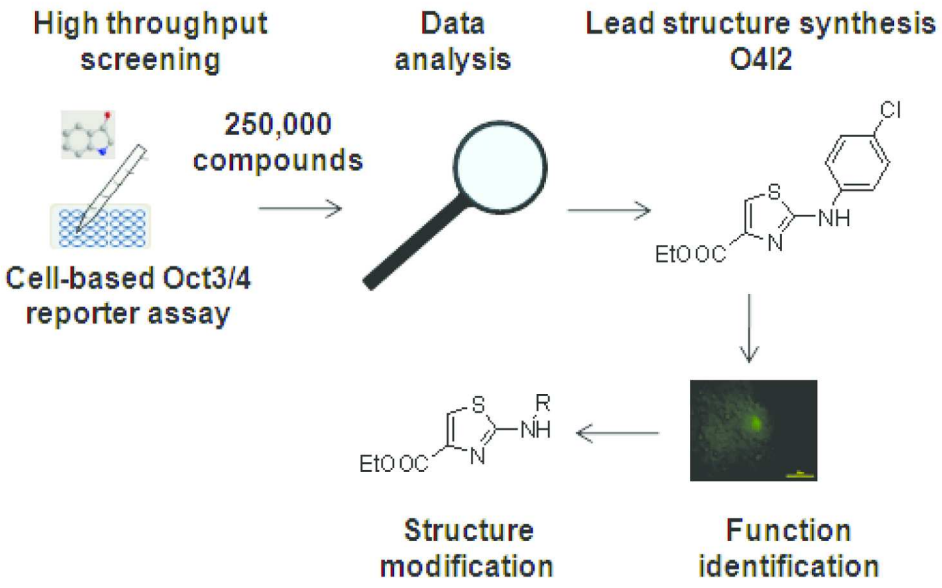
25. Cantz, T.; Key, G.; Bleidissel, M.; Gentile, L.; Han, D. W.; Brenne, A.; Scholer, H. R. Absence of OCT4 expression in somatic tumor cell lines. *Stem Cells* **2008,** 26, 692-697.

26. Baell, J. B.; Holloway, G. A. New substructure filters for removal of pan assay interference compounds (PAINS) from screening libraries and for their exclusion in bioassays. *J Med Chem* **2010,** 53, 2719-40.

27. Cheng, X.; Holenya, P.; Can, S.; Alborzinia, H.; Rubbiani, R.; Ott, I.; Wolfl, S. A TrxR inhibiting gold(I) NHC complex induces apoptosis through ASK1-p38-MAPK signaling in pancreatic cancer cells. *Mol Cancer* **2014,** 13, 221-236.

Table of Contents graphic





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