University of Glasgow

Hayatigolkhatmi, K., Padroni, G., Su, W., Fang, L., Gómez-Castañeda, E., Hsieh, Y.C., Jackson, L., Pellicano, F., Burley, G.A. and Jørgensen, H.G.(2019) An investigation of targeted inhibition of transcription factor activity with pyrrole imidazole polyamide (PA) in chronic myeloid leukemia (CML) blast crisis cells. Bioorganic and Medicinal Chemistry Letters, 29(18), pp. 2622-2625.

There may be differences between this version and the published version. You are advised to consult the publisher's version if you wish to cite from it.

http://eprints.gla.ac.uk/191641/

Deposited on: 1 August 2019

Enlighten – Research publications by members of the University of Glasgow
http://eprints.gla.ac.uk

Elsevier Editorial System(tm) for Bioorganic & Medicinal Chemistry Letters

Manuscript Draft

Manuscript Number: BMCL-D-19-00202R3

Title: An investigation of targeted inhibition of transcription factor activity with pyrrole imidazole polyamide (PA) in chronic myeloid leukemia (CML) blast crisis cells

Article Type: Short Communication

Keywords: Chronic Myeloid Leukemia, Blast Crisis CML, Pyrrole-imidazole Polyamide, E2F Transcription Factor 1, Leukemia Stem Cells, Tyrosine Kinase Inhibitor.

Corresponding Author: Dr. Heather G. Jorgensen, Ph.D.

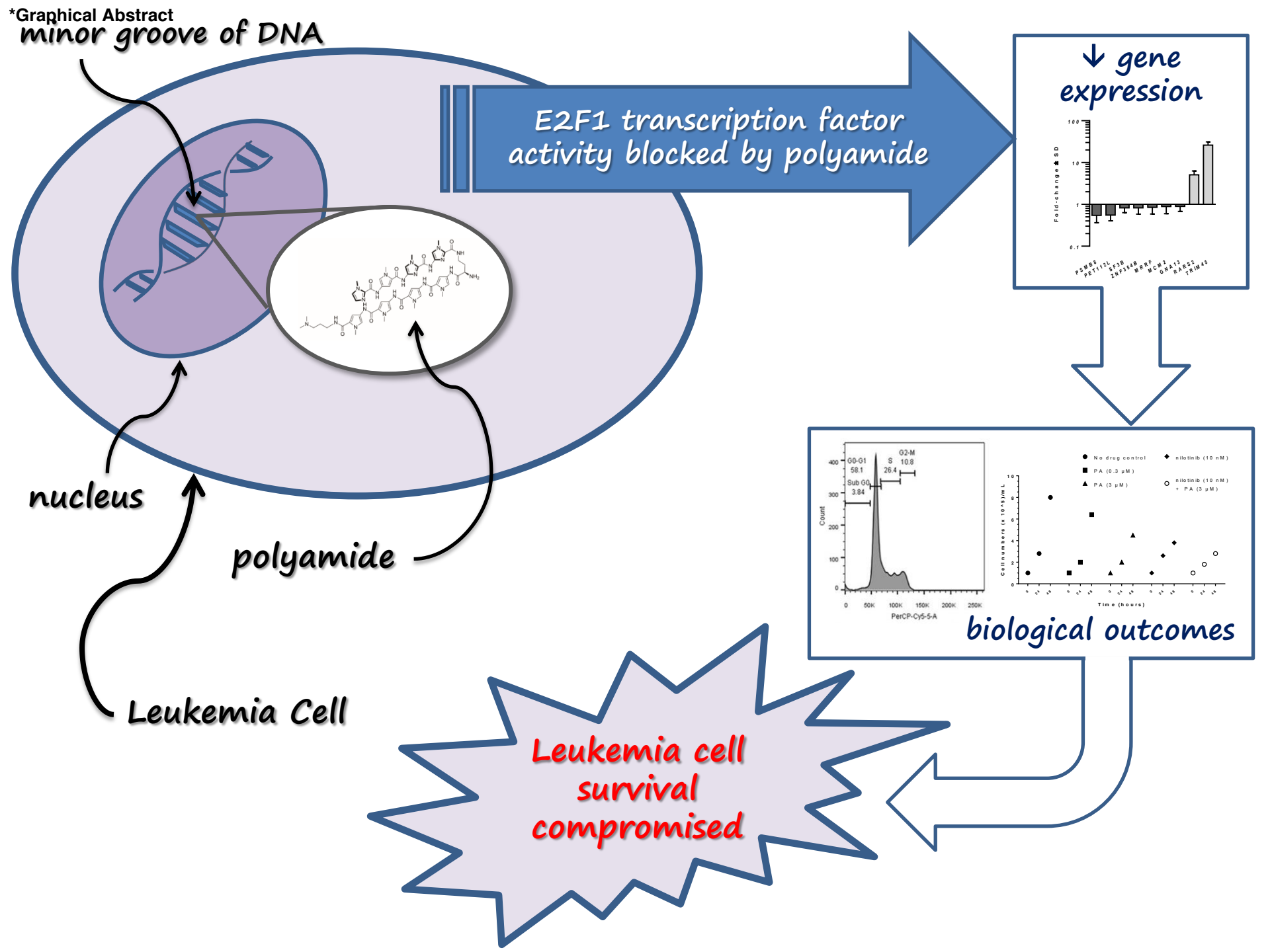
Corresponding Author's Institution: University of Glasgow

First Author: Kourosh Hayatigolkhatmi, MSc Medical Genetics and Genomics

Order of Authors: Kourosh Hayatigolkhatmi, MSc Medical Genetics and Genomics; Giacomo Padroni; Wu Su; Lijing Fang; Eduardo Gómez-Castañeda; Ya-Ching Hsieh; Lorna Jackson; Francesca Pellicano; Glenn A Burley; Heather G Jørgensen

Abstract: Tyrosine kinase inhibitor (TKI) therapy is the standard treatment for chronic phase (CP)-chronic myeloid leukemia (CML), yet patients in blast crisis (BC) phase of CML are unlikely to respond to TKI therapy. The transcription factor E2F1 is a down-stream target of the tyrosine kinase BCR-ABL1 and is up-regulated in TKI-resistant leukemia stem cells (LSC). Pyrrole imidazole polyamides (PA) are minor groove binders which can be programmed to target DNA sequences in a gene-selective manner. This manuscript describes such an approach with a PA designed to down-regulate E2F1 controlled gene expression by targeting a DNA sequence within 100 base pairs (bp) upstream of the E2F1 consensus sequence. Human BC-CML KCL22 cells were assessed after treatment with PA, TKI or their combination. Our PA inhibited BC-CML cell expansion based on cell density analysis compared to an untreated control after a 48-hour time-course of PA treatment. However, no evidence of cell cycle arrest was observed among BC-CML cells treated with PA, with respect to their no drug control counterparts. Thus, this work demonstrates that PAs are effective in inhibiting E2F1 TF activity which results in a temporal reduction in BC-CML cell number. We envisage that PAs could be used in the future to map genes under E2F1 control in CML LSCs.

*Graphical Abstract



*Manuscript
Click here to view linked References

Scientific Communication

An investigation of targeted inhibition of transcription factor activity with pyrrole imidazole polyamide (PA) in chronic myeloid leukemia (CML) blast crisis cells

Abstract:

Tyrosine kinase inhibitor (TKI) therapy is the standard treatment for chronic phase (CP)-chronic myeloid leukemia (CML), yet patients in blast crisis (BC) phase of CML are unlikely to respond to TKI therapy. The transcription factor E2F1 is a down-stream target of the tyrosine kinase BCR-ABL1 and is up-regulated in TKI-resistant leukemia stem cells (LSC). Pyrrole imidazole polyamides (PA) are minor groove binders which can be programmed to target DNA sequences in a gene-selective manner. This manuscript describes such an approach with a PA designed to down-regulate E2F1 controlled gene expression by targeting a DNA sequence within 100 base pairs (bp) upstream of the E2F1 consensus sequence. Human BC-CML KCL22 cells were assessed after treatment with PA, TKI or their combination. Our PA inhibited BC-CML cell expansion based on cell density analysis compared to an untreated control after a 48-hour time-course of PA treatment. However, no evidence of cell cycle arrest was observed among BC-CML cells treated with PA, with respect to their no drug control counterparts. Thus, this work demonstrates that PAs are effective in inhibiting E2F1 TF activity which results in a temporal reduction in BC-CML cell number. We envisage that PAs could be used in the future to map genes under E2F1 control in CML LSCs.

Keywords: Chronic Myeloid Leukemia, Blast Crisis CML, Pyrrole-imidazole Polyamide, E2F Transcription Factor 1, Leukemia Stem Cells, Tyrosine Kinase Inhibitor.

Background: Knowledge is accumulating on how best to modulate transcription factor (TF) activity, an emerging area for development of new therapeutics [1]. Classical small molecule inhibitors, which disrupt protein activity through binding to small hydrophobic clefts, can suffer from off-target effects, whereas peptidomimetics have low bioavailability [1]. Pyrrole-imidazole polyamides (PAs) offer an alternative approach to these strategies by binding to DNA sequences and down-regulating expression of target genes [2]. PAs can be programmed to bind to target sequences according to established pairing rules [2-4] enabling PAs to inhibit transcriptional elongation of RNA polymerase II and TF-DNA binding [2-5].

E2F1 is a well-known TF, which controls cell cycle progression and regulates tumor suppressor proteins. E2F1 is upregulated in chronic myeloid leukemia (CML) leukemia stem cells (LSC) downstream of the BCR-ABL1 signaling pathway [6]. There is an unmet clinical need to gain a greater understanding of the molecular determinants of CML patients developing resistance to tyrosine kinase inhibitor (TKI) therapy in the blast crisis (BC) phase [7]. In this study we investigated the ability of a PA to disrupt E2F1 function in BC-CML, having previously shown activity in chronic phase (CP)-CML cells [8].

Methods: Our approach was to use our designed PA (Fig. 1A), targeting WGWGGW (where W = T/A) (Fig. 1B) sequences found 100 base pairs (bp) upstream of the consensus E2F1 binding site (TTTCGC) in the promoter region of a suite of E2F1 target genes in BC-CML cells [8]. In this way, our PA is able to halt RNA polymerase addition to the transcription machinery, through interfering with its binding elements at the promoter of each target gene [9].

PA was prepared by automated solid phase synthesis using a CS336X synthesizer as previously described [10,11]. The synthesizer was programmed in the standard hardware configuration for DIC/HOBt (or HBTU/DIEA) protocols. Reagent position 1 was DMF, reagent position 2 was dry DMF, reagent position 3 was piperidine/DMF (20%), reagent position 4 was DCM, reagent position 5 was TFA/phenol/H_2O (92.5:5:2.5), reagent position 6 was DIEA/dry DMF (10%), reagent position 7 was 2,4,6-collidine/dry THF (15%), and reagent position 8 was dry THF.

As phosgene and CO_2 gas were evolved, the peptide synthesizer was placed in a fume hood and the waste gas was treated with aqueous 20% NaOH solution to destroy the excess phosgene. The synthesis was carried out on a 0.06 mmol scale (400 mg of the resin; 0.15 mmol/g). Each cycle of amino acid addition involved deprotection, amino acid activation and coupling. Two successive coupling cycles are employed when coupling pyrrole amino acids to imidazole amines; all other couplings are performed with single coupling cycles.

When the coupling of all heterocycles was complete, resin was washed twice with DCM (5 mL, calibrated delivery from reagent bottle 4) and deprotected twice (for 2 min and 20 min, respectively) with the mixture of TFA/phenol/H_2O (5 mL, calibrated delivery from reagent bottle 5). The RV was drained and the resin was treated with two DCM washes (5 mL from reagent bottle 4), a DMF wash (5 mL from reagent bottle 1), a DIEA/dry DMF wash (5 mL from reagent bottle 6, bypassing the AA reservoir), a dry THF wash (to wash the needle, 5 mL from reagent

bottle 8, bypassing the AA reservoir), a DMF wash (5 mL from reagent bottle 1), and a dry DMF wash (5 mL from reagent bottle 2). The resin containing the fully-formed PA was treated with $Cu(OAc)_2$ in dimethylaminopropylamine (Dp, 20 µL), the suspension was shaken at 55°C for 16h. After cooling to room temperature, the crude PA was collected by centrifugation and the blue solution was diluted with 10% MeCN/H_2O/0.1% TFA and purified by semi-preparative RP-HPLC (Fig. 1C).

Cell viability and density of human KCL22 cells (DSMZ-German Collection of Microorganisms and Cell Cultures, DSMZ # ACC 519) when exposed to our PA, TKI (nilotinib), and their combination were investigated by trypan blue dye exclusion counting in standard Neubauer haemocytometer chambers. The KCL22 cells were seeded at 1 x 10^5 cells/mL in serum supplemented RPMI640. The PA was tested in the micromolar concentration range; nilotinib was used at 10 nM which is around its IC_{50} in our hands for this cell line. For synergy, Combination Indices (CI) were calculated manually using the Bliss Independence model according to $CI = ((E_A + E_B) – E_A.E_B) / E_{AB}$; where E_A is the effect of drug A alone, E_B is the effect of drug B along and E_{AB} is the effect of the combination. $CI < 1$ is synergistic; $CI = 1$ is additive; $CI > 1$ is antagonistic.

Cell cycle investigation was done using propidium iodide (PI) staining. Briefly, after fixation in 70% ice-cold ethanol, cells were washed twice in PBS and stained with 2 µL of 100 µg/mL RNase (to ensure only the DNA is stained) in 200 µL of 50 µg/mL PI. Samples were analysed by flow cytometry in the PE/PerCP channel; data analysis was done using FlowJo software (TreeStar). RNA extractions were done using the RNeasy plus micro or mini kits (QIAGEN). cDNA samples were made using applied Biosystems High-Capacity RNA-to-cDNA™ Kit (ThermoFisher). Gene expression analysis was done using EvaGreen® on the BioMark HD System, using flex six IFC (Fluidigm) based on manufacturer's protocols. Housekeeping gene (*GAPDH* and *RNF20*) cycle threshold (C_t) values were used to normalise the data of genes of interest, generating ΔC_t values. ΔC_t values of drug treated samples were subtracted from ΔC_t of the relevant no drug control, generating $\Delta\Delta C_t$ values to calculate relative levels of gene expression (fold change, $2^{-\Delta\Delta Ct}$).

Results & Discussion: Our polyamide follows established pairing rules for aromatic pyrrole (Py) and imidazole (Im) amino acids whereby Py/Py recognises A·T/T·A, while Im/Py targets G·C pairings and Py/Im the reverse (C·G).[10] For more in-depth structural information concerning how hairpin polyamides bind to a target sequence, please refer to [12,13]. It may be presumed that a PA, with a short half-life, binds and inhibits TF activity within 24 hours in culture whereas the biological consequences of altered transcription may lag behind. Hence, we looked at gene expression at 24h, and observed cellular process effects by 48 hours. As can be seen in Fig. 2A, the presence of our PA in the culture held back KCL22 cell expansion compared to no drug control. Combining a PA with nilotinib seemed to show a greater effect than nilotinib alone at 48 hours on cell counts, however the CI was not synergistic. In addition, PI staining was used to investigate the cell cycle status. No difference between PA-treated cells and no drug control at the same time point was found (Fig. 2B). This suggested that our PA had not caused

cell cycle arrest. BC-CML cells acquire more genetic lesions with disease progression so are no longer unilaterally dependent on BCR-ABL1 like CP-CML cells, so they might show more insensitivity to PA, as can develop to TKIs. Contrary to our hypothesis, the complex network of cell survival signaling in BC-CML cells due to the additional genetic alterations eluded to earlier may help BC-CML cells overcome cell cycle arrest induced consequent to the PA inhibiting E2F1 activity. Reduction in cell numbers without apparent cell cycle arrest suggests inhibition of E2F1 TF activity by PA in BC-CML cells had induced cell death.

Our PA targets sequences within 100 bp upstream of the 5′ end of the consensus E2F1 binding site in the promoter region of E2F1 target genes meaning 42 candidates of 4,300 putative E2F1 target genes were chosen. 17 of these 42 genes with highest expression in $CD34^{+}$ hematopoietic stem/progenitor cells (based on the Gerber *et al*., publicly available dataset provided by the Stemformatics website [14]), were selected as biomarkers of PA activity by gene expression analysis. After complementary DNA (cDNA) primer efficiency testing, 9 genes were selected for further study: *MRRF*, *MCM2*, *SF3B4*, *GNA13*, *PSMB8*, *PET112L*, *TRIM45*, *ZNF354B*, and *RARS2*. The gene expression results suggested that the PA disrupts E2F1 function by down-regulating the expression of 7 of the 9 selected E2F1 regulated genes (Fig. 3). Thus, this PA could have the potential for development as a chemical probe to inhibit E2F1's transcriptional role of its target genes among BC-CML cells. Interestingly, two genes, namely *TRIM45* and *RARS2* were upregulated in KCL22 PA treated cells in opposition to our hypothesis of down-regulation. While E2F1 TF activities were targeted by our PA with 7 of 9 E2F1 target genes being down-regulated, some KCL22 cells persisted without the apparent need for those gene products for their overall survival, so here we might have enriched for viable, resistant KCL22 cells.

Our PA was efficacious as a single agent at 3 μM concentration limiting KCL22 cell number in culture. However, when combined with TKI, despite apparent cooperativity in restricting cell numbers, there was statistically no greater effect than either agent alone (synergy) after 48 hours in culture, possibly owing to the fact that E2F1 expression itself is controlled by BCR-ABL1. Hence, inhibition of the E2F1 would give no added benefit in the context of simultaneous targeting of BCR-ABL1 tyrosine kinase activity with TKI. Going forward, we would investigate the effect of scheduling drug administration to explore how altering E2F1-controlled gene expression by a PA would best contribute to pharmacological 'synthetic lethality' improving outcomes with TKI alone.

Conclusion: In summary, we have demonstrated that our PA down-regulates the expression of E2F1-controlled genes, which causes a restriction in the growth of KCL22 BC-CML cells. Taking both our *in vitro* studies together (in CP-CML and BC-CML), and should *in vivo* effects be demonstrated, we would envisage that our PA approach could be further developed as an adjuvant therapy for CP-CML more than BC-CML, or, as a general chemical biological probe of E2F1-controlled expression to further investigate TFs such as E2F1 as novel drug targets in cancer.

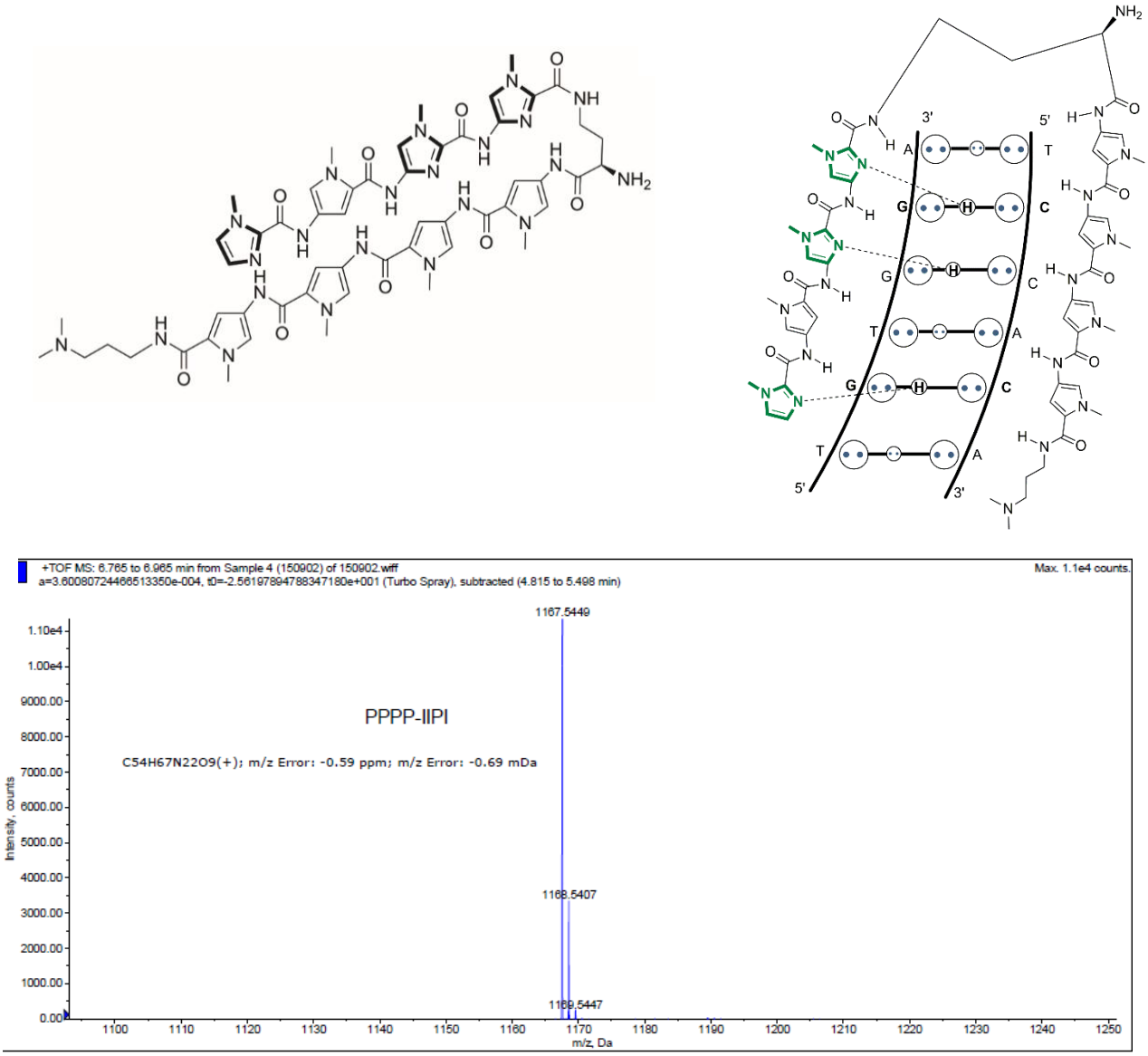


Figure 1. Structure and analysis of synthesized pyrrole imidazole polyamide.

A: Structure of the pyrrole imidazole polyamide (PA) designed against E2F1 TF activity in this study. **B:** Schematic preview of our PA binding to the WGWGGW sequence in the minor groove of the target DNA, where W = T/A. **C:** Representative Reverse Phase (RP)-HPLC chromatogram of synthesized PA. HRMS (ESI, +ve mode): 1167.5449 [M+H]+.

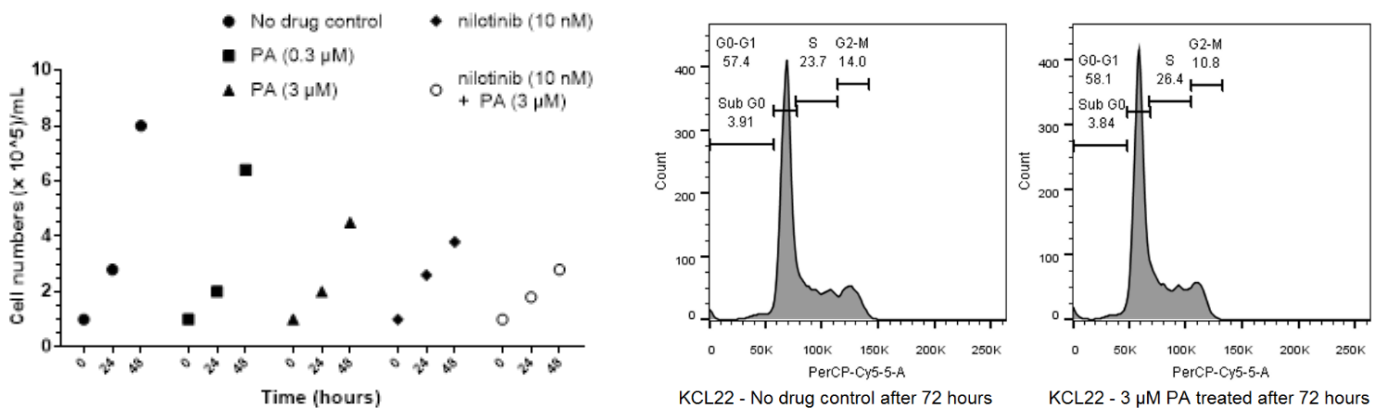


Figure 2. **KCL22 cell proliferation and cell cycle status after being treated with PA**.
A: Cell density, as investigated by trypan blue staining, of KCL22 cells during 48h time-course after being treated with PA, nilotinib or a combination of both. PA alone reduced proliferation of KCL22 cells. The PA effect alone at 48h was not as pronounced as nilotinib alone or in combination with nilotinib yet CI for the combination was >1 at 48h. **B:** Flow cytometry histograms of PI staining for cell cycle of KCL22 cells treated with 3 µM PA or no drug control at 72 h. There is almost the same proportion of cells at the different cell cycle stages (G1, S, G2, M) among those treated with 3 µM PA or no drug control. This is suggestive of PA not having any cell cycle arrest effect. Hence the difference in cell counts seen between untreated and treated cells in (**A**) is unlikely to be explained by cell cycle arrest and consequent restriction of proliferation.

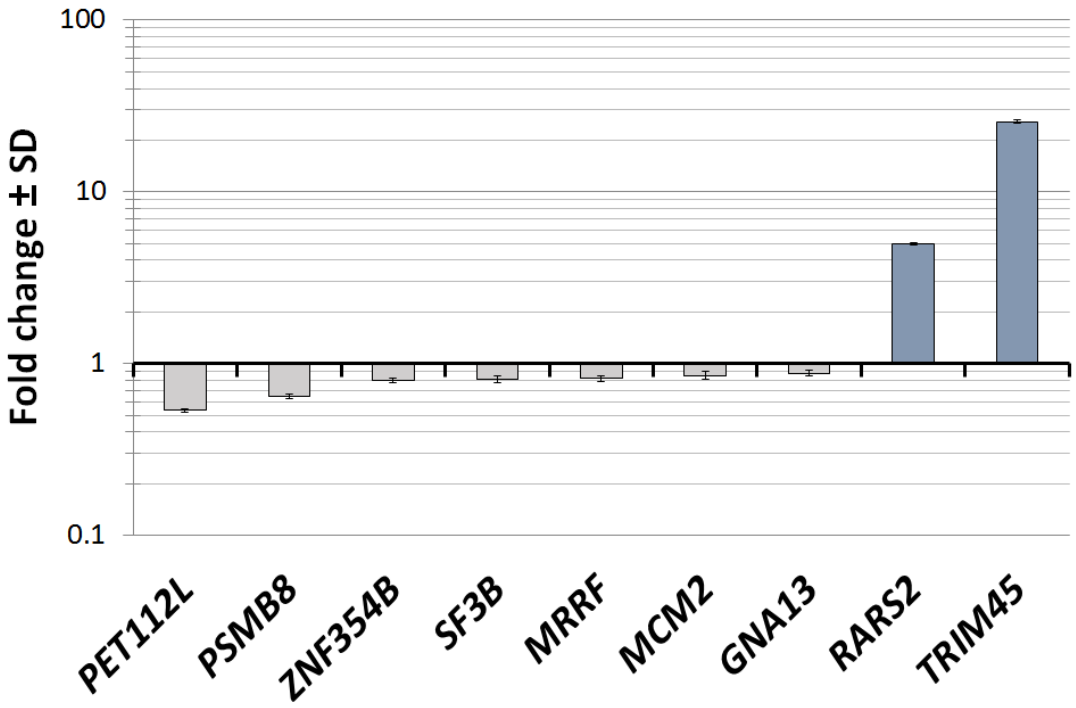


Figure 3. **RT-qPCR gene expression results for KCL22 cells treated with 3 µM PA for 24 hours**. Gene expression was determined using Fluidigm platform with EvaGreen® dye at 24 hours after addition of PA (3 µM) to the cultures. Cycle threshold (C_t) values were normalised by two housekeeping genes i.e., *GAPDH* and *RNF20*. Gene expression levels are presented as fold-change ($2^{-\Delta\Delta Ct}$) in the sample versus untreated control. This is suggestive that 7 out of 9 target genes are down-regulated after 24 hours of PA treatment. Mean ± SD of three technical replicates are shown.

Authors and Affiliations

Hayatigolkhatmi K.[a], Padroni G.[b], Su W.[c], Fang L.[c], Gómez-Castañeda E.[a], Hsieh Y.C.[a], Jackson L.[a], Pellicano F.[d], Burley G.A.[b], Jørgensen, H.G.[a]

[a] Paul O'Gorman Leukaemia Research Centre, Institute of Cancer Sciences, College of Medical, Veterinary and Life Sciences, University of Glasgow, UK.

[b] Department of Pure and Applied Chemistry University of Strathclyde, Thomas Graham Building, Glasgow, UK.

[c] Guangdong Key Laboratory of Nanomedicine, Institute of Biomedicine and Biotechnology, Shenzhen Institutes of Advanced Technology, Chinese Academy of Sciences, Shenzhen, Guangdong, P. R. China.

[d] Drug Discovery Program, Cancer Research UK Beatson Institute, Garscube Estate, Glasgow, UK.

Correspondence to: Heather G. Jørgensen, Paul O'Gorman Leukemia Research Centre, Institute of Cancer Sciences, College of Medical, Veterinary and Life Sciences, University of Glasgow, 21 Shelley Road, Glasgow, G12 0ZD, UK. Email: heather.jorgensen@glasgow.ac.uk

Contribution

KH designed and performed the research, analysed the data and drafted the manuscript; GP performed the research and drafted the manuscript; WS and LF made the PA; EG-C, Y-CH, LJ, FP designed the research; GB and HGJ designed the research, analysed the data, drafted and finalized the manuscript.

Competing interest

The authors declare no competing financial interests.

Funders

KH was self-funded; GP is funded by a University of Strathclyde PhD studentship; HGJ is supported by Friends of Paul O'Gorman Leukaemia Research Centre, Glasgow.

References

[1] Fontaine, F, Overman, J and François, M. Pharmacological manipulation of transcription factor protein-protein interactions: opportunities and obstacles. *Cell Regeneration* (London) (2015); 4.1:2. doi: 10.1186/s13619-015-0015-x

[2] Yang, F, et al. Antitumor activity of a pyrrole-imidazole polyamide. *Proc Natl Acad Sci USA* (2013); 110.5:1863. doi: 10.1073/pnas.1222035110.

[3] Withers, JM, et al. DNA minor groove binders as therapeutic agents. *Comprehensive Supramolecular Chemistry II* (2017); 149-178. doi: 10.1016/B978-0-12-409547-2.12561-2.

[4] Kawamoto, Y, Bando, T and Sugiyama, H. Sequence-specific DNA binding Pyrrole–imidazole polyamides and their applications. *Bioorganic Med Chem* (2018); 26.8:1393. doi: 10.1016/j.bmc.2018.01.026.

[5] Chenoweth, DM, and Dervan, PB. Structural basis for cyclic Py-Im polyamide allosteric inhibition of nuclear receptor binding. *J Am Chem Soc* (2010); 132.41:14521. doi: 10.1021/ja105068b

[6] Pellicano, F, et al. hsa-mir183/EGR1–mediated regulation of E2F1 is required for CML stem/progenitor cell survival. *Blood* (2018); 131.14:1532. doi: 10.1182/blood-2017-05-783845.

[7] Hehlmann, R, et al. Management of CML-blast crisis. *Best Pract Res Clin Haematol* (2016); 29.3:295. doi: 10.1016/j.beha.2016.10.005.

[8] Hayatigolkhatmi, K, et al. Investigation of a minor groove-binding polyamide targeted to E2F1 transcription factor in chronic myeloid leukaemia (CML) cells. *Blood Cells Mol Dis* (2018); 69:119-122. doi: 10.1016/j.bcmd.2017.11.002.

[9] Dervan, PB, and Edelson, BS. Recognition of the DNA minor groove by pyrrole-imidazole polyamides. *Curr Opin Struct Biol* (2003); 13.3:284. doi.org/10.1016/S0959-440X(03)00081-2

[10] Fang, L, et al. Fully automated synthesis of DNA-binding Py-Im polyamides using a triphosgene coupling strategy. *Org Lett* (2015);17.1:158 doi: 10.1021/ol503388a

[11] Pauff, SM, et al. Pyrrole-imidazole polyamides: manual solid-phase synthesis. *Curr Protoc Nucleic Acid Chem* (2015); 63.1:8.10.1. doi:10.1002/0471142700.nc0810s63

[12] Padroni, G, et al. Structural basis of DNA duplex distortion induced by thiazole-containing hairpin polyamides. *Nucleic Acids Res* (2018); 46.1:42 doi.org/10.1093/nar/gkx1211

[13] Rottschäfer, G, et al. A phosphorus analogue of p-quinodimethane with a planar P4 ring: A metal-free diphosphorus source. *Chem Eur J* (2019); 25:2757. doi.org/10.1002/chem.201805932

[14] Wells, CA, et al. Stemformatics: visualisation and sharing of stem cell gene expression. *Stem Cell Res* (2013); 10.3:387. doi: 10.1016/j.scr.2012.12.003.