Letter to the Editor

Investigation of a minor groove-binding polyamide targeted to E2F1 transcription factor in Chronic Myeloid Leukaemia (CML) cells

To the Editor,

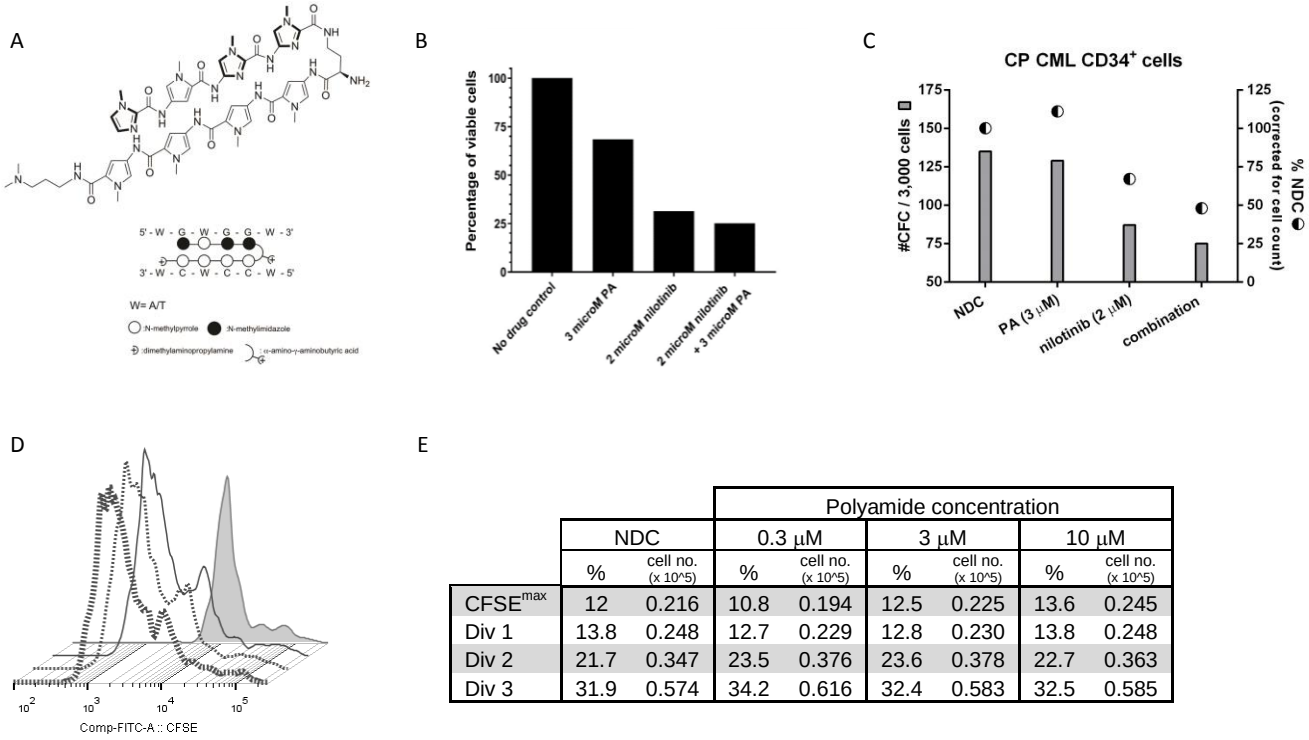
The current treatment for chronic myeloid leukaemia (CML) is tyrosine kinase inhibitors (TKI), which inhibit BCR-ABL1 phosphorylation activity. Despite success of TKI therapy in causing complete cytogenetic response (i.e., 0% Philadelphia (Ph) chromosome-positive cells detected by cytogenetics in the bone marrow), in the majority of chronic phase (CP) CML patients, nearly all patients have minimal residual disease detectable by sensitive Q-PCR owing to the inability of TKI agents to eliminate persistent, primitive, leukemic stem cells (LSC) [1,2]. We believe E2F1 is a critical transcription factor (TF) for CML LSC survival. E2F1 binds to retinoblastoma protein in a cell-cycle dependent manner so is responsible for regulating cell proliferation and apoptosis. In CML, BCR-ABL1 signaling leads to an up-regulation of E2F1.The process starts with BCR-ABL1 driven up-regulation of hsa-mir183 in Ph^+ cells which then down-regulates its direct target, EGR1 subsequently upregulating E2F1 (personal communication). Although our studies suggest that inhibition of E2F1 itself may be promising therapeutically, transcription factors are notoriously challenging as drug targets.

Small synthetic oligomers composed of aromatic amino acids, called polyamides (PA) are able to bind to predetermined DNA sequences in the minor groove of DNA. PA molecules are resistant to cellular proteases and can target genomic DNA of cancer cells *in vitro* and *in vivo*. PA molecules inhibit gene expression through either competitive-binding to TF binding sites or inhibition of RNA polymerase [3-6]. The PA used in this study was designed to recognize the general sequence WGWGGW (where W = A or T). To inhibit E2F1 transcriptional activity, we explored targeting a PA binding site GTGGA within 100 base pairs (bp) of the 5' end of the consensus E2F1 binding site (TTTCGC) in the promoter region of the TF's target genes. To determine the ability of our PA to disrupt E2F1 activity, we asked: how would our PA affect CP CML cell cycle, proliferation and apoptosis; how would it alter cell function, e.g. colony formation capacity, of CP CML $CD34^+$ cells; and how would it change E2F1 target gene expression alone or in combination with TKIs?

Primary $CD34^+$ cells from CP CML patients stored in the University of Glasgow's Paul O'Gorman Leukemia Research Centre hematological cell research bank with informed consent (West of Scotland Research Ethics Committee (REC) reference 15-WS-0077) were chosen samples for this study. CP CML $CD34^+$ cells were seeded at $2x10^5$/mL in physiological growth factor supplemented serum free medium [7]; PA was tested at three different concentrations (0.3, 3 or 10 μM); nilotinib was used at 2 μM.

Viability was determined by flow cytometry by staining cells with Annexin V / DAPI. Our data were suggestive of our PA (Fig. 1A) having some effect on restricting cell viability with respect to untreated control after 72h in culture (Fig. 1B). However, this effect was not as great as with nilotinib on its own. Interestingly, the combination of PA (3 μM) and nilotinib (2 μM) showed a trend towards a co-operative negative effect on number of surviving CP CML cells. Indeed this was mirrored in the colony forming assay (Fig. 1C). As our hypothesis is predicated on BCR-ABL1 TK activity driving E2F1 expression, this was an unexpected result worthy of further investigation. We propose residual E2F1 function may explain the apparently increased effect with the combination; and suggests pulsed dosing of one agent before the other might be more beneficial.

CP CML CD34$^+$ cells were also stained with carboxyfluorescein succinimidyl ester (CFSE), and further identified by anti-human anti-CD34 surface staining, to assess the ability of these cells to divide (Fig. 1D) as a reduced number of viable cells could come about through the processes of increased cell death and/or control of cell cycle and division. PA had no effect on CP CML CD34$^+$ cell potential to divide in the time frame tested (72h) (Fig. 1D & E) hence reduced numbers of viable cells must be through impairment of survival.



	Polyamide concentration							
	NDC		0.3 μM		3 μM		10 μM	
	%	cell no. (x 10^5)	%	cell no. (x 10^5)	%	cell no. (x 10^5)	%	cell no. (x 10^5)
CFSE^{max}	12	0.216	10.8	0.194	12.5	0.225	13.6	0.245
Div 1	13.8	0.248	12.7	0.229	12.8	0.230	13.8	0.248
Div 2	21.7	0.347	23.5	0.376	23.6	0.378	22.7	0.363
Div 3	31.9	0.574	34.2	0.616	32.4	0.583	32.5	0.585

Figure 1. Analysis of CP CML CD34$^+$ cell viability and proliferation 72 h after being treated with PA, nilotinib or the combination. **A.** Schematic representation of the PA used to inhibit DNA-binding of E2F1 in the target binding site. **B.** Comparative analysis of PA and nilotinib on CP CML CD34$^+$ cell viability *in vitro*. **C.** The number of colony forming cells (CFC) per 3,000 live cells plated after 12 days culture in semi-solid medium was scored (grey bars, left hand axis). To determine the absolute number of CFC, a correction factor was applied allowing for the drug effect, so multiplying to find the number of CFC produced had all remaining cells in the dish been plated (right hand axis) and normalizing to the no drug control (NDC). **D.** Tracking of cell division with CFSE staining of CD34$^+$ cells surviving 72h drug treatment in liquid culture suggested no effect of PA on the ability of the cells to proliferate with respect to the untreated control. Front to back**,** 0.3μM (thick dotted line), 3μM, (thin dotted line), 10 μM (unbroken line), CFSE^{max} (cells arrested with Colcemid®, filled curve; peak with mean fluorescence intensity in the FITC channel of approximately 10^4) **E.** Calculating the absolute number of cells per division as the product of the percentage gated times viable cell count confirmed the lack of effect of PA alone on the ability of CP CML CD34$^+$ cells to divide (according to the method of Prost *et al*., Nature 2015; doi:10.1038/nature15248).

We next investigated the modulation of gene expression by our PA in these cells. From a list of 4,300 possible target genes, 42 were identified with PA binding sequence (5'-GTGGA-3') within 100 bp and 5' to the E2F1 consensus binding sequence. Levels of expression of these genes were then investigated among normal and CP CML CD34$^+$ hematopoietic stem/progenitor cells (HSC/HPC) within a publically available dataset on the Stemformatics portal [8] (Fig. 2). Primers were designed for 17 of the 42 most

differentially expressed genes in CP CML CD34^{+} cells. Nine of those 17 primer pairs (*MRRF, MCM2, SF3B4, GNA13, PSMB8, PET112L, TRIM45, ZNF354B, RARS2*) presented efficiencies ranging between 88 and 110% (Table 1) and were used to investigate gene expression changes by qPCR induced by our PA (Fig. 3). After 24h, *RARS2* and *TRIM45* genes showed down-regulation with all PA three concentrations (0.3, 3 and 10 μM) (Fig. 3). *RARS2* is involved in mitochondrial protein translation while *TRIM45* has an established role in proliferation, development, oncogenesis, and apoptosis.

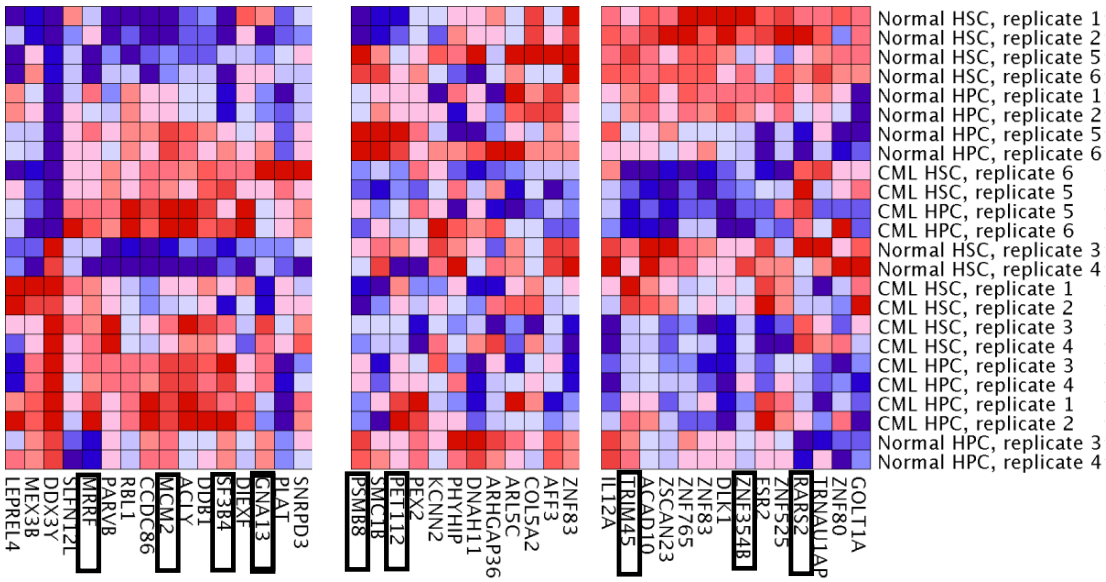


Figure 2. Heat-map showing expression values of genes having a PA binding sequence 5' to E2F1 transcription factor consensus binding sequence. Gene expression values for 42 candidate genes within Gerber *et al.*, publically available dataset accessible via the Stemformatics website, of normal and CML hematopoietic stem (HSC) and hematopoietic progenitor cells (HPC). Darker blue shows lower, while darker red shows higher expression levels with respect to housekeeping genes. Screenshots captured from LEUKomics portal of Stemformatics [8].

Gene name	DNA concentration (ng/mL)	Slope	Efficiency (%)
MRRF	3.125 - 100	-3.547	91
MCM2	3.125 - 100	-3.109	110
SF3B	3.125 - 100	-3.515	93
GNA13	3.125 - 100	-3.530	92
PSMB8	3.125 - 100	-3.257	103
PET112L	3.125 - 100	-3.654	88
TRIM45	6.25 - 100	-3.618	89
ZNF354B	6.25 - 50	-3.535	92
RARS2	3.125 - 100	-3.479	94

Table 1: Primer efficiencies. In the primer efficiency test for genes of interest based on their qPCR standard curve (by SYBR® green detection based on Qiagen RT^{2} Profiler PCR Arrays kit) the resultant slope of the line was used in the equation [-1+10$^{(-1/slope)}$] x 100 whereby the primer efficiency as a percentage for each primer pair can be determined. The nine genes from among 17 that had acceptable efficiencies between approximately 90 and 110% and were taken forward are shown.

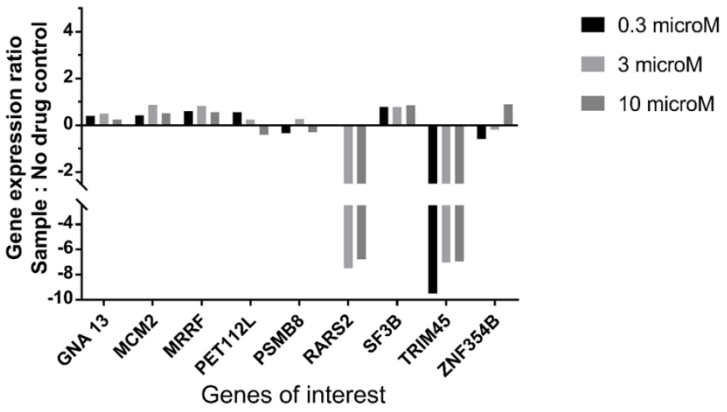


Figure 3. RT-qPCR gene expression results for CP CML CD34^{+} cells after 24h *in vitro* treatment with PA. *TRIM45* and *RARS2* showed down-regulation with all PA three concentrations (0.3, 3 and 10 μM). Ct values were normalized to *GAPDH* and *RNF20* housekeeping genes. Relative gene expression levels were calculated by the standard $2^{-\Delta\Delta Ct}$ method.

In summary, we showed a minor-groove binding PA inhibits the transcriptional activity of E2F1, which in turn caused a reduction in CP CML cell viability and function in combination with TKI. Studies are currently underway to systematically survey DNA sequences up/downstream from the E2F1 consensus sequence using a PA compound library and establish an 'interactome' map of E2F1 function in CML cells. A deeper understanding of this TF could ultimately translate into the development of TF inhibitors as a novel anti-cancer drug target.

Contribution

KH designed and performed the research, analysed the data and drafted the manuscript; GP performed the research; EG-C, Y-CH, and LJ analysed the data and reviewed the manuscript; FP designed the research and reviewed the manuscript; TLH designed the research; GB and HGJ designed the research, analysed the data, drafted and finalised the manuscript.

Conflict-of-interest disclosure

The authors declare no competing financial interests.

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