The gene expression signature of anagrelide provides an insight into its mechanism of action and uncovers new regulators of megakaryopoiesis

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<th>Journal:</th>
<th>Journal of Thrombosis and Haemostasis</th>
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<td>JTH-2014-01244.R1</td>
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<tr>
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<td>Original Article - Platelets</td>
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<td>Date Submitted by the Author:</td>
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<td>Complete List of Authors:</td>
<td>Ahluwalia, Maninder; Cardiff Metropolitan University, School of Health Sciences Butcher, Lee; Cardiff Metropolitan University, School of Health Sciences Donovan, Hannah; Cardiff Metropolitan University, School of Health Sciences Killick-Cole, Clare; Cardiff Metropolitan University, School of Health Sciences Jones, Paul; Cardiff Metropolitan University, School of Health Sciences Erusalimsky, Jorge; Cardiff Metropolitan University, School of Health Sciences</td>
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Please select Five Mandatory Key Words from the Medical Subject Headings List: anagrelide, megakaryocyte, activating transcription factor 4, eukaryotic initiation factor-2 alpha subunit, thrombocytosis
The gene expression signature of anagrelide provides an insight into its mechanism of action and uncovers new regulators of megakaryopoiesis

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Running title: The transcriptional signature of anagrelide

Abstract word count: 212

Main text word count: 3897

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Summary

Background: Anagrelide is a cytoreductive agent used to lower platelet counts in essential thrombocytopenia. Although the drug has been known to selectively inhibit megakaryopoiesis for many years, the molecular mechanism accounting for this activity is still unclear.

Objectives and Methods: To address this issue we have compared the global gene expression profiles of human hematopoietic cells treated ex-vivo with and without anagrelide while growing under megakaryocyte differentiation conditions, using high density oligonucleotide microarrays. Gene expression data were validated by the quantitative polymerase chain reaction and mined to identify functional subsets and regulatory pathways.

Results: We identified 328 annotated genes differentially regulated by anagrelide, including many genes associated with platelet functions and with the control of gene transcription. Prominent among the latter was TRIB3, whose expression increased in the presence of anagrelide. Pathway analysis revealed that anagrelide up-regulated genes which are under the control of the transcription factor ATF4, a known TRIB3 inducer. Notably, immunoblot analysis demonstrated that anagrelide induced the phosphorylation of eIF2α, which is an upstream regulator of ATF4, and increased ATF4 protein levels. Furthermore, salubrinal, an inhibitor of eIF2α dephosphorylation, increased the expression of ATF4-regulated genes and blocked megakaryocyte growth.

Conclusions: These findings link signalling through eIF2α/ATF4 to the anti-megakaryopoietic activity of anagrelide and identify new potential modulators of megakaryopoiesis.

Keywords: anagrelide, ATF4, eIF2α, megakaryocyte, thrombocythaemia.
Introduction

Anagrelide is a platelet lowering agent used in the clinic as part of the armamentarium to treat essential thrombocythemia (ET) [1;2]. Although originally developed as an inhibitor of platelet aggregation [3] initial studies in humans demonstrated that the drug had profound thrombocytopenic effects which had not been manifested during preclinical studies in animal models [4]. Further studies demonstrated that the platelet lowering effects were due to a potent inhibition of megakaryopoiesis [5;6]. Subsequent efforts to explain the differential responses in rodents and humans attributed the anti-megakaryopoietic activity to a metabolite of the drug [7] or to species-specific inhibition of thrombopoietin (TPO) receptor-stimulated tyrosine phosphorylation [8]. However these claims could not be reconciled with results from our own laboratory comparing between the biological activities of anagrelide and its human metabolites [9] or analysing the effects of the parental drug on TPO receptor-stimulated early signal transduction events [10]. On the other hand, further work showed that anagrelide inhibits the expression of various key transcription factors involved in megakaryocyte (MK) development and platelet production, namely GATA-1, FOG-1, FLI-1 and NF-E2 [10]. Anagrelide also inhibits a Type III phosphodiesterase (PDE III) found in platelets and the myocardium [11]. This activity underlies the anti-platelet aggregation effect of the drug but does not appear to be related to its anti-megakaryopoietic properties [9;10]. Thus, despite these advances in the understanding of the mode of action of the drug, to date the primary target accounting for the platelet-lowering effect has remained elusive.

Gene expression profiling using DNA microarrays has been successfully used for over a decade to elucidate mechanisms underlying biological pathways and to generate testable hypothesis on the mode of action of poorly characterised pharmacological agents [12;13]. Therefore, in an attempt to gain further insights into the mechanism of action of anagrelide we have compared the transcriptional profiles of primary cultures of developing human MKs growing in the absence or presence of the drug. Here we report the outcome of this approach and the results of ensuing biochemical studies which indicate that anagrelide
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inhibits MK differentiation concomitantly with the activation of the eIF2α/ATF4 signalling axis. In addition, this study uncovers new potential regulators of MK development.

Methods

Drugs

Anagrelide hydrochloride was purchased from Tocris Bioscience (Bristol, UK). Salubrinal, cilostamide and MG-132 were from Calbiochem (Nottingham, UK). Stock solutions were made in DMSO, stored in aliquots at -20°C and when required diluted in culture medium immediately before experiments.

Cell culture and drug treatments

MKs were generated ex-vivo from human neonate hematopoietic cells using a two-step liquid culture system as previously described [10]. In brief, umbilical cord blood-derived CD34+ cells (Stem Cell Technologies, London, UK) were expanded for 4 days in Stemspan™ medium (Stem Cell Technologies) supplemented with 2% human umbilical cord blood plasma and a mixture of hematopoietic growth factors consisting of TPO (Insight Biotechnology, Wembley, UK), SCF, Flt3 ligand and IL-3 (all from R&D Systems, Abingdon, UK). To promote terminal megakaryocytic differentiation the cells were washed and then sub-cultured in fresh medium supplemented with 2% cord blood plasma and 40 ng/ml TPO only, for different lengths of time. Where required, drugs or an equivalent amount of vehicle were added at the beginning of the differentiation step or at various time points after its initiation as indicated. Cultures were maintained at 37°C in a humidified incubator under 5% CO₂/95% air.

Oligonucleotide microarrays

Sample preparation: CD34+ cells obtained from three different donors were expanded for 4 days and then differentiated for a further 4 days in the absence or presence of 0.3 μM anagrelide as described above. RNA was then extracted with RNeasy reagent (Qiagen,
Crawley, UK) according to the manufacturer’s instructions and assessed for purity and integrity with the Agilent 2100 Bioanalyser (Agilent Technologies, Stockport, UK). On-column DNAse digestion (Qiagen) was performed to eliminate genomic DNA contamination. RNA samples of vehicle-treated cells and anagrelide-treated cells from each of the three donors were then subjected to gene expression profiling on Affimetric Human Genome U133 Plus 2.0 oligonucleotide chips (Affymetrix UK Ltd., High Wycombe, UK). Preparations of labelled cRNA, hybridization and scanning, were performed according to the manufacturer’s standard protocols.

**Data analysis:** Raw signals were processed by GeneSpring software (Agilent Technologies) with normalisation to the median value performed to allow comparisons across arrays. To reduce noise, probe sets were excluded if the signal strength did not significantly exceed background values or if it did not reach a threshold value for reliable detection in all the samples according to the relaxed Affymetrix MAS 5.0 probability of detection (p<0.1), as previously described [14]. The remaining data were analysed further to select those genes which showed a statistically significant (p<0.05) change in the level of expression as a result of anagrelide treatment. Lists of differentially expressed genes were subjected to further computational analysis using DAVID [15] (v6.7, http://david.abcc.ncifcrf.gov/) and Reactome [16] (http://www.reactome.org/) bioinformatics tools. Individual gene inspection was performed with the aid of the GeneCards Human gene database (http://www.genecards.org/) and PubMed (http://www.ncbi.nlm.nih.gov/pubmed/).

Gene expression data for the 6 microarrays have been submitted to Gene Expression Omnibus.

**RNA expression**

Cellular RNA was extracted, reverse transcribed and analysed by the quantitative polymerase chain reaction (Q-PCR) using gene-specific TaqMan probes (Applied
Biosystems, Warrington, UK) as previously described[10]. Probes are listed in Table S1. β-glucuronidase (GUSB) and TATA-box binding protein (TBP) were used as internal controls.

**Flow cytometry**

MK-specific antigen expression was monitored by flow cytometry using fluorescein isothiocyanate-conjugated anti-CD61 (clone Y2/51, Dako, Ely, UK) and allophycocyanin-conjugated anti-CD42b (clone HIP1, BD Biosciences, Oxford, UK) antibodies as previously described [9].

**Western blotting**

Aliquots of ~2 x 10^6 cells were washed with ice-cold phosphate buffered saline and re-suspended by adding 70 μL ice-cold lysis buffer (10 mM Tris HCl, pH 7.4, 100 mM NaCl, 10% glycerol, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulphate, 1 mM EDTA, 1 mM EGTA, 20 mM Na₃P₂O₇, 2 mM Na₃VO₄, 1 mM NaF, 100 nM Calyculin A, 1 mM phenylmethylsulphonyl fluoride and Halt Protease Inhibitor Cocktail (Thermo-Fisher Scientific, Basingstoke, UK)). The resultant lysates were further disrupted by sonication for 10 seconds at an amplitude of 35% using a VCX500 Ultrasonic Processor (Sonics & Materials, Newtown, CT, USA) and then centrifuged at 14000xg for 5 minutes to remove particulate material. Proteins (~25-50 μg) in samples of the supernatant fraction were separated by LDS-10% polyacrylamide gel electrophoresis under reducing conditions and transferred to nitrocellulose membranes (Invitrogen, Paisley, UK). Immunoblotting was carried out with rabbit monoclonal antibodies (Cell Signaling Technology, Danvers, MA) against phosphorylated eIF2α (clone D9G8), total eIF2α (clone D7D3) or ATF4 (clone D4B8). A rabbit IgG against actin (Sigma-Aldrich) was used to control for variations in protein loading. Immunoreactive bands were detected with a horseradish peroxidase-labelled anti-rabbit IgG (Cell Signaling Technology) and enhanced chemiluminescence. The relative intensity of the bands was quantified by scanning densitometry using the Image J software programme (available at http://rsb.info.nih.gov/).
Results

*The global gene expression signature of anagrelide*

To distinguish transcriptional changes related to the anti-megakaryocytic activity of anagrelide we compared the genome-wide expression profiles of cells treated with TPO for 4 days in the presence of the drug or an equivalent amount of vehicle. Q-PCR analysis confirmed that under these conditions anagrelide reduced markedly the rise in the expression of a subset of representative genes associated with MK development (Fig. S1), as previously reported [10].

Pairwise comparisons between the microarray signals from control and anagrelide-treated samples using a 2 fold-change cut-off and a statistical threshold set at $p<0.05$, identified 412 gene tags fitting these criteria. These represented 328 annotated genes, 214 of which were down-regulated and the rest were up-regulated (Table S2). Functional enrichment analysis of Gene Ontology (GO) terms revealed that among the down regulated genes there was a statistically significant overrepresentation of categories related to the components and function of platelets, such as integrin binding, cell adhesion, coagulation, response to wounding and secretory granules (Fig. 1). In contrast, for the up-regulated genes there was a significant enrichment in terms associated with immune and inflammatory responses, and cytokine/chemokine activity (Fig. 1). Genes present in the different overrepresented functional categories are listed in Tables S3 and S4.

Transcriptional control is fundamental to the orchestration of the MK differentiation process. Hence, the list of 412 gene tags was also inspected to identify functional subsets of genes that might potentially affect transcriptional responses. This inspection revealed that anagrelide affected the expression of 23 genes corresponding to proteins which could be directly involved in the regulation of gene transcription (Table 1). Twelve of these transcripts were up-regulated and the rest were down-regulated. Eleven genes in this category and the cytokine IL8 were selected for further verification by Q-PCR. In addition, the effect of the
PDEIII inhibitor cilostamide on their expression was examined; this compound was chosen because among various pure PDEIII inhibitors it has a similar potency to anagrelide for this activity. This analysis confirmed the differential expression as a result of anagrelide treatment and also demonstrated a lack of effect of cilostamide (Fig. 2). Furthermore, as shown in Fig. S2, a very strong correlation ($r=0.97$, $p < 0.0001$) between the values obtained from the microarray analysis and those measured by Q-PCR was observed, thus endorsing the reliability of the microarray data.

**Anagrelide increases the expression of genes activated by ATF4**

Among the genes listed in Table 1, the human homolog of Drosophila tribbles 3 (TRIB3) stood out as being strongly up-regulated by anagrelide. TRIB3 expression is known to be regulated by the transcription factor ATF4 [17;18]. In line with this notion, pathway enrichment analysis of the microarray data set against the curated database of biological pathways Reactome ranked the activation of ATF4 as one of the top three statistically significant over-represented events ($p<0.01$). Indeed, this analysis uncovered five additional genes under the transcriptional control of ATF4 which were up-regulated by anagrelide (Table 2).

To assess in more depth the role of ATF4 in the response to anagrelide, we examined the effect of a short window of exposure to the drug on the expression of ATF4 mRNA and three of its target genes (ATF3, DDIT3 and TRIB3). To this end cells were treated with anagrelide for a 24 hour interval, starting at different time points after the initiation of the differentiation process. As shown in Fig. 3, this treatment schedule revealed that during the first 2 days of differentiation there was little or no response to the drug. However, following this refractory phase TRIB3, DDIT3 and ATF3 expression was seen to increase sharply, the response becoming stronger the longer the pre-incubation time, even though the period of exposure to the drug remained the same. In contrast, ATF4 expression only increased at a much later stage and to a lesser degree. Thus, these results suggest that anagrelide stimulates ATF4-
mediated transcription, by a mechanism that does not involve an increase in ATF4 mRNA levels.

The transcriptional response to anagrelide may depend on the megakaryocyte differentiation state

To ascertain whether the timing of exposure to anagrelide also influenced the degree of change in the expression of genes other than those directly controlled by ATF4, we examined the levels of the transcriptional regulators JUN, MAX, TAL1 and NFIB in the above experimental setting. As shown in Fig. S3, in the case of JUN and MAX, the effect of anagrelide was clearly larger if the drug was added at later time points. In the case of TAL1 and NFIB, the response to the 24h treatment with the drug was quite small, but nevertheless a similar trend was observed. Taken together, these findings suggest that the transcriptional response to anagrelide is orchestrated via a mechanism which may be dependent on the differentiation state of the cell.

Anagrelide stimulates signalling through eIF2α/ATF4

In other systems activation of ATF4-mediated transcription is triggered by the upstream phosphorylation of the α-subunit of the eukaryotic translation initiation factor-2 (eIF2α) which in turn promotes an increase in ATF4 translation [19]. Consistent with this notion, anagrelide stimulated a rapid phosphorylation of eIF2α (Fig. 4A) and an increase in ATF4 protein levels; the latter however, could only be unravelled in the presence of the proteasomal inhibitor MG132 (Fig. 4B).

The phosphorylation of eIF2α is connected to the inhibition of megakaryocyte growth

To obtain evidence that the phosphorylation state of eIF2α was related to the anti-MK activity of anagrelide, we studied the response to salubrinal, a known inhibitor of eIF2α dephosphorylation. Salubrinal was tested in MK cultures at 75 μM as this is the
concentration reported to have cellular effects in other systems [20]. As shown in Fig. 5 at this concentration salubrinal mimicked the effects of anagrelide, causing a rise in the level of phosphorylated eIF2α, increasing the expression of ATF4-regulated genes, and inhibiting MK growth.

Discussion

In the present study we have identified the global gene expression signature of anagrelide in a further attempt to advance our understanding on its mode of action. To this end we have used a well-established ex-vivo model system of megakaryopoiesis in which human CD34+-derived hematopoietic progenitor cells were treated with the drug at a concentration of 0.3 µM for a period of four days under conditions which would otherwise promote terminal MK differentiation. These treatment conditions were selected on two accounts: 1) the chosen dose of anagrelide does not affect the proliferation of hematopoietic progenitors or their differentiation into other lineages [21]; 2) at this dose, a four days exposure constitutes the earliest time point at which a consistent transcriptional response to the drug is observed when examining the changes in expression of a selected group of genes [10]. Using this approach we found 328 genes that were differentially expressed as a result of anagrelide treatment. Close to two thirds of the genes in this data set were down-regulated. Among these we detected a significant over-representation of functional categories associated with the contents of platelets and their functions, altogether encompassing forty four genes. These included genes encoding for proteins involved in platelet activation, secretion, adhesion, aggregation, clot formation and wound healing. These results are consistent with the overall action of anagrelide as an inhibitor of thrombopoiesis, therefore endorsing the validity of the transcriptome analysis. In addition, we also identified 114 genes in our data set that were up-regulated. The latter included a significantly high proportion of genes associated with immune and inflammatory responses and with cytokine/chemokine activity. This might suggest that by stalling the MK differentiation programme, the cell acquires
phenotypic characteristics of other myeloid lineages. Alternatively, the possibility that in-vivo, the residual thrombopoietic activity that takes place in the presence of anagrelide could produce MKs and platelets with altered functional properties cannot be discounted. One gene present in this group was IL8, a pro-inflammatory cytokine also found to be elevated in primary myelofibrosis [22]. In this respect, our findings could be relevant to concerns raised by the PT-1 trial where an increased frequency of myelofibrotic transformation [23] and a greater increase in reticulin fibrosis over time [24] were seen in ET patients treated with anagrelide when compared to those treated with hydroxyurea. However, whether anagrelide actually promotes fibrosis is currently unknown; this question could only be addressed by comparing its effect against an untreated cohort.

In accordance with previous work suggesting that anagrelide affects the transcriptional control of MK differentiation [10], we also found significant changes in the expression of 23 genes that encode putative transcriptional regulators. However, unlike in our previous Q-PCR-based expression study where we found that the drug treatment led to a decrease in the mRNA levels for the transcription factors GATA1, FOGL, FL1 and NFE2 [10], we did not find these genes listed in our microarray data set. Nevertheless, we could confirm by Q-PCR that their expression was also reduced by anagrelide in the current samples, although by less than 2-fold, which was the cut-off of the microarray analysis.

It has been recently reported that anagrelide inhibits megakaryopoiesis owing to its PDEIII inhibitory activity by stimulating cAMP/PKA signalling and subsequently targeting the transcription factor E2A (TCF3), down-regulating its mRNA levels [25]. Nevertheless, both our microarray data set and our Q-PCR analysis showed no evidence that anagrelide reduced TCF3 mRNA levels at the concentration tested. Furthermore, of all the genes that we subsequently verified by PCR, none was found to respond to the PDEIII inhibitor cilostamide, suggesting that the majority of the gene expression changes induced by anagrelide were not due its PDEIII inhibitory activity. Indeed, this interpretation is consistent with previous findings showing that pure PDEIII inhibitors have negligible effects on
megakaryopoiesis [9] and is also in keeping with another study showing that cAMP signalling enhances MK development rather than inhibiting it [26].

A previous global gene expression study of MK cultures derived from human mobilized peripheral blood CD34⁺ cells identified 199 putative transcriptional regulators of megakaryopoiesis [27]. Five transcripts identified in that study (IRX3, NFIB, MAX, SLC2A4RG and TAL1) were also shown to be strongly (>2 fold change) affected by anagrelide in the current work. In addition, genes in this category, including IRX3, TRIB3, NLK, MAX and JUN have been listed in microarray datasets related to studies of differentiation [28;29], malignancy [30], or signal transduction [31] in the MK lineage. However, with the exception of JUN [32], TAL1 (reviewed in [33;34]), and more recently NFIB [35], the expression or role of these genes in megakaryopoiesis has not been studied further. In our study, the gene most strongly down-regulated by anagrelide in this category was IRX3. This gene encodes a homeobox transcription factor previously implicated in the development of the nervous system [36]. Future work will determine whether this gene is also a major player in MK development.

Prominent among the genes up-regulated by anagrelide was TRIB3, a member of an emerging group of pseudo-kinases, increasingly implicated in the regulation of cellular metabolism, stress responses, cell proliferation and differentiation (reviewed in [37;38]). In the context of haematopoiesis TRIB3 has been reported to undergo up-regulation by erythropoietin and has been associated with erythroid progenitor cell survival [39] and differentiation [40]. In contrast, in line with our findings, TRIB3 has been previously cited in a microarray data set among the down regulated genes of a TPO-response signature [41]. However, its role in megakaryopoiesis is yet to be established. The finding in the present study that it was up-regulated by anagrelide raises the possibility that it may act as a repressor of this process. In this respect it is noteworthy that in other systems TRIB3 has been shown to act as a transcriptional repressor by interacting with a number of transcription factors, including ATF4 [42], DDIT3 [18], C/EBPβ [43] and PPARγ [44]. In addition, it has
been reported to bind and inhibit the protein kinases AKT [45] and MEK1 [46] as well as the E3 ubiquitin-protein ligase SMURF1 [47]. Whether any of these interactions might play a role in the inhibition of megakaryopoiesis remains to be determined.

A major finding of the present study was that anagrelide up-regulated the expression of genes that are under the transcriptional control of ATF4, TRIB3 being one of them. In other systems the gene expression changes orchestrated by ATF4 occur as a result of a selective increase of its translation [48]. The increase in ATF4 translation occurs as a consequence of the phosphorylation of eIF2α, an event which allows the scanning ribosomes to bypass an inhibitory upstream open reading frame (ORF) of the mRNA, and re-initiate translation from the downstream coding ORF [19]. In the present study initial experiments to detect ATF4 in immunoblots obtained from MK cultures treated with anagrelide were unsuccessful. ATF4 is an unstable protein readily degraded by the proteasome [49], raising the possibility that the failure to detect this protein was due to its rapid degradation. To test for this possibility, cultures were pre-treated with the proteasomal inhibitor MG132, and in this case a clear increase in ATF4 protein levels was detected after treatment with anagrelide. These results suggest that the drug causes a transient increase in ATF4 that is readily detected only when the proteasome is inhibited. Importantly, in our study anagrelide also caused a rapid increase in the phosphorylation of eIF2α. The phosphorylation state and activity of this protein are known to be negatively regulated by the phosphoprotein phosphatase PP1 [50]. A compound called salubrinal has been shown to inhibit the ability of PP1 to dephosphorylate eIF2α, thus diminishing the negative effect exerted by this mechanism [20]. Accordingly, we found that salubrinal increased eIF2α phosphorylation and mimicked the anti-MK activity of anagrelide. Taken together our findings indicate that anagrelide acts upstream of eIF2α on a pathway that leads to its activation.

The phosphorylation of eIF2α constitutes the point of convergence of a number of upstream stress signalling protein kinases. Concomitantly with the increase in ATF4 protein levels this
phosphorylation also leads to a transient attenuation of global protein synthesis in an attempt to restore cellular homeostasis [48]. Because of the integrative nature of this pathway, its downstream consequences were termed the “integrated stress response” (ISR) [51]. The ISR is also activated by diverse chemical entities, including cannabinoids [52], certain phenylmaleimides [53] and the plant alkaloid derivative halofuginone [54]. Interestingly, the latter is known for its selective inhibition of helper T cell differentiation [54]. Thus, like the effects of these substances, our findings raise the possibility that anagrelide inhibits MK differentiation by activating an arm of the ISR. In this respect, bioinformatic and literature searches currently do not point to an obvious pathway linking individual downstream mediators of the ISR with specific MK regulators. This might indicate that the previously reported reduction in the expression of MK transcription factors [10] reflects a partial blockage of MK differentiation imposed by anagrelide through the more general inhibitory effects of the ISR.

Our previous studies have underscored the selectivity of anagrelide for the MK lineage [9;10;21]. A possibility to explain this behaviour of the drug is that its primary target is a MK-specific factor which is induced exclusively as part of this lineage differentiation programme. The finding that upon a short exposure to anagrelide the magnitude of the increase in the expression of ATF4-regulated genes as well as the changes in the expression of other key transcription factors, escalate when the drug is added after the process of differentiation has already started, is entirely consistent with this possibility. These findings are also in keeping with our previous report showing that virtually no transcriptional response to anagrelide is observed within the first two days of continuous exposure to the drug [10].

In summary in this work we have demonstrated that anagrelide induces a rapid phosphorylation of eIF2α and an increase in ATF4 downstream gene expression. Together, these two events constitute the central signaling axis of the ISR. In addition, we have shown that salubrinal, a known positive modulator of the ISR, mimics the anti-megakaryopoietic action of anagrelide, providing further independent evidence for the involvement of this
pathway in the activity of the drug. A proposed model for the mechanism of action of anagrelide is shown in Fig. 6. In this model we hypothesise that anagrelide inhibits MK differentiation in the process of eliciting a cellular stress response. Whether inhibition of the MK gene expression programme results from the concerted effect of a reduction in global protein translation and/or from yet to be discovered targeted interactions of ATF4 and/or ATF4-regulated genes with the MK transcriptional machinery, is currently unknown. The present study also emphasises the value of anagrelide as an experimental tool to uncover potential new modulators of MK differentiation and to dissect the mechanisms that control this process.

Addendum

M. Ahluwalia and L. Butcher designed and performed experiments and analysed the data; H Donovan, P.M. Jones and C. Killick-Cole performed experiments and analysed the data; J.D. Erusalimsky conceived and supervised the study, analysed the data and wrote the paper. All the authors interpreted the data and revised the manuscript for content and accuracy.

Acknowledgements

We thank Dr Richard Franklin, Dr Stephen Damment and Dr Paul Wade for their valuable advice at different stages of this work. We are particularly indebted to Dr Mike Hubank and Ms Nipurna Jina from the Microarray Facility of the Institute of Child Health, at University College London for their assistance in running the microarrays and in the initial analysis of the results. This work was supported in part by research funding from Shire UK to JDE.

Conflict of interest disclosure

JDE is a co-inventor in a patent application (number US2011011966A1, WO2011044162) owned jointly by Cardiff Metropolitan University and Shire Pharmaceuticals that relate to the use of TRIB-3 for the screening of anti-megakaryocytic agents. MA is a co-inventor in the
said patent. This patent complies with Patent Policies implemented by Cardiff Metropolitan University. All other authors declare no competing financial interests.

References


Table 1: Genes that regulate transcription modulated by anagrelide

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<td>JUN</td>
<td>jun oncogene</td>
<td>2.0</td>
</tr>
</tbody>
</table>

List of differentially expressed genes identified by DAVID-assisted functional annotation clustering of GO terms related to transcription and then curated using GeneCards and PubMed resources. Transcripts are ranked based on the fold change in expression between vehicle and 0.3 µM anagrelide-treated cells as determined by microarray analysis.
Table 2: Genes under the transcriptional control of ATF4 up-regulated by anagrelide

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Gene Name</th>
<th>Fold change</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCL2</td>
<td>chemokine (C-C motif) ligand 2</td>
<td>7.5</td>
</tr>
<tr>
<td>IL8</td>
<td>interleukin 8</td>
<td>5.1</td>
</tr>
<tr>
<td>TRIB3</td>
<td>tribbles homolog 3 (Drosophila)</td>
<td>4.2</td>
</tr>
<tr>
<td>ATF3</td>
<td>activating transcription factor 3</td>
<td>2.8</td>
</tr>
<tr>
<td>ASNS</td>
<td>asparagine synthetase</td>
<td>2.7</td>
</tr>
<tr>
<td>DDIT3</td>
<td>DNA damage-inducible transcript 3</td>
<td>2.7</td>
</tr>
</tbody>
</table>

Transcripts are ranked based on the fold change in expression between vehicle and 0.3 µM anagrelide-treated cells as determined by microarray analysis.
Figure Legends

**Fig. 1. Functional annotation analysis of genes affected by anagrelide.** Functional enrichment analysis of GO terms was carried out using DAVID tools. Lists of up- and down-regulated genes were analysed separately. Fold enrichment is depicted by the bar length. The number of differentially expressed genes in each functional category and the FDR-adjusted \( p \)-values representing the significance of the enrichment are shown next to the corresponding bars. Only annotations with a significant FDR-adjusted \( p \)-value of \( \leq 0.05 \) are shown. The key represents the three domains covered by GO.

**Fig. 2. Comparison between the effects of anagrelide and cilostamide on the expression of selected genes.** Cells were cultured for four days under MK differentiation conditions in the presence of the indicated drugs (0.3 µM) or an equivalent amount of vehicle. mRNA levels for the indicated target genes were determined by Q-PCR and fold changes are expressed relative to the values in vehicle-treated cells (set to 1 and represented by the broken lines). Negative values refer to down-regulation. Data are presented as mean ± SD of replicate cultures from 4 different donors (n=8). ** \( p < 0.01 \) and *** \( p < 0.001 \) by ANOVA with Dunnet’s post-hoc test against vehicle-treated cells.

**Fig. 3. Effect of a short exposure to anagrelide on the induction of ATF4, TRIB3, DDIT3 and ATF3 mRNA expression.** Cells were cultured under MK differentiation conditions for the indicated days. Anagrelide (1 µM) or an equivalent amount of vehicle were added 24 h prior to cell harvesting. Relative gene expression levels were determined by Q-PCR and are expressed relative to the respective transcript levels at the initiation of the culture period. Results are representative of two independent experiments. Error bars denote SD of technical replicates (n=3).

**Fig. 4. Anagrelide increases eIF2α phosphorylation and ATF4 protein expression in differentiating MKs.** (A) Four day MK cultures were treated for the indicated length of time
with 1.0 µM anagrelide or (B) for 2 hours with or without anagrelide in the absence or
presence of 10 µM MG132 (added 15 min prior to the addition of anagrelide). Polypeptides
bands of interest were detected by immunoblotting and quantified by scanning densitometry.
Blots are representative of three experiments. The values under the images represent the
intensities of the P- eIF2α and ATF4 bands normalized to the corresponding total eIF2α or
actin bands and are expressed relative to the level of eIF2α or ATF4 measured in the
sample without anagrelide (A) or in the sample with MG132 alone (B). RI, relative intensities;
nd, not detected.

**Fig. 5: Salubrinal mimics the anti-megakaryocytic activity of anagrelide.** (A, B) Four
day MK cultures were treated with 75 µM salubrinal or 1 µM anagrelide for 2 h (A) or for 24 h
(B). In (A) the phosphorylation eIF2α was quantified as described in Fig. 4 and the values
under the image represent the intensities of the P-eIF2α bands expressed relative to the
intensity level measured in vehicle-treated cells. In (B) relative gene expression levels were
determined as described in Fig. 3. Error bars denote SD of technical replicates (n=6). (C)
Cells were grown for 7 days in the absence or presence of the indicated compounds. The
number of MKs is expressed as a percentage of the number of CD61 bright cells in vehicle-
treated cultures. Data are presented as mean ± SD of replicate cultures (n=3) *** p < 0.001
by ANOVA with Dunnet’s post-hoc test against vehicle-treated cells.

**Fig. 6: Proposed model for the mechanism of action of anagrelide.** The diagram shows
the site of action of salubrinal and the putative point of action of anagrelide.
Fig 1
Fig 2

254x190mm (96 x 96 DPI)
Fig 3

254x190mm (96 x 96 DPI)
Fig 4
Fig 5
Fig 6

254x190mm (96 x 96 DPI)