Bortezomib (PS-341; Velcade) is a reversible inhibitor of the 26S proteasome that produces significant clinical responses in both newly diagnosed and advanced multiple myeloma (MM). However, only 40% of patients respond to bortezomib as a single-agent therapy, and most of those will become resistant. Consequently, understanding the mechanism of action of bortezomib is of great interest. Although the inhibitory activity of bortezomib is clearly defined, the downstream mechanisms of cytotoxicity remain poorly understood and at times controversial.1

There is now emerging evidence that microRNAs (miRNAs) are important factors in anticancer drug activity and resistance.2 We therefore decided to investigate the potential involvement of miRNAs in the action of bortezomib on MM cells.

Three well-characterised MM cell lines (RPMI-8226, JJN3 and Thiel) were treated with bortezomib for 72 h (10 nM, LC laboratories, Woburn, MA, USA). Compared with diluent-only controls (0.008% dimethyl sulfoxide), levels of apoptosis (Cell Death Detection ELISAplus kit; Roche Diagnostics, Lewes, UK) in controls (0.008% diluent) were decreased 10.5- (JJN-3), 33.3- (Thiel) and 8.4-fold (RPMI-8226) (data not shown).

Total RNA was extracted by Trizol (Invitrogen, Paisley, UK) from three biological triplicate experiments and the RNA used for whole transcriptome (Affymetrix U133plus2.0) and miRNome (Affymetrix Genechip miRNA v.1.0) microarray analyses (Affymetrix, Santa Clara, CA, USA). Differences in cell line identity followed by bortezomib treatment were discriminatory features for both mRNA and miRNA expression levels as demonstrated by unsupervised cluster analyses (Supplementary Figure S1). Each treated sample was normalised against its respective paired non-treated sample, and probes (mRNA or miRNA) filtered for a median ± fold-change of > 2 between triplicates. This resulted in 1516 and 4136 genes that were up- and downregulated, respectively, in JJN3 cells in response to bortezomib treatment; 824 and 1864 genes in RPMI-8226 cells; and 1292 and 5183 genes in Thiel cells (Supplementary Figure S2). There were 228 and 883 genes commonly upregulated and downregulated respectively in all three MM cell lines (Supplementary Figure S2). Those were 228 and 883 genes commonly upregulated and downregulated respectively in all three MM cell lines (Supplementary Figure S2). The association analysis revealed that members of the protein ubiquitination canonical pathway were significantly enriched in the genes that were differentially expressed in response to bortezomib in each of the three MM cell lines individually, as well as for those genes that were commonly deregulated in all three lines (Supplementary Table S1). Interestingly, the most significant canonical pathway of differentially expressed genes in two of the cell lines (Thiel and JJN3) was the ‘role of BRCA1 in DNA damage response’; recently it has been suggested that bortezomib induces ‘BRCAness’ in MM cells.3

Many of the most deregulated genes that were common to all three cell lines (Supplementary Tables S2 and S3) had previously been associated with bortezomib treatment including upregulation of heat-shock proteins (HSP6, HSPA1, AP-1 complex (Jun), cell stress markers (ATF3),6 redox haemostasis genes (HMOX1, DDIT3),5 and the antiapoptotic protein BAG3,3 and downregulation of survivin (BIRC5), topoisomerase and MYB- oncogene.7

As with the gene expression data the miRNA microarray data were normalised between paired samples. This resulted in 37 and 75 miRNAs that were up- and downregulated, respectively, in JJN3 cells (median fold-change > 2); 23 and 26 miRNAs in RPMI-8226 cells; and 30 and 98 miRNAs in Thiel cells (Supplementary Figure S3). Nine and 14 miRNAs were commonly upregulated and downregulated, respectively, in all three MM cell lines (Supplementary Figure S3 and Supplementary Table S4). Four of these miRNAs (miR-92a-5p, let-7f, miR-27a-5p and miR-188 (Xp11.23)) were validated by qualitative reverse transcriptase-PCR (Supplementary Figure S4). Interestingly, the most downregulated miRNA was miR-92a-5p, which along with miR-18a (also down-regulated), are encoded by the miR-17~92 cluster whose overexpression has been linked to tumorigenesis and poor prognosis in MM.8 Let-7f has been demonstrated to promote angiogenesis by targeting antiangiogenic genes.9 Administration of anti-Let-7f was found to increase apoptosis and decrease cell proliferation levels in MM cell lines and to significantly reduce size of tumours in an MM xenotransplant model.10 Four of the fourteen (29%) commonly downregulated miRNAs are encoded within a single cluster (Xp11.23) that contains a further four members, also downregulated in response to treatment (data not shown). The Xp11.23 cluster is encoded within intron 3 of the CLCN5 gene. Intriguingly, mutations in CLCN5 (including mutations involving intron 3) are frequently found in patients with Dent’s disease, a rare renal tubular disorder characterised by progressive renal failure, that share many clinical manifestations with MM including presentation of Fanconi syndrome.11

Recently cyclin-depedent kinase 5 (CDK5) was identified as being a major modulator of bortezomib sensitivity in both MM cell lines and patient tumour cells.12 We noted that miR-27a-5p, one of the miRNAs identified as being downregulated by all three MM cell lines in response to bortezomib treatment (Supplementary Table S4 and Supplementary Figure S4), was predicted to target CDK5 (Figure 1a). This putative interaction was tested by cloning the 3′-untranslated region sequence of CDK5 into a luciferase reporter vector. Transfection of miR-27a-5p significantly reduced luciferase output compared with a scrambled sequence (Figure 1b). Furthermore, we found that endogenous levels of CDK5 mRNA decreased significantly in MM cells transfected with miR-27a-5p (Figure 1c), but did not find a concordant change in CDK5 protein levels (data not shown). JJN3 cells transfected with miR-27a-5p appeared to have increased sensitivity to bortezomib activity as demonstrated by an increase in apoptosis levels (Figure 1d), although little or no difference was detected in proliferation levels. These findings are consistent with previous observations that downregulation of CD5 (via siRNAs) increased the sensitivity of MM cell lines (and patient tumour cells) to bortezomib treatment.12 Consequently, it is tempting to suggest that miR-27a levels could be associated with the bortezomib-refractory status of MM patients; this is an area we...
are currently pursuing. Consistent with this hypothesis it has been observed that **miR-27a** levels are downregulated in bortezomib-resistant MM cell lines,\(^{13}\) and more generally downregulation of **miR-27a** has been associated with chemotherapy resistance.\(^ {14}\)

In order to ascertain whether deregulation of miRNAs by bortezomib could explain the pattern of differential gene expression observed, we used the TargetScan predictive algorithm\(^ {15}\) (as implemented in Ingenuity Pathway Analysis software suite (Ingenuity, Redwood City, CA, USA)) to identify putative target genes that were also deregulated >2-fold in the opposite sense to their respective miRNA regulator. Of the Affymetrix miRNA v.1.0 human probe set \((n = 1101)\), 393 miRNAs are included in the TargetsCan database, along with 16,245 genes of the Affymetrix U133plus2.0 probe set \((n = 42661)\). In JJN3 cells, 96% \((67/70\text{ deregulated miRNAs in TargetsCan})\) of miRNAs were predicted to target 1 or more of 2558 genes \((51\%\text{ of 5010 deregulated genes in database})\); RPMI-8226 cells, 100% \((32/32\text{ deregulated miRNAs in TargetsCan})\) of miRNAs were predicted to target 1 or more of 2447 \((42\%\text{ of 5764 deregulated genes in database})\). Of the 12 miRNAs in the TargetsCan database that were commonly differentially expressed by all three cell lines in response to bortezomib treatment, all were predicted to target 1 or more of 302 genes \((30\%\text{ of 1000 deregulated genes})\).

Surprisingly, ontogeny analysis of these potentially miRNA-regulated genes once again showed a significant enrichment for genes that form part of the protein ubiquitination pathway (Supplementary Table S5). These results suggest that in addition to targeting the proteasome directly, bortezomib alters the expression of specific miRNAs that target downstream...
components of the protein ubiquitination pathway presumably acting in a synergistic fashion. How the miRNAs are themselves deregulated by bortezomib remains unclear, but nevertheless represents a hitherto unknown function for miRNAs and provides additional information, and targets for improving the efficacy of this promising treatment for MM patients.

CONFLICT OF INTEREST
The authors declare no conflict of interest.

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