Gene expression-based prediction of myeloma cell sensitivity to histone deacetylase inhibitors

J Moreaux1,2, T Reme1,2, W Leonard2, J-L Veyrune1,2, G Requirand1, H Goldschmidt3,4, D Hose3 and B Klein*,1,2,5

1CHU Montpellier, Institute of Research in Biotherapy, Montpellier, France; 2INSERM-UM1 U1040, Institute for Research in Biotherapy, CHU Montpellier, Av Augustin Fliche, Montpellier 34197, France; 3Medizinische Klinik und Poliklinik V, Universitätsklinikum Heidelberg, Heidelberg, Germany; 4Nationales Centrum für Tumorerkrankungen, Heidelberg, Germany and 5Université Montpellier1, UFR Médecine, Montpellier, France

Background: Multiple myeloma (MM) is still a fatal plasma cell cancer. Novel compounds are currently clinically tested as a single agent in relapsing patients, but in best cases with partial response of a fraction of patients, emphasising the need to design tools predicting drug efficacy. Histone deacetylase inhibitors (HDACi) are anticancer agents targeting epigenetic regulation of gene expression and are in clinical development in MM.

Methods: To create a score predicting HDACi efficacy, five MM cell lines were treated with trichostatin A (TSA) and gene expression profiles were determined.

Results: The expression of 95 genes was found to be upregulated by TSA, using paired supervised analysis with Significance Analysis of Microarrays software. Thirty-seven of these 95 genes had prognostic value for overall survival in a cohort of 206 newly diagnosed MM patients and their prognostic information was summed up in a histone acetylation score (HA Score); patients with the highest HA Score had the shorter overall survival. It is worth noting that MM cell lines or patients’ primary MM cells with a high HA Score had a significant higher sensitivity to TSA, valproic acid, panobinostat or vorinostat.

Conclusion: In conclusion, the HA Score allows identification of MM patients with poor survival, who could benefit from HDACi treatment.
in the cytoplasm only. Class III HDACs include the sirtuin family, which does not act primarily on histones, and class IV includes HDAC11 (Lane and Chabner, 2009; Neri et al., 2012). Histone deacetylase inhibitors (HDACi) are now being used in the treatment of some hematologic malignancies (Kelly et al., 2010). Histone deacetylase inhibitors are classified into four classes according to their chemical structure: aliphatic acids (valproic acid (VPA) and sodium phenylbutyrate), hydroxamates (panobinostat, trichostatin A (TSA), vorinostat, belinostat (PXD101), NVP-LAQ824 and givinostat (ITF2357)), cyclic peptide (romidepsin (depsipeptide)) and benzamides (MS-275, MGCD0103) (Neri et al., 2012). Histone deacetylase inhibitors include inhibitors specific to class I HDACs (MGCD0103, romidepsin and MS-275) and pan-HDACi (TSA, panobinostat, vorinostat and belinostat) (Neri et al., 2012). Multiple myeloma (MM) is a plasma cell (PC) neoplasm characterised by the accumulation of malignant PCs, termed MM cells (MMCs), primarily within the bone marrow (BM). Despite the recent introduction of novel agents such as bortezomib or lenalidomide, MM remains an almost incurable disease (Moreau, 2012). Multiple myeloma arises through progressive accumulation of multiple genetic abnormalities that include primarily overexpression of a D-type cyclin gene, CCND1 (cyclin D1) in the case of t(11;14)(q13;32.2) translocation, CCND3 (cyclin D3) in the case of the rare t(6p23;14q32) translocation or CCND2 (cyclin D2) on the background of a t(14q32;16q23) translocation involving CMAF or t(4;14)(p16.3;q32.3) involving WHSC1/FGFR3. CCND genes are also overexpressed in hyperdiploid MM patients because of gene amplification or downregulation of miRNAs that target CCND genes (Bergsagel and Kuehl, 2005; Rio-Machin et al., 2013). Secondary genetic abnormalities include NRAS or KRAS mutations, TP53 monoallelic deletion and mutations, MYC alterations, mutations of genes coding for NF-κB pathway (Hideshima et al., 2004; Bergsagel and Kuehl, 2005; Morgan et al., 2012). Histone deacetylase inhibitors have already been evaluated in MM including TSA (Lavelle et al., 2001), vorinostat (Mitsiades et al., 2003, 2004), depsipeptide (Khan et al., 2004), KD5170 (Feng et al., 2008), NVP-LAQ824 (Catley et al., 2003), VPA (Kaiser et al., 2006; Neri et al., 2008) and panobinostat (Neri et al., 2012). Histone deacetylase inhibitors induce G1 cell cycle arrest in MMCs through dephosphorylation of retinoblastoma protein and increase expression of p53 and p21 (Lavelle et al., 2006; Neri et al., 2008) and panobinostat (Neri et al., 2012). Histone deacetylase inhibitors induce apoptosis by downregulation of Bcl-2 family members (Mitsiades et al., 2003; Khan et al., 2004) and overcome drug resistance mediated by the BM environment (Mitsiades et al., 2003). Furthermore, glucose-regulated protein 78 (GRP78) was recently identified as a novel non-histone target of HDACi (Rao et al., 2010; Kahali et al., 2011). Glucose-regulated protein 78 has a central role in the unfolded protein response (UPR). Glucose-regulated protein 78 acetylation following HDACi treatment was described to activate UPR and contributes to the antitumour activity of HDACi. Class I HDACs binding to GRP78, within the endoplasmic reticulum (ER), represent a novel mode of UPR regulation and an interesting mechanism of HDACi action (Kahali et al., 2012). The ER of normal PCs and MMCs is well developed to accommodate the production and secretion of large amounts of immunoglobulins. That is why association of HDACi with proteasome inhibitors could be promising in MM treatment (Hideshima and Anderson, 2013). When used as a single agent in patients with relapsing/refractory MM, HDACi have shown modest antitumour activity (Richardson et al., 2008; Niesvizky et al., 2011). In combination with other anti-MM treatments, HDACi can induce durable antitumour responses (Badros et al., 2009; Harrison et al., 2011).

To improve the clinical testing of the efficacy of novel agents, a major stake is identify patients who could benefit from treatment by finding biomarkers predictive for sensitivity of MMCs to HDACi. We recently reported the development of a gene expression-based risk score predicting the sensitivity of MMCs to DNA methylation inhibitors (Moreaux et al., 2012). In this study, we used the same strategy to build a histone acetylation (HA) score, based on genes whose expression is deregulated by HDACi in MMCs. Histone acetylation score score makes it possible to identify a subgroup of 42% of patients with short overall survival (OS), whose MMCs are highly sensitive to HDAC inhibition.

Human myeloma cell lines. Human myeloma cell lines (HMCLs, N = 40) were obtained as described previously (Zhang et al., 1994; Rebouroussou et al., 1998; Tarte et al., 1999; Gu et al., 2000; Moreaux et al., 2011). Human myeloma cell lines’ phenotypic and molecular characteristics have been described previously (Moreaux et al., 2011). Human myeloma cell lines’ microarray data have been deposited in the ArrayExpress public database (accession numbers: E-TABM-937 and E-TABM-1088).

Primary MMCs and gene expression profiling. Patients presenting with previously untreated MM (N = 206) or monoclonal gammapathy of undetermined significance (N = 5) at the university hospitals of Heidelberg and Montpellier as well as seven healthy donors have been included in the study with the approval of the ethics committee of Montpellier and Heidelberg after obtaining written informed consent in accordance with the Declaration of Helsinki. Clinical parameters and treatment regimens of the MM patients included in the Heidelberg–Montpellier cohort were described previously (Moreaux et al., 2012).

Normal BM PCs and myeloma cells were purified as published previously (Moreaux et al., 2012) and whole genome gene expression profiling assayed with Affymetrix U133 2.0 plus microarrays (Affymetrix, Santa Clara, CA, USA) (ArrayExpress accession number E-MTAB-372). Affymetrix data of an independent cohort of 345 MM patients from the University of Arkansas for Medical Sciences (UAMS, Little Rock, AR, USA) were also used (Gene Expression Omnibus accession number GSE26585, http://www.ncbi.nlm.nih.gov/geo/). T(4;14) translocation was evaluated using MMSSET spike expression (Kassambara et al., 2012b) and del17p13 surrogated by TP53 probe set signal (Xiong et al., 2008) for UAMS-TT2 patients.

Change in gene expression profile of myeloma cell lines by HDACi. Five HMCLs (XG-5, XG-6, XG-7, XG-20 and LP1) were treated without or with 0.33 μmol l−1 TSA (Sigma, St Louis, MO, USA) for 24 h in RPMI 1640, 10% fetal bovine serum supplemented with IL-6 for IL-6-dependent HMCls (Moreaux et al., 2011, 2012). Whole genome gene expression profiling was assayed with Affymetrix U133 2.0 plus microarrays (Affymetrix).

Sensitivity of myeloma cell lines and primary myeloma cells to HDACi. Human myeloma cell lines were cultured with graded TSA, VPA (Sigma), vorinostat (SAHA) (Sigma) or panobinostat (LBH-589) (Sigma) concentrations. Human myeloma cell lines’ cell growth was quantified with a Cell Titer Glo Luminescent assay (Promega, Madison, WI, USA) as described (Moreaux et al., 2012). The half inhibitory concentration (IC50) was determined using GraphPad Prism (http://www.graphpad.com/scientific-software/prism/).

Primary myeloma cells of 13 patients were cultured with or without graded concentrations of TSA and MMC cytotoxicity was evaluated using anti-CD138-PE mAb (Immunotech, Marseille, France) as described (Mahtouk et al., 2004; Moreaux et al., 2012).

Bioinformatics and statistics. Gene expression data were analysed using SAM (Significance Analysis of Microarrays)
RESULTS

Identification of prognostic genes whose expression is upregulated by TSA treatment of MMCs. Genes upregulated by TSA treatment of MMCS were identified by treating five HMCLs with 0.33 μM TSA for 24 h, a concentration-inducing histone acetylation in mammalian cells, in particular in myeloma cells in vitro (Yoshida et al, 1990; Heller et al, 2008). In addition, this TSA treatment did not affect myeloma cell viability (Supplementary Table S1) (Heller et al, 2008). Using SAM supervised paired analysis, expression of 95 genes was found significantly upregulated by TSA treatment (FDR <5%; Supplementary Table S2). Trichostatin A-regulated genes are significantly enriched in genes related to ‘Immunological disease and Inflammatory disease’ pathway (P<0.05; Ingenuity pathway analysis, data not shown). It is worth noting that TSA-deregulated genes were also found to be upregulated by Panobinostat treatment in MMC (Supplementary Figure S1). We next investigated associations of TSA-deregulated genes with OS using Maxstat R function. The aim was to identify genes regulated by HDAC that have potentially important disease-modulating functions. The results of our analysis were corrected for multiple testing using the Benjamini–Hochberg algorithm. Investigating the expression of these 95 TSA-regulated genes in primary MMcs of a cohort of 206 newly diagnosed patients (HM cohort), 16 genes had a bad prognostic value and 21 a good one (Table 1). The prognostic information of HDACi-regulated genes was gathered within an HA Score as indicated in Materials and Methods. The value of HA Score in normal, premalignant or malignant PCs is displayed in Figure 1. Cells from MGUS patients had a significantly higher HA Score than normal BMPCs (P<0.001), MMcs of patients had a significantly higher HA Score than normal BMPCs or PCs from MGUS patients (P<0.001), and HMCLs had the highest score (P<0.001) (Figure 1). Figure 2B shows the contribution of the 16 bad prognostic and the 21 good prognostic genes for HA score. Bad prognostic genes are highly expressed in patients with high HA score and the reverse for good prognostic genes. Investigating the HA Score in the eight groups of the molecular classification of MM (Zhan et al, 2006), HA Score was significantly higher in the proliferation subgroup (P<0.001) associated with a poor prognosis and significantly lower in the CD2 subgroup (P<0.001) (Zhan et al, 2006) (Figure 3). Among the 37 genes of the HA Score, 7 code for proteins that have been described as lysine acetylation target proteins and 18 have been identified as HDACi targets (Choudhary et al, 2009; Niesen and Blanck, 2009; Bantscheff et al, 2011; Iwahashi et al, 2011) (Supplementary Tables S5 and S6). Gene expression profiles of HA Score genes in purified MMC and normal BM subpopulations are listed in Supplementary Figure S2. Supplementary Figure S2 shows a highly variable expression of each of the 37 genes in primary MMcs of the patients, indicating they all contribute to unravel disease heterogeneity.

Prognostic value of HA score in two independent cohorts of patients. When used as a continuous variable, HA Score had prognostic value (P<10−4, results not shown). Using Maxstat R function, a maximum difference in OS was obtained with an HA Score = −11.3, splitting patients into a high-risk group of 42.7% patients (HA Score > −11.3) with a 43.5 months median OS and a low-risk group of 57.3% patients (HA Score ≤ −11.3) with not reached median survival (Figure 2A). High-risk patients are characterised by a higher expression of the 16 bad prognostic genes and a lower expression of the 21 good ones used for HA Score building (Figure 2B). Using univariate Cox analysis, HA Score, UAMS-HRS, IFM-score and GPI had prognostic value as
well as t(4;14), del17p, albumin and ISS using the HM patient cohort (Supplementary Table S3). When these parameters were compared two by two, HA Score tested with β2m and t(4;14) remained significant. When tested together, HA Score, β2m, t(4;14) and GPI kept prognostic value. The HA Score, computed using HM cohort parameters, is also prognostic in an independent cohort of 345 patients from UAMS (UAMS-TT2 cohort). The median OS of patients with high HA Score was 71.4 months and not reached for patients with low HA Score (P < 0.0001) (Figure 2A). Using Cox univariate analysis, UAMS-HRS, IFM and GPI scores as well as t(4;14) and del17p had prognostic value. Serum concentrations of β2m or albumin are not publicly available for this cohort. When analysed two by two, HA Score remained significant compared with UAMS-HRS, IFM, GPI, t(4;14) and del17p in the UAMS-TT2 cohort (Supplementary Table S3). When these parameters were tested together, HA Score, UAMS-HRS, t(4;14) and del17p kept prognostic value in UAMS-TT2 cohort.

Histone acetylation score allows identification of HMCLs or patients' primary MMCs sensitive to TSA in vitro. We investigated whether HA Score could predict for the sensitivity of HMCLs to HDAC inhibitors. Ten out of 40 HMCLs (Moreaux et al, 2011) with the highest or lowest HA Score were selected to assay for TSA sensitivity. The five HMCLs with the highest HA Score displayed

Figure 1. Histone acetylation score in normal and malignant PCs. Histone acetylation score in normal BMPCs (N = 7), in premalignant PCs of patients with monoclonal gammopathy of undetermined significance (MGUS, N = 5), in MM cells of patients with intramedullary MM (N = 206) and in HMCLs (N = 40). ** indicate that the score value is significantly different with a P-value < 0.01.

Figure 2. Prognostic value of HA Score in MM. (A) Patients of the HM cohort were ranked according to increased HA Score and a maximum difference in OS was obtained with HA Score = −11.3 splitting patients into high-risk (42.7%) and low-risk (57.3%) groups. Histone acetylation score also had a prognostic value of an independent cohort of 345 patients from University of Arkansas for Medical Science (UAMS) treated with TT2 therapy (UAMS-TT2 cohort). The parameters to compute the HA Score of patients of UAMS-TT2 cohort and the proportions delineating the two prognostic groups were those defined with HM cohort. (B) Clustergram of HA Score genes ordered from best to worst prognosis. The level of the probe set signal is displayed from low (deep blue) to high (deep red) expression. MM patients (N = 206) were ordered by increasing GE-based risk score.
a significant ($P = 0.0004$) fivefold higher sensitivity to TSA (median $IC_{50} = 10.97 \text{nM}$; range: $6.32–17.4 \text{nM}$) than the five HMCLs with low HA Score (median $IC_{50} = 52.33 \text{nM}$; range: $29.49–57.74 \text{nM}$) (Figure 4). No difference in recurrent genetic abnormalities was found between HMCLs with the highest or lowest HA Score (Table 2). Histone acetylation score could also predict for sensitivity of patients’ primary MMCs, cocultured with BM environment, to TSA. The TSA concentrations used to treat primary MM samples were chosen to cover the range of TSA concentrations yielding 50% inhibition of the growth of the 10 HMCLs displaying high and low HA Scores (Figure 4).

Primary MMCs of eight patients with an HA Score above the Maxstat cutoff ($> – 11.3$; Figures 1 and 2) exhibited significant ($P < 0.05$) 2.4-fold higher TSA sensitivity than MMCs of five patients with HA Score $\leq – 11.3$ (Figure 5).

Histone acetylation score is predictive for sensitivity of human myeloma cells to other clinical grade HDACi in vitro. We sought to determine whether HA Score could predict for the sensitivity of myeloma cells to other clinical grade HDAC inhibitors (Neri et al., 2012). The five HMCLs with the highest HA Score exhibited a significant higher sensitivity to panobinostat, VPA or vorinostat (median $IC_{50} = 1.16 \text{nM}$, $0.28 \mu M$ and $528 \text{nM}$, respectively) than the five HMCLs with lowest HA Score ($P = 0.007$, $P = 0.009$ and $P = 0.02$; median $IC_{50} = 3.16 \text{nM}$, $0.43 \mu M$ and $897 \text{nM}$, respectively) (Figure 6A–C).
In this study, we have identified a gene expression-based HA Score, which is predictive for patients’ survival and for the *in vitro* sensitivity of HMCLs or patients’ primary myeloma cells to the pan-HDACi TSA and also to other three clinical-grade HDACi. Histone deacetylase inhibitors have been investigated for treating patients with MM, either as a single agent (Richardson *et al*, 2008; Niesvizky *et al*, 2011; Neri *et al*, 2012) or in combination with current drugs used in MM, such as dexamethasone, lenalidomide or bortezomib (Badros *et al*, 2009; Harrison *et al*, 2011; Neri *et al*, 2012). Panobinostat, used as a single agent, demonstrated limited activity in patients with MM, refractory to at least two lines of treatment (Wolf *et al*, 2012). In association with melphalan, panobinostat yielded a 33% overall response rate in a phase I study, including 12 patients with relapsed/refractory MM (Offidani *et al*, 2012). Association of panobinostat and bortezomib could be promising as proteasome inhibition affects the UPR pathway, leading to increased HDAC-mediated aggregates formation (Hideshima *et al*, 2011). Phase IB and II studies have shown that association of panobinostat to bortezomib and dexamethasone could yield to objective response in relapsing patients refractory to bortezomib therapy (Siegel *et al*, 2008; San-Miguel *et al*, 2011; Richardson *et al*, 2011). Given the encouraging *in vitro* and *in vivo* data, association of panobinostat, bortezomib and dexamethasone is now being evaluated in a large phase III randomised trial (San-Miguel *et al*, 2011). Combination of vorinostat and bortezomib was also investigated in a phase II trial, including patients with MM refractory or ineligible to bortezomib or IMiD therapy (Siegel *et al*, 2011) and showed a 17% overall response rate and 6 months median response duration. These data also prompt investigating the interest of a combination of vorinostat and bortezomib in phase III trial, including 637 patients with relapsed/refractory MM (Dimopoulos *et al*, 2011). Final results of the trial remain awaited, but interim results did not demonstrate a marked therapeutic benefit of vorinostat (Dimopoulos *et al*, 2011). These trials suggest that HDACi could have some benefit for MM. However, their efficacy could be underestimated because it was limited to a subgroup of patients. The current HA Score could be promising to investigate whether the best response to HDACi is found in patients with MMCs displaying a high HA Score.

Only upregulated genes were identified in the HMCLs treated with TSA compared with untreated HMCLs. This may be explained by the 24-h treatment of cell lines with HDACi according to usual protocols. This short treatment makes it possible to release the transcriptional suppressor activity of HDAC yielding to overexpressed genes. However, it is likely too short to
get an indirect repression of genes because of the HDACi-induced overexpression of an inhibitor of these genes. Among the current 95 genes deregulated by TSA treatment in HMCLs, 24 genes were commonly identified by Heller et al. (2008) (Supplementary Table S4). Seven out of 37 HA Score genes code for proteins that have been described as lysine acetylation target proteins and 18 out of 37 HA Score genes have been identified as HDACi targets (Choudhary et al., 2009; Niesen and Blanck, 2009; Bantscheff et al., 2011; Iwahashi et al., 2011) (Supplementary Tables S5 and S6).

Why HA Score which is built using 37 HDACi-upregulated and prognostic genes could predict for the sensitivity of MMCs to HDACi? Patients with high HA score, and poor survival, are characterised by a higher expression of the 16 bad prognostic genes and a lower expression of the 21 good ones in MMCs (Figure 2B). Thus, one can speculate that primary MMCs of patients with high HA score have a high tumour metabolism and growth, which can be efficiently targeted by the upregulation of gene products encoded by genes upregulated by HDACi, in particular the 21 good prognostic genes. At the opposite, MMCs of patients with a low HA score could be in a more quiescent state and less sensitive to HDACi. However, a full understanding of the reason why HA score could predict for HDACi sensitivity will be provided by an extensive study of the function of the products encoded by HDACi-regulated genes in promoting MMC survival and/or proliferation. Some genes could highlight pathways involved in MM and we comment below the putative roles of NFKBIZ (nuclear factor of kappa light polypeptide gene enhancer in B-cell inhibitor zeta), BASPI (brain acid-soluble protein 1) or QKI (Quaking), whose expression in MMCs is induced by HDACi treatment and is associated with good prognosis. NFKBIZ is a member of IκB family.

Figure 6. Histone acetylation score predicts for sensitivity of HMCLs to panobinostat, valproic acid (VPA) or SAHA HDACi. Human myeloma cell lines with a high HA Score (N = 5) exhibit significant higher sensitivity to panobinostat, VPA or SAHA compared with HMCLs with a low HA Score (N = 5). Human myeloma cell lines were cultured for 4 days in 96-well flat-bottom microtitre plates in RPMI 1640 medium, 10% fetal calf serum, 2 ng/ml IL-6 culture medium (control) and graded concentration of panobinostat (A), VPA (B) or SAHA (vorinostat) (C). Data are mean values ± standard deviation (s.d.) of five experiments determined on sextuplet culture wells.
(Totzke et al, 2006), localised in the nucleus, where it interacts with and regulates nuclear NF-κB activity. Suppression of endogenous NFKBIZ renders cells more resistant to apoptosis, whereas its overexpression induces cell death (Yamazaki et al, 2001; Totzke et al, 2006). This is of interest because NF-κB pathway is frequently activated through various gene mutations in MM (Annunziata et al, 2007; Keats et al, 2007). More recently, it was demonstrated that NFKBIZ inhibits the transcriptional activity of STAT3, leading to cell growth inhibition and apoptosis induction mediated by the downregulation of a known STAT3 target, Mcl-1 (Wu et al, 2009). Mcl-1 is the major antiapoptotic protein for MMs, involved in IL-6-mediated survival of MMs (Derenne et al, 2002; Jourdan et al, 2003). Brain acid-soluble protein 1 is repressed in Myc-transformed cells, and conversely has a strong potential to inhibit cell transformation induced by Myc (Hartl et al, 2009). The inhibition of Myc-induced fibroblast cell transformation by BASP1 also prevents the transcriptional activation or repression of known Myc target genes. Brain acid-soluble protein 1 appears to be a potential tumour suppressor in cancer (Hartl et al, 2009). Myc protein is frequently highly expressed in primary MMs (Skopelitou et al, 1993) and a vicious circle involving IRF4 and Myc was identified yielding to deregulation of MMC growth (Shaffer et al, 2008). Histone deacetylase inhibitors could be useful to target NF-κB or Myc activation in MMs through the upregulation of NFKBIZ and BASP1 expression. The RNA-binding protein QKI belongs to the evolutionarily conserved signal-transduction and activator of RNA family. It has been demonstrated that overexpression of QKI induced the G1 cell cycle arrest in oligodendrogliocyte progenitor cells (Larocque et al, 2005). Furthermore, QKI inhibits colon cancer cell growth, acting as a tumour suppressor (Yang et al, 2010). It was demonstrated that QKI protein is directly transcribed by E2F1, which in turn negatively regulates the cell cycle by targeting multiple cell cycle regulators including p27, cyclin D1 and c-fos (Yang et al, 2011). These results demonstrated that a better understanding of the cellular response to epigenetic-targeted treatments will increase our knowledge of MM development and progression and will provide potential therapeutic advances. Epigenetic therapies could be combined with conventional therapies to develop personalised treatments in MM and render resistant tumours responsive to treatment. These advances may limit the side effects of treatment, improving compliance with dosing regimens and overall quality of life. Our methodology could be extended to other anti-MM treatments.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.
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