Published in final edited form as:

Xenotransplantation. 2013 November ; 20(6): . doi:10.1111/xen.12052.

Minimal Effect of Bortezomib in Reducing Anti-Pig Antibodies in HLA-Sensitized Patients: A Pilot Study

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Abstract

Background—Bortezomib, a proteasome inhibitor used to treat multiple myeloma, has been administered (+/- plasma exchange +/- intravenous immunoglobulin [IVIg]) in attempts to reduce antibodies against human leukocyte antigens (HLA) in sensitized patients undergoing organ transplantation. To our knowledge, bortezomib has not been investigated for its effect on natural anti-pig antibodies. If bortezomib could reduce the production of anti-pig antibodies, this would likely be beneficial to the outcome of pig organ grafts in primates.

Methods—Nine patients received bortezomib either to reduce anti-HLA antibody levels before organ allotransplantation or to treat antibody-mediated rejection. Patients at the Mayo Clinic (Group 1; n=4) received bortezomib alone, whereas at the UPMC (Group 2; n=5) this was combined with plasmaphereses +/- IVIg in some cases. Anti-pig IgM and IgG levels against wild-type (WT) and α 1,3-galactosyltransferase gene-knockout (GTKO) pig aortic endothelial cells (flow cytometry – relative MFI) and anti-Gal IgM and IgG (ELISA – OD_{480nm}) were measured pre- and post-bortezomib therapy.

Results—Mean anti-pig IgM levels were 11.2 (WT) and 1.9 (GTKO) pre-bortezomib and 9.4 (WT: P=0.02) and 1.7 (GTKO: P=0.33) post-bortezomib. Mean anti-pig IgG levels were 4.3 (WT) and 1.5 (GTKO) pre-bortezomib and 3.6 (WT: P=0.21) and 1.4 (GTKO: P=0.20) post-bortezomib. Mean anti-Gal IgM and IgG levels were 0.7 and 1.1, respectively, pre-treatment, and 0.6 (P=0.03) and 1.1 (NS), respectively, post-treatment. When the data were analyzed in Groups 1 and 2 separately, there were no significant differences between the pre- and post-bortezomib levels of anti-pig, anti-nonGal, or anti-Gal IgM or IgG.

Conclusion—From this limited study, we conclude that bortezomib might reduce anti-Gal IgM levels in primates, but, in this respect alone, is unlikely to have any significant effect on the outcome of GTKO pig organ transplantation.

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Disclosure of Conflict of Interest: David Ayares is an employee of Revivacor Inc. No other author has a conflict of interest

Introduction

Bortezomib (Velcade, Millenium Pharmaceuticals, Cambridge, MA) is a proteasome inhibitor that has been approved by the United States Food and Drug Administration (FDA) for the treatment of patients with multiple myeloma.¹⁻⁴ Proteasome inhibition results in apoptosis of plasma cells; its mechanisms of action have been reviewed.^{5,6} It has also been administered for certain other conditions.^{7,8}

In recent years, there has been considerable interest in the value of bortezomib in reducing the production and level of antibodies against human leukocyte antigens (HLA) in sensitized patients undergoing organ transplantation.^{5,9-38} It has been administered in addition to conventional biologic and/or pharmacologic immunosuppressive therapy, and also combined with courses of plasma exchange and/or intravenous immunoglobulin (IVIg). The results in allotransplantation models have been variable and difficult to interpret, partly because of the clinical circumstances (when some patients are treated before organ transplantation to reduce the level and anti-HLA antibodies, and others are treated post-transplantation because they are undergoing antibody-mediated rejection of an allograft) and partly because of the various other therapies with which it has been combined, e.g., increased conventional immunosuppressive therapy, such as anti-thymocyte globulin (ATG), and/or anti-CD20 monoclonal antibody (mAb), plasma exchange, and/or the administration of IVIg.

To our knowledge bortezomib has not been investigated for its effect on natural preformed anti-pig antibodies.

Although the availability of $\alpha 1,3$ -galactotransferase gene-knockout (GTKO) pigs has abrogated the effect of antibodies directed to galactose- $\alpha 1,3$ -galactose (Gal) antigens on xenograft survival, there remain other anti-pig antibodies, known as anti-nonGal antibodies, that are also detrimental to the survival of a pig xenograft.³⁹⁻⁴² The targets for these anti-nonGal antibodies remain largely unknown.⁴³ Although their effect is to some extent abrogated by expression of one or more human complement-regulatory proteins on the pig vascular endothelium⁴⁴⁻⁴⁷, this is not entirely protective, particularly in regard to vascular endothelial cell activation which may be an initiating factor in the development of coagulation dysregulation.⁴⁸⁻⁵⁵ This may take the form of the development of a thrombotic microangiopathy or a consumptive coagulopathy. If an agent was available that would prevent or restrict production of these anti-nonGal antibodies, it would likely become easier to maintain survival of a pig xenograft, and possibly prevent the development of coagulation disturbances. In addition to conventional immunosuppressive agents, various other agents have been studied in an effort to reduce the level of anti-pig or anti-nonGal antibodies without great success.⁵⁶⁻⁶¹ For example, although anti-CD20mAbs almost totally deplete B cells, they do not reduce the number of plasma cells, and therefore natural anti-pig antibody production continues unimpeded.⁵⁷

We have investigated a small number of patients who have received bortezomib either to reduce anti-HLA antibody levels before organ allotransplantation or in the treatment of antibody-mediated rejection following organ allotransplantation. Our findings from this limited initial study are that, although bortezomib was associated with a reduction in the levels of anti-wild-type (WT) pig IgM and anti-Gal IgM, it had no effect on the levels of anti-nonGal IgM or anti-WT pig, anti-nonGal, or anti-Gal IgG.

Methods

Clinical data

Nine patients received treatment with bortezomib by the i.v. route (Table 1). In 4 patients who were awaiting an organ transplant (at the Mayo Clinic; Group 1) no plasmaphereses were carried out, no IVIg was administered, and no patient received any additional immunosuppressive agents. Details of dosage and timing of bortezomib administration are indicated in Table 1.

In 5 patients who had undergone organ transplantation (at the University of Pittsburgh; Group 2) the course of bortezomib was combined with repeated plasmapheresis and/or IVIg in some cases as well as the administration of additional therapies in the form of ATG, anti-CD20mAb, methylprednisolone, and mycophenolate mofetil (Table 1). The number of doses of the drug administered, the total drug dose for each patient, and the duration of the course are listed in Table 1. There was no clear correlation between timing of bortezomib administration and the administration of other drugs or of plasmaphereses and/or IVIg infusion. Sera were not available to us to determine whether there was any depletion of anti-pig antibody immediately after each plasmapheresis.

Sources of human sera

After approval from the Mayo Clinic (Group 1, n=4) and University of Pittsburgh (Group 2, n=5) Institutional Review Boards, blood was drawn from the 9 patients. The period between the pre-bortezomib and post-bortezomib blood tests are indicated in Table 1. The post-treatment blood sample that was tested was drawn between 1 and 31 days after the last dose of bortezomib (Table 1).

Sera were prepared (heat-inactivated at 56°C for 30min) and stored at -80°C until use. The sera were monitored retrospectively for anti-pig and anti-nonGal antibody levels by flow cytometry, and for anti-Gal antibody levels by ELISA. Details of these patients, the course of bortezomib, and adjunctive therapy are summarized in Table 1. The number of patients and sera that could be tested was limited by the availability of sufficient stored sera. All assays were carried out by the same personnel in a single laboratory (STI).

Pig peripheral blood mononuclear cells

To detect the presence of anti-pig (anti-Gal+anti-nonGal) and anti-nonGal antibodies in the serum, peripheral blood mononuclear cells were isolated from wild-type (WT) and GTKO pigs (pPBMC; Revivicor, Blacksburg, VA), as previously described.⁴⁰ Two pigs (one WT and one GTKO) which were of nonA (O) blood type were used in all experiments to reduce variability.

Binding of human serum IgM and IgG to pig PBMCs (flow cytometry)

Binding of human xenoreactive antibodies (IgM and IgG) to pig cells (WT and GTKO) was measured by flow cytometry using relative mean fluorescence intensity (MFI), as previously described.⁴⁰

Measurement of anti-Gal IgM and IgG (ELISA)

Anti-Gal antibodies in serum samples were detected by an anti-Gal ELISA for both IgM and IgG, as previously described.⁴³

Statistical analysis

All results are expressed as mean $\pm$ SEM. A paired *t* test was used to compare the data of anti-pig and anti-Gal antibodies before and after bortezomib treatment using GraphPad Prism version 4 (Graphpad Software, San Diego, CA). A *P* value of <0.05 was considered to be statistically significant.

Results

Anti-pig and anti-nonGal IgM and IgG levels (flow cytometry)

Mean anti-pig IgM levels in the 9 patients (combined Groups 1+2) before the course of treatment were 11.2 (WT) and 1.9 (GTKO), respectively, and at the end of the treatment period were 9.4 (WT: *P*=0.02) and 1.7 (GTKO: *P*=0.32), respectively (Figure 1A). Mean anti-pig IgG levels before treatment were 4.3 (WT) and 1.5 (GTKO), respectively, and at the end of the treatment period were 3.6 (WT: *P*=0.21) and 1.4 (GTKO: *P*=0.20) (Figure 1B).

The data have been analyzed separately for each of the two centers that contributed sera to this study - the Mayo Clinic (Group 1) (Figure 2A) and the University of Pittsburgh (Group 2) (Figure 2B). When the data were analyzed separately, there were no significant differences between the pre- and post-bortezomib levels of anti-pig or anti-nonGal IgM or IgG (Table 2A and 2B).

Anti-Gal IgM and IgG levels (ELISA)

Mean anti-Gal IgM and IgG levels (OD_{480nm}) in the 9 patients (combined Groups 1+2) before the course of treatment were 0.7 $\pm$ 0.1 and 1.1 $\pm$ 0.1, respectively, and at the end of treatment period were 0.6 $\pm$ 0.1 (*P*=0.03) and 1.1 $\pm$ 0.28 (*P*=0.25), respectively (Figure 3A). When the data were analyzed separately for the two Groups, there were no significant differences between the pre- and post-bortezomib levels of anti-Gal IgM or IgG (Table 2 and Figure 3).

Discussion

In patients with multiple myeloma, bortezomib successfully inhibits proteasomes, resulting in apoptosis of plasma cells and prolongation of survival.¹⁻⁴ More recently, treatment with bortezomib has been advocated to reduce anti-HLA antibodies in patients with pre-existing sensitization to potential allografts or in those with an allograft undergoing antibody-mediated rejection.^{5,9-38} A course of therapy has often been combined with increased conventional immunosuppressive therapy, and/or a course (or courses) of plasmapheresis and/or IVIg administration. The results, however, have been variable, with some groups reporting significant reduction in antibodies, but others not. The exact contribution of bortezomib to the clinical outcome has often been difficult to determine as many of these patients have received multiple forms of therapy. Although treatment with bortezomib can be associated with moderate-to-severe side effects, these would not preclude its administration in xenotransplantation models if it could be demonstrated to be beneficial. Its potential value in xenotransplantation in reducing the production of anti-pig antibodies in primates might be immense, and is certainly an area that would seem worthy of exploration. It was with this aim that the present limited study was carried out.

We conclude that bortezomib in the regimens that were used in these patients had a modest effect in reducing natural IgM antibodies directed to WT pig cells and that these were exclusively directed to Gal antigens. However, the agent had no significant effect on the level of anti-nonGal IgM or on the levels of anti-WT pig, anti-nonGal, or anti-Gal IgG.

We would emphasize that the study is limited by the small number of patients tested, and is also possibly complicated by various factors such as the presence or absence of a kidney or intestinal allograft and the administration of biologic and pharmacologic immunosuppressive agents. The lack of multiple serum samples to test during the course of therapy, e.g., after each plasmapheresis, is also a limiting factor. Furthermore, on occasions the blood samples that were available to us for testing had not been drawn immediately before the course of bortezomib was initiated and/or were not drawn immediately after the course had been completed. However, in all but two cases the blood was tested within 14 days of the start of therapy, and in all but one case within 21 days of the termination of therapy. If plasma cell production of anti-pig antibody had been significantly suppressed by bortezomib, it would be expected that antibody levels would remain low for several weeks or months after cessation of therapy. From our limited observations, this was clearly not the case.

The heterogeneity of the therapy administered in the Group 2 patients is perhaps particularly problematic. Nevertheless, it would be anticipated that the other immunosuppressive agents administered concomitantly (as in Group 2) would enhance, rather than inhibit, the effect of bortezomib in reducing antibody, and therefore we do not believe this is a factor influencing the results significantly.

However, in some patients in Group 2, the effect of bortezomib +/- the other therapeutic measures taken could be at least in part affected by the administration of IVIg, as this would undoubtedly contain normal or high levels of anti-pig antibodies^{62,63}. In a study by Buhler et al⁶³, weekly treatment with IVIg (500mg/kg) significantly reduced anti-HLA panel-reactive antibody (PRA) levels in patients with a high PRA, but had no effect on anti-Gal antibody levels or serum cytotoxicity to pig aortic endothelial cells. However, in the present study, some patients did not receive IVIg, and yet bortezomib was still associated with a minimal effect.

Indeed, neither in Group 1 (in which no plasmapheresis or IVIg administration was carried out) nor in Group 2 (in which these additional therapies were carried out in some patients) was there any significant reduction in anti-pig, anti-nonGal, or anti-Gal IgM or IgG levels, although the reduction in anti-Gal IgM in Group 1 almost reached significance ($p=0.07$). When the data from Groups 1+2 were combined ($n=9$), however, significant reductions in anti-pig IgM and anti-Gal IgM (both $p<0.05$) were seen following the course of bortezomib. This suggests that, if larger numbers of patients had been studied, bortezomib therapy might have been found to result in a greater and more consistent reduction in antibody. It also indicates that it was primarily the level of anti-Gal IgM that was reduced; this is unlikely to be beneficial when GTKO pig organs are transplanted.

Both pre- and post-bortezomib anti-Gal IgM and IgG levels were significantly higher (both $p<0.05$) in the Group 1 than Group 2 patients, and this is difficult to explain. There may be some variation in expression of gastrointestinal microorganisms⁶⁴, though this seems unlikely. The fact that 4 of the 5 patients in Group 2 had undergone intestinal allotransplantation may be a factor as intestinal pathology may have resulted in a change in intestinal flora. However, it is most likely that the lower levels of antibody in Group 2 patients were associated with the fact that these patients had been receiving conventional immunosuppressive therapy for some time before bortezomib therapy was initiated, whereas those in Group 1 had not.

It is uncertain whether serum antibody levels are maintained by a stable or a constantly-replenishing plasma cell population. If plasma cells are continuously replenished from the memory B cell population, changes in antibody levels may not be observed if antibody half-

lives are long. The half-life of antibodies can indeed be long (weeks or months) and so plasma cell depletion may not be evident for a considerable period of time after bortezomib (or other) treatment. Furthermore, in the absence of pheresis, serum antibody levels, if not sampled at appropriate time-points, may not appear to diminish if the plasma cell depletion is less than modest. In the Group 1 patients, the period between pre-treatment and post-treatment sampling extended from 77 to 119 days, and in Group 2 from 26 to 318 days. It might have been expected that these periods were sufficient to detect some significant decrease in antibody levels in some cases.

We have not compared our data with the response to bortezomib of anti-HLA antibodies in the patients in the study. As the reported effect of bortezomib on the level of anti-HLA antibodies has been so variable, for the purposes of the present investigation we believe the effect on anti-HLA antibodies is irrelevant. We do not consider that any effect on anti-HLA antibodies can be used as a 'control' against which changes in natural anti-pig antibodies can be compared.

There are significant differences between anti-HLA antibodies and natural anti-pig antibodies. Anti-HLA antibodies are invariably elicited antibodies directed to protein antigens resulting from stimulation or activation of the adaptive immune response and develop in response to blood transfusions, pregnancy, or the presence of an organ or cell allograft. They are clearly T cell-dependent. In contrast, natural anti-pig antibodies are at least in part directed to carbohydrate antigens, develop during infancy, and are believed to be a response to colonization of the gastrointestinal tract by various viruses, microorganisms, and parasites⁶⁴. Their development has generally been believed to be associated with the innate immune response and to be T cell-independent⁶⁵, although data from studies in mice⁶⁶ and from infant baboons⁶⁷ suggest that development of these antibodies may be T cell-dependent, and therefore likely to be related to the adaptive immune response.

Of particular relevance to the present study are the findings of Boothpur et al, who reported that bortezomib therapy was unsuccessful in reducing the level of anti-A/B antibodies¹. This present report provides further evidence suggesting that this agent may not be particularly effective in suppressing production of natural anti-carbohydrate antibodies.

From the results of the present admittedly limited study, we would conclude that bortezomib will not be greatly beneficial in reducing anti-pig antibody levels in primates about to receive a pig xenograft, particularly if a GTKO pig organ is transplanted (which will almost certainly be the case) and therefore may not facilitate pig organ or cell xenotransplantation. However, the course of bortezomib given to the patients reported here was limited to a maximum of 119 days and it is possible that a prolonged course, possibly in association with an anti-B cell agent, might eventually reduce plasma cell production of these antibodies. On the basis of the information provided by the present small study, however, we would suggest that bortezomib may be of no or very limited benefit in GTKO pig organ xenotransplantation.

Acknowledgments

Hidetaka Hara MD, PhD is supported in part by NIH grant # 1RO3A1096296-01. Mohamed Ezzelarab is supported in part by the Joseph A. Patrick research Fellowship in Transplantation of the Thomas E. Starzl Transplantation Institute of the University of Pittsburgh. Studies on xenotransplantation at the Thomas E. Starzl Transplantation Institute are supported in part by CMRF (competitive medical research funding) from the University of Pittsburgh Medical Center, NIH grants #1U19AI090959-01, #U01A1066331, and #1PO1 HL107152, by an Ocular Tissue Engineering and Regenerative Ophthalmology (OTERO) Postdoctoral Fellowship, and by Sponsored Research Agreements between the University of Pittsburgh and Revivicor, Inc., Blacksburg, VA.

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Figure 1.

Serum anti-pig antibody levels by flow cytometry pre- and post-bortezomib treatment in the combined Groups 1 and 2.

(A) Although a significant difference ($p < 0.02$) was seen in anti-WT IgM between pre- and post-bortezomib, there were no significant differences in anti-nonGal IgM. (B) There were no significant differences in anti-WT IgG or anti-nonGal IgG between pre- and post-bortezomib. All results ($n=9$) are expressed as mean $\pm$ SEM. A paired t test was used to compare the data pre- and post-bortezomib.

There were no significant differences in serum antibody levels for anti-WT IgM, anti-WT IgG, anti-GTKO IgM, and anti-GTKO IgG between pre- and post-bortezomib treatment (Line graph [A] and bar graph (mean $\pm$ SEM) [B]). A paired t test was used to compare the data pre- and post-bortezomib treatment.

Figure 2.

Serum anti-pig antibody levels by flow cytometry pre- and post-bortezomib treatment in (A) Group 1 (n=4) and (B) Group 2 (n=5).

There were no significant differences in anti-WT IgM or IgG, or anti-nonGal IgM or IgG between pre- and post-bortezomib in either Group 1 (A) or Group 2 (B). A paired *t* test was used to compare the data pre- and post-bortezomib.

Figure 3.

Serum anti-Gal antibody levels by ELISA (absorbance [OD]) pre- and post-bortezomib in

(A) the combined Groups 1 and 2 ($n=9$) (B) Group 1 ($n=4$) and (C) Group 2 ($n=5$).

In the combined Groups 1 and 2 (A), although there was a significant difference ($p<0.03$) in anti-Gal IgM pre- and post-bortezomib, there was no significant difference in serum anti-Gal IgG. In the individual groups (Group 1 (B) or Group 2 (C)), there was no significant difference in anti-Gal IgM or IgG. The results are expressed as mean $\pm$ SEM. A paired t test was used to compare the data pre- and post-bortezomib treatment.

Table 1

Summary of Bortezomib Treatment

Patient (age, gender, organ transplanted)	#Treatments (# of doses)	Total drug (mg)	Day of blood draw pre-bortezomib	Duration of course (days)	Day of blood draw post-bortezomib	Duration between samples (days)	Other therapy*
Group 1 (n=4)							
1: 37, F, K	16	37.2	-3	73	+1	77	-
2: 51, F, K	16	39.2	-61	73	+1	135	-
3: 46, F, K	16	36.0	-3	73	+1	77	-
4: 45, M, K	16	39.8	-14	73	+32	119	-
Group 2 (n=5)							
1: 52, F, I	5	11.0	0	25	+1	26	IVIg/ATG/MP/anti-CD20mAb
2: 30, M, I	6	13.8	0	52	0	52	-
3: 47, M, I	3	7.5	-1	41	+1	43	IVIg/ATG/MP
4: 45, F, K	4	9.6	-9	10	+7	26	PP/IVIg/MP/anti-CD20mAb/MMF
5: 14, M, I	11	18.7	161	136	+21	318	PP/IVIg/ATG/MP/anti-CD20mAb/MMF

* Additional to conventional immunosuppressive therapy; ATG = anti-thymocyte globulin; F = female; I = intestine; IVIg = intravenous immunoglobulin; K = kidney; M = male; MP = methylprednisolone; PP = plasmapheresis.

Table 2A
Anti-pig, Anti-nonGal, and Anti-Gal IgM and IgG Levels Pre- and Post-Bortezomib Treatment in Group 1 (Mean +/- SEM)

	Pre-bortezomib	Post-bortezomib	Statistical Significance (p)
Flow Cytometry (Relative MFI)			
Anti-pig IgM	17.6+/-2.5	15.7+/-3.2	0.10
Anti-pig IgG	6.0+/-1.8	5.0+/-1.4	0.38
Anti-nonGal IgM	2.8+/-0.9	2.4+/-0.5	0.39
Anti-nonGal IgG	2.0+/-0.5	1.8+/-0.4	0.29
ELISA (OD_{480nm})			
Anti-Gal IgM	1.0+/-0.1	0.9+/-0.2	0.07
Anti-Gal IgG	1.4+/-0.1	1.3+/-0.1	0.15

Table 2B
Anti-pig, Anti-nonGal, and Anti-Gal IgM and IgG Levels Pre- and Post- Bortezomib
Treatment in Group 2 (Mean +/- SEM)

	Pre-bortezomib	Post-bortezomib	Statistical Significance (p)
Flow Cytometry (Relative MFI)			
Anti-pig IgM	6.0+/-1.7	4.3+/-1.0	0.17
Anti-pig IgG	2.9+/-0.7	2.4+/-0.4	0.48
Anti-nonGal IgM	1.2+/-0.1	1.2+/-0.1	0.82
Anti-nonGal IgG	1.2+/-0.1	1.1+/-0.1	0.59
ELISA (OD_{480nm})			
Anti-Gal IgM	0.4+/-0.1	0.3+/-0.1	0.30
Anti-Gal IgG	0.9+/-0.1	0.9+/-0.1	0.88