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Chapter 7: Use of immunosuppressive regimens to reduce humoral immunogenicity generated by primary AAV vector gene delivery

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7.1 Introduction

Recombinant adeno-associated viral vectors (AAVs) show great promise for gene therapy in a variety of different genetic disorders (91;161;323). AAV vectors have an excellent safety profile, as demonstrated in numerous non-clinical and clinical studies (324;325). Furthermore, AAV vectors were shown to mediate a stable therapeutic transgene expression in several non-clinical studies (323;326-329) and more recently in clinical studies (92;330-332). However, the majority of the transgenic proteins are expressed by episomally retained double stranded genomes that persist in post-mitotic tissues (333). This characteristic raises the possibility that transgene expression will decline over time with the natural turnover of the transduced cells. Therefore, repeated administration may become necessary to maintain expression of the transgenic protein within therapeutic levels. One major challenge for a successful re-administration of AAV vectors is the presence of neutralizing antibodies (nAb) that are developed after the first administration. Those neutralizing serotype-specific antibodies, directed towards the viral capsid proteins, prevent efficient gene transfer with rAAV of the same serotype (334;335). Therefore, the generation of a humoral immune response does not permit the use of a “vector of choice” more than once, which is a concern for life-long disorders, for which re-administration has to be considered. Hence strategies to decrease existing anti-AAV nAb titers need to be explored.

Bortezomib, a selective inhibitor of the 26S proteasome (176;177) has been shown to significantly decrease AAV specific humoral immune responses after AAV-based gene delivery in mice (178). However, this inhibition was only partially effective and insufficient to allow subsequent re-administration with a recombinant AAV vector of the same serotype (178). This limitation was shown to be due to the combination of residual antibody levels and the inability of Bortezomib to completely deplete the memory B cells (178).

In this chapter, the potential of a poly-therapy combining Bortezomib with an anti-CD20 monoclonal antibody, targeted against pre-B cells, mature B cells and memory B cells (179;180), to reduce efficiently AAV specific nAb, thereby enabling AAV vector re-administration was explored. Additionally, the efficacy of the combined immunosuppressive regimen on the antibody response against the immunogenic protein ovalbumine (OVA) as a transgene delivered by AAV was monitored.
7.2 Results & Discussion

7.2.1 Experimental set-up
Mice were injected intramuscularly with $1 \times 10^{13}$ gc/kg of AAV1 (CMV-OVA) or PBS. All the mice were followed for 21 days (3 weeks) for the development of nAb against the AAV1 capsid and antibodies against OVA transgene product. From day 22, immunosuppressive (IS) therapy was initiated for 2 weeks, mono-therapy with Bortezomib or anti-CD20, and poly-therapy with both Bortezomib and anti-CD20 ([Figure 7.1](#)). The drugs were administered three times intravenously at a dose of 0.75mg/kg for the Bortezomib (day 28, 33 and 36 of the experiment) and two times intraperitoneally at a dose of 100 $\mu$g for anti-CD20 (day 22 and 27 of the experiment).

![Figure 7.1 Experimental outline](#)

7.2.2 Humoral response against OVA
To assess the influence of Bortezomib and anti CD20 on the humoral immune response against the OVA transgene, the levels of specific anti-OVA antibodies generated were measured over time ([Figure 7.2 A-C](#)). The data obtained show a significant inhibitory effect of Bortezomib (67.19 % on day 28 and 71.18 % on day 35) ([Figure 7.3](#)) and anti-CD20 (61.51 % on day 28 and 44.38 % on day 35) ([Figure 7.3](#)) on the humoral response raised against the OVA protein when compared to the control without IS. No significant additive inhibitory effect on anti-OVA antibody level was observed when anti-CD20 and Bortezomib treatment were combined ([Figure 7.2 C](#)). The inhibitory effect of the IS treatment on the humoral response was transient as the antibodies level increased at day 42 of the experiment ([Figure 7.2 A-C](#)).
Figure 7.2 to assess the influence of Bortezomib and anti CD20 on the humoral immune response against the OVA transgene, the levels of specific anti-OVA antibodies generated, were assessed over time.
To assess the influence of Bortezomib and anti CD20 on the humoral immune response against the OVA transgene, the levels of specific anti-OVA antibodies generated, were assessed at day 28 and 35. The data obtained show a significant inhibitory effect of Bortezomib (67.19% on day 28 and 71.18% on day 35) and anti-CD20 (61.51% on day 28 and 44.38% on day 35) on the humoral response raised against the OVA protein when compared to the control without IS. *P value <0.05, **P value, <0.01, ***P value <0.001, ***P value <0.0001 vs AAV (CMV-OVA).

Figure 7.3 to assess the influence of Bortezomib and anti CD20 on the humoral immune response against the OVA transgene, the levels of specific anti-OVA antibodies generated, were assessed at day 28 and 35. The data obtained show a significant inhibitory effect of Bortezomib (67.19% on day 28 and 71.18% on day 35) and anti-CD20 (61.51% on day 28 and 44.38% on day 35) on the humoral response raised against the OVA protein when compared to the control without IS. *P value <0.05, **P value, <0.01, ***P value <0.001, ***P value <0.0001 vs AAV (CMV-OVA).
7.2.3 Humoral response against AAV1- Neutralizing antibodies (nAb) against the AAV1 vector capsid

To determine the effect of Bortezomib and anti-CD20 on the levels of neutralizing antibodies against the AAV1 viral capsid, a neutralizing antibody assay was performed on mice plasma samples obtained at days 0, 14, 28, 42, 56 and 84 (Figure 7.4). After Bortezomib administration (days 28, 33 and 36), a decrease was observed in the levels of the nAb titers against the AAV1 capsid at days 42 (13.6%), 56 (26.3%) and 84 (38.7%) when compared to the control without IS (Figure 7.4A). After anti-CD20 administration (days 22, and 27), a decrease was observed in the levels of the nAb titers against the AAV1 capsid (day 84) when compared to the control without IS (35.5%) (Figure 7.4B). The delayed response to the anti-CD20 antibody treatment is consistent with the mechanism of action of this antibody (336). The combination of both IS drugs did not show an additive effect on the reduction of anti-AAV1 nAb levels (Figure 7.4C). The effect of the IS treatment on the humoral response was sustained until the end of the observation period, as the neutralizing antibodies levels remained low until day 84 for both Bortezomib and anti CD-20. However, the anti-AAV nAb titer obtained after treatment (a titer of 2592) was not low enough to perform re-administration, as previously reported (178).

We conclude that a combined treatment with Bortezomib and anti-CD20 antibody, when a high titer (~3550) of anti-AAV1 nAb has already been established, cannot reduce the level of those antibodies sufficiently to permit re-administration. Therefore other combination of treatments that include additional cellular targets should be explored in the future.
Figure 7.4 to determine the effect of Bortezomib and anti-CD20 on the levels of neutralizing antibodies against the AAV1 viral capsid, a neutralizing antibody assay was performed on mice plasma samples obtained at days 0, 14, 28, 42, 56 and 84. After Bortezomib administration (days 28, 33 and 36), a decrease was observed in the levels of the nAb titers against the AAV1 capsid at days 42 (13.6%), 56 (26.3%) and 84 (38.7%) when compared to the control without IS. After anti-CD20 administration (days 22, and 27), a decrease was observed in the levels of the nAb titers against the AAV1 capsid (day 84) when compared to the control without IS (35.5%). The combination of both IS drugs did not show an additive effect on the reduction of anti-AAV1 nAb levels. The effect of the IS treatment on the humoral response was
sustained until the end of the observation period as the neutralizing antibodies levels remained low until day 84 for both Bortezomib and anti CD-20.

7.2.4 Safety

No weight loss or signs of illness were observed in the mice receiving the immunosuppressive drugs alone or in combination.

It has been described previously that a dose of 1 mg/kg Bortezomib was associated with toxicity in 15% of the animals treated (178), suggesting a narrow therapeutic window of this drug. We report that administration of a dose of 0.75mg/kg Bortezomib associated or not with anti-CD20 (100 μg per injection) did not appear to induce toxicity in mice.

Additionally thymus, spleen, bone marrow and PBMCs were collected at sacrifice, stained for B and T cell markers and analyzed with the use of flow cytometry. We did not observe any significant differences in expression of CD19, CD25 or CD138 markers (data not shown) which demonstrate the absence of long term effects on the adaptive immune system.

7.2.5 Conclusions and future prospects

Overall, this study shows that a poly-therapy combining Bortezomib with an anti-CD20 monoclonal antibody was not sufficient to lower a high titer of pre-existing antibodies against the AAV1 capsid to a level that will enable an efficient AAV1 re-administration. The same drugs alone or in combination were, by contrast, efficient in preventing immune response against the immunogenic protein (OVA) delivered as a transgene by AAV1. This observation can be related to the differences in the kinetic and mechanism of development of the humoral immune response against the AAV capsid and the transgene product delivered by AAV (337).

The two immunosuppressive agents used in this study, Bortezomib and anti-CD20, are mainly targeting pre-B cells, mature B cells and memory B cells (179;180). Additional cellular inhibitors, as T cell inhibitors, may be required in order to bring down the humoral response to the AAV1 capsid. Further studies are needed in order to evaluate the efficacy of combination treatment that targets both B and T cells population as well as the therapeutic benefit in relation to different nAb levels.
7.3 Materials & methods

7.3.1 Study Design
C57BL/6 mice (males, age 6-8 weeks) were obtained from Harlan Laboratories, the Netherlands (n=6 per group). The experimental protocol was approved by the ethical committee for animal welfare of the AMC (Academic Medical Center, Amsterdam, the Netherlands). The general procedure is summarized in Figure 7.1. Mice received intramuscular injection of AAV1 (CMV-OVA) at day 0, at 1x10^{13} gc/kg or PBS/5% sucrose. Blood was drawn weekly and at sacrifice. Treatment was initiated with three different experimental regiments. A group receiving mono-therapy with Bortezomib (Velcade, company) at day 28, 33 and 36, a group receiving mono-therapy with anti-CD20 antibody (eBioscience, clone: AISB12) at day 22 and 27, a group receiving poly-therapy with both Bortezomib (day 28, 33 and 36) and anti-CD20 (Day 22 and 27). Anti-CD20 was administered intraperitoneal at 100µg/mouse; Bortezomib was administered intravenously at 0.75mg/kg. The control groups were mice injected with PBS and mice injected with AAV1 (CMV-OVA) only.

7.3.2 AAV vector production and characterization
AAV vectors were produced (technology adapted from A. Negrete and R.M. Kotin (294)) containing the OVA protein, under the control of the cytomegalovirus (CMV) promoter. AAV was purified with an anion column using the ÄKTAexplorer system (GE-Healthcare). After purification, presence of AAV capsid proteins (VP1, VP2 and VP3) was determined by polyacrylamide gel electrophoresis and concentration of vector genomes (gc/ml) determined by qPCR.

7.3.3 Virus neutralizing assay
HEK293 cells were seeded in a 96 wells plate (Corning) coated with 0.25% poly L-lysine at a density of 1 x10^5 cells/well in 100µl DMEM with 10% FBS and antibiotics (Penicillin/Streptomycin Solution). Cells were incubated overnight at 37°C. Medium was then removed and the following mix was added: AAV1 (CMV-eGFP) incubated with heat-inactivated plasma sample in a total volume of 100µl of DMEM with antibiotics. The mix was kept for 1 hour at 4°C before being added. Medium of the HEK293 cells was removed by aspiration and the mix added for 20 hr at 37°C. Two serial dilutions of test plasma were applied; 1:100, 1:200, 1:400, 1:800, 1:1600, 1:3200, 1:6400, and 1:12800 and 1:10, 1:20, 1:40, 1:80, 1:160, 1:320, 1:640, and 1:1280. As a positive control, cells without plasma were analyzed. The cells were washed with PBS and collected after trypsinisation. GFP expression of the cells was analyzed by fluorescence-activated cell sorting (FACS, Becton Dickinson) in channel FL1 at a wavelength of 530 nm. The analysis was performed with the Cellquest software. The percentage of inhibition was calculated related to GFP expression measured in rAAV1 HEK293 infected cells (no inhibition, 100% expression).

Plasma samples were considered to have neutralizing activity if the lowest plasma dilution inhibited vector transduction by at least 50%, as described before (170-172;311).
7.3.4 Assessment of anti-OVA antibody level

Level of anti-OVA antibody in mouse plasma was measured by anti-OVA specific ELISA. Nunc MaxiSorp® flat-bottom 96-well plates (ThermoScientific) were coated with 0.5 μg/ml OVA protein and anti-OVA antibody level in samples was detected with 1:1000 rabbit-anti-mouse-HRP (DAKO).

7.3.5 Statistical analysis

Results are presented as means (+/-standard error of the mean (SEM). Statistical analyses were performed using Prism 5.0 (GraphPad). Data were analyzed using a 2 way ANOVA, followed by Bonferroni post hoc test for multiple comparisons.