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Intravenous administration of Factor VIII - O-Phospho-L-Serine (OPLS) complex reduces immunogenicity and preserves pharmacokinetics of the therapeutic protein

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Abstract

Hemophilia A is a bleeding disorder caused by the deficiency of an important coagulation factor; Factor VIII (FVIII). Replacement therapy using exogenously administered recombinant FVIII is the most commonly used method of treatment. However, approximately 30% of Hemophilia A patients develop neutralizing antibodies (Nabs) against the recombinant protein. Nabs abolish FVIII activity and drastically influence efficacy of the protein. The immunogenic epitopes of FVIII reside predominantly in the C2 domain of FVIII. However, the C2 domain also contains a lipid binding region. O-Phospho-L-Serine (OPLS) which is the head-group moiety of phosphatidylserine, interacts with the lipid binding region of FVIII. Previous studies have shown that FVIII complexed with OPLS lowered Nab development against FVIII following subcutaneous administration. In dendritic cell – T-cell co-culture studies, OPLS treatment increased the secretion of immunosuppressive cytokines (Transforming Growth Factor- β and Interleukin-10), and simultaneously decreased pro-inflammatory IL-17 cytokine. Here, we investigated FVIII immune response and pharmacokinetics upon intravenous administration of FVIII-OPLS complex. We studied the effect of FVIII-OPLS complex on the interaction between a professional antigen presenting cell; dendritic cell and T-cell, and T-cell clonal expansion. Pharmacokinetics parameters were estimated following intravenous administration of FVIII and FVIII-OPLS. The results suggest that OPLS lowers FVIII immune response following intravenous administration. OPLS also hinders FVIII-specific T-cell clonal proliferation and preserves FVIII PK profile. Thus, the ease of protein-lipid complexation, preservation of FVIII activity and *in vivo* behavior, and improved *in vitro* FVIII stability, makes OPLS an attractive excipient in the preparation of next generation or biosimilar FVIII products with improved safety profile.

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Keywords

Factor VIII; O-Phospho-L-Serine; OPLS; neutralizing antibody; pharmacokinetics; T cell; dendritic cell; cytokine; TGF β ; IL 10; IL 17

1. INTRODUCTION

Factor VIII is a multi-domain protein (1) consisting of six domains viz. NH₂-A1-A2-B-A3-C1-C2-COOH. It is an important co-factor in the blood clotting cascade (2). Deficiency or lack of an active form of FVIII leads to a debilitating bleeding disorder called Hemophilia A. Although, recombinant human Factor VIII (FVIII) is commercially available as the option of choice to treat hemophiliacs, the protein is inflicted with several drawbacks including protein aggregation, short circulation half-life and adverse immune response. All these issues render the protein less efficacious and negatively impact patients' quality of life. The C2 domain of FVIII is critical for the protein *in vivo* activity (3). Upon release in to the systemic circulation, FVIII rapidly associates with its carrier protein called von Willebrand factor (vWF) (4). Association with vWF prolongs FVIII plasma survival by protecting FVIII from degradation by circulating enzymes (5). Additionally, vWF also prevents FVIII endocytosis by dendritic cells (DC) (6). Studies have shown that regions within the C2 domain are involved in FVIII - vWF interaction (7). FVIII clearance is mediated by low-density lipoprotein receptor related protein (LRP) (8, 9), and C2 domain is involved in the interaction with LRP (10). Additionally, it has been recognized that T-lymphocytes are essential for FVIII immune response (11, 12). Upon interaction with professional antigen-presenting cells such as dendritic cells (DCs) that present immunogenic epitopes on their surface, activated antigen-specific T-cells subsequently interact with and activate B-cells which further differentiate into anti-drug antibody secreting plasma cells (13–19). It was observed that most of the T-cell immune epitopes reside within the C2 domain of FVIII (20). Thus, the C2 domain of FVIII is not only involved in the protein activity, but also involved in FVIII aggregation (21), clearance and immunogenicity (22).

Hence, a clinical benefit would be with development of next generation FVIII replacement therapy that can overcome these aforementioned issues. Interestingly, the C2 domain also comprised of a lipid binding region (LBR). Phosphatidylserine; an endogenously present anionic lipid binds to the LBR via its head-group O-Phospho-L-Serine (OPLS) (23, 24). Previous studies have investigated the utility of OPLS as an excipient in FVIII preparations. The results indicate that OPLS improved the physical stability to FVIII following FVIII-OPLS complexation, thereby reducing subcutaneously administered FVIII immune response in naïve HA mice (25, 26). Moreover, co-culture of FVIII-specific splenic CD4⁺ T-cells with DC pre-exposed to FVIII-OPLS resulted in the secretion of immunosuppressive Transforming Growth Factor (TGF)- β and Interleukin (IL)-10 cytokines and concomitant decrease in IL-17 pro-inflammatory cytokine level (27). Additional studies were carried out to further our understanding of the beneficial effects of OPLS in FVIII therapy. Currently FVIII is administered via the intravenous (I.V.) route in the clinic. Therefore, here we investigated the effect of FVIII-OPLS on FVIII immune response following I.V. administration. As it is important to know the effect of OPLS on T-cells, we also studied

OPLS effect on FVIII-specific CD4⁺ T-cell clonal expansion *in vitro*. It is imperative that OPLS complexation do not cause any detrimental effect on FVIII pharmacokinetics (PK). Hence, we studied the effect of OPLS complexation on FVIII PK in naïve HA mice following I.V. administration.

The results indicate that FVIII-OPLS reduce FVIII immune response possibly due to its effect on reducing FVIII-specific T-cell activation and proliferation, as well as, due to the previously reported secretion of immunoregulatory cytokines in the cell-culture micro-environment. Additionally, complexation with OPLS maintained FVIII PK and retained FVIII *in vivo* activity. Thus, OPLS could be utilized as a next-generation excipient in FVIII preparations to minimize the undesired drawbacks associated with FVIII and improve FVIII therapy.

2. MATERIALS AND METHODS

Excipient-free, full-length, recombinant human Factor VIII was a generous gift from the Western New York Hemophilia Foundation. O-Phospho-L-Serine (OPLS) and Phosphocholine chloride calcium salt tetrahydrate (PChg) was purchased from Sigma Aldrich (St. Louis, MO). Sterile water for injection, sterile syringes, needles, isoflurane and other surgical items were purchased from Henry Schein Inc. (Melville, NY). Sterile 0.22 µm syringe filters were procured from Millipore (Billerica, MA). Buffer salts were purchased from Fischer Scientific (Fair Lawn, NJ). Sterile cell strainers, petri dishes, NUNC maxisorb ELISA plates and tissue culture micro-well plates were purchased from VWR Inc. (Bridgeport, NJ). aPTT reagents were obtained from T-coag Ireland Ltd. (Ireland). ESH8 standard antibody was purchased from American Diagnostica Inc. (Stamford, CT) and Goat anti-mouse IgG detection antibody was purchased from Southern Biotech (Birmingham, AL). PNPP substrate reagent kit was purchased from KPL Inc. (Gaithersburg, MD). Recombinant murine Granulocyte Macrophage Colony Stimulating Factor (GM-CSF) was purchased from Peprotech Inc. (Rocky Hill, NJ). Sterile cell culture media, sterile distilled water and Dynabeads CD4⁺ negative isolation kit were obtained from Invitrogen Inc. (Carlsbad, CA). Sterile, heat-inactivated fetal bovine serum was obtained from Lonza Ltd. (Switzerland). Chromogenix Coamatic FVIII activity determination kit was purchased from DiaPharma Group Inc., (West Chester, OH).

Animals

C57BL/6J strain of mice with target deletion of Exon 16 of the f8 gene (HA mice) was used for the studies. All animal handling and experiments were performed according to an approved protocol by the Institutional Animal Care and Use Committee (IACUC) of University at Buffalo, NY.

2.1.1 Preparation of OPLS or Phosphocholine solution

Tris buffer was prepared using sterile water for injection. Solubility of OPLS (mol.wt. 185.07) and PChg (mol.wt. 329.73) in water is 50mg/mL and 100mg/mL respectively. 10 mM OPLS or Phosphocholine chloride calcium salt tetrahydrate (henceforth referred to as OPLS/PChg) solution was prepared by adding appropriately weighed quantity of OPLS/

PChg to 10 mL of Tris buffer. pH was adjusted to 7.0 and the resulting solution was sterile filtered through 0.22 μ m syringe filter under aseptic conditions.

2.1.2 Preparation of FVIII-OPLS/PChg complexes

Required quantity of FVIII was added to 10 mM OPLS/PChg solution and complexed by incubating for 30 min at room temperature as previously described (26). This incubation condition is found to be optimal for complexation.

2.1.3 Endotoxin Test

The prepared complexes were analyzed for endotoxin level by using Endosafe® Endochrome-K endotoxin assay kit (Charles River Inc., MA). Any endotoxin, if present, was below the detection limit of the assay kit. The samples were subsequently used for further studies.

2.2 FVIII or FVIII-OPLS/PChg immune response

Naïve HA male mice were used for the study. The animals were divided into three groups with 6 animals in each group and were administered four consecutive once-a-week I.V. doses of FVIII or FVIII-OPLS/ PChg (2 ug FVIII /animal /injection) via the penile vein. During the course of study, one animal in the FVIII group died for unknown reason. On the sixth week, all remaining animals were sacrificed and blood was collected by cardiac puncture in 10 % acid citrate dextrose (ACD) anti-coagulant buffer. Plasma was isolated by centrifugation of blood samples at 5000 g at 4 °C for 5 min. The plasma samples were stored at –80 °C until further analysis.

2.3 Anti-FVIII Nab titer determination

Level of neutralizing antibodies (Nabs) against FVIII was determined by activated partial thromboplastin time (aPTT) FVIII residual activity assay following Nijmegen's modification (28). Plasma samples were serially diluted using FVIII-deficient human plasma. The resulting diluted samples were incubated with normal pooled human plasma in 1:1 ratio and incubated at 37 °C for 2 h. Later, the samples were analyzed using Coag-a-mate®-XM instrument after addition of Platelin-L and CaCl₂ reagents. The clotting time in seconds was noted. A routinely used approach of serially diluted plasma sample that showed 50 % decreased FVIII activity when compared to a reference FVIII standard curve was considered as the Nab titer for that sample.

2.4 Anti-FVIII total binding antibody titer determination

Total binding antibody level towards FVIII in the plasma samples was determined by ELISA assay as previously described (29). Standard monoclonal ESH8 anti-FVIII antibody was used to generate a standard curve.

2.5 Isolation of dendritic cells

Naïve HA mice were sacrificed and bone marrow from the femurs and tibiae was collected. Bone marrow cells were cultured with recombinant murine GM-CSF according to the method of Lutz (30) to differentiate them into bone marrow-derived dendritic cells (DCs).

2.6 CD4⁺ T-cell proliferation

Three groups of naïve HA mice were administered once-a-week doses of FVIII or FVIII-OPLS/PChg for two consecutive weeks via the subcutaneous route (2 µg of FVIII /animal / injection). In parallel, a naïve HA mice was sacrificed to culture DCs. Day 9 DCs were exposed to 2 µg/ml of FVIII for 24 h at 37 °C and 5 % CO₂. On day 10, which was also the third day after the second weekly immunization, all immunized animals were sacrificed and their spleens were collected. Splenic CD4⁺ T-cells were isolated using Dynabeads® CD4⁺ negative isolation kit (Invitrogen Inc.) as described earlier (27). Alongside, after 24 h of incubation, the DC were harvested, pooled and washed by centrifugation. 2×10^5 CD4⁺ T-cells from each spleen were co-cultured with 7×10^4 FVIII pre-exposed DC in quadruplicate for T-cell proliferation study in 96-well sterile tissue culture plate and incubated for 72 h at 37 °C and 5 % CO₂. OPLS/PChg was not present in the cell-culture micro-environment. After 72 h, ³H-Thymidine (1 µCi/well) was added to the plate and incubated for an additional 16 h at 37 °C and 5 % CO₂. After 16 h, the plate was harvested and radioactivity was measured using liquid scintillation technique. Stimulation index (SI) was calculated as a ratio of the 'average count of the sample' divided by the 'average count of the negative control'.

2.7 Pharmacokinetic study

Naïve male HA mice were aged between 8–12 weeks old were utilized for the study. The average weight was 24.3 g and the weight range was between 22.8–26.4 g. Animals were administered an i.v. bolus dose of 400 IU/kg of free FVIII or 400 IU/kg of FVIII-OPLS via the penile vein. The animals (2–4 mice / time point) were sacrificed at 0.083, 0.5, 1, 2, 4, 8, 16, 24 and 36 h and blood was collected by cardiac puncture in 10 % acid citrate dextrose (85 mM sodium citrate, 110 mM D-glucose and 71 mM citric acid) anti-coagulant buffer. The samples were centrifuged at 5000 g at 4 °C for 5 min and the plasma was collected. The plasma samples were stored at –80 °C until further analysis. A chromogenic assay kit (DiaPharma Group) was used to determine FVIII activity in the plasma samples following manufacturer's protocol (31, 32). Approximately, 100 % of the injected FVIII dose was detected in the 5 min post-injection plasma samples indicating that the entire injected dose reached the systemic circulation. Basic PK parameters such as half-life ($t_{1/2}$), systemic clearance (CL), area under the plasma activity curve (AUAC) and mean residence time (MRT) were determined by Non-compartmental analysis (NCA) using Phoenix WinNonlin 6.2 software (Pharsight Inc., Cary, NC).

2.8 Statistical analysis

Statistical analysis was performed using GraphPad Prism (GraphPad Software Inc., La Jolla, CA) statistical software. One-way ANOVA with Tukey's post-hoc analysis was used to comparison. $P < 0.05$ was considered a statistically significant difference.

3. RESULTS

Effect of OPLS on FVIII immune response

Immune response against FVIII consists of development of anti-drug antibodies. These antibodies include FVIII neutralizing or also called as inhibitory antibodies, as well as other FVIII binding antibodies. Anti-FVIII Nabs abrogate the activity of the protein, thereby resulting in loss of FVIII efficacy (33). Although, other binding antibodies do not neutralize FVIII activity, they can potentially be involved in FVIII clearance. Therefore, these antibodies can influence FVIII PK and reduce FVIII half-life. As a consequence, higher and more frequent doses of FVIII become necessary upon onset of the immune response.

Previously, it was observed that subcutaneous administration of FVIII-OPLS to HA mice interfered with the development of anti-FVIII immune response when compared to the administration of free FVIII in this mice model (26). However, FVIII is currently administered via the I.V. route in the clinic. Hence, we investigated the effect of OPLS on FVIII immune response upon I.V. administration of FVIII-OPLS in naïve HA mice (Fig. 1). Following administration of free FVIII, a robust anti-FVIII Nab response was observed (447 ± 24 ; Mean $\pm$ SEM) (Fig. 1. A). In comparison, FVIII-OPLS treated animals developed significantly lower anti-FVIII Nab titers (227 ± 16 ; Mean $\pm$ SEM). Administration of FVIII-PChg also reduced the Nab titers (292 ± 39 ; Mean $\pm$ SEM). Overall, the data indicates that OPLS can lower clinically relevant FVIII inhibitor titers in this pre-clinical animal model when administered via the I.V. route. It is important to mention here that upon determination of total anti-FVIII antibody titers, the FVIII-treated animals developed the highest titer levels (5468 ± 1018 ; Mean $\pm$ SEM) among all groups (Fig. 1. B). In comparison, FVIII-OPLS treated animals exhibited reduced total titers (3158 ± 936 ; Mean $\pm$ SEM). Lowered anti-FVIII total titers compared to free FVIII group were also observed in the FVIII-PChg treated animals (4240 ± 1079 ; Mean $\pm$ SEM). Although FVIII-OPLS treated group showed reduced total titers, a statistical significance could not be established ($p > 0.05$).

Effect of OPLS on T-cell clonal expansion

It is well-recognized that immune response against FVIII needs CD4⁺ T-cell help. CD4⁺ T-cells recognize many immunogenic epitopes present in the C2 domain of FVIII. However, the C2 domain also encompasses the lipid-binding region (LBR). Previous studies have shown that PS interacts with the LBR and thereby renders the immunogenic epitopes cryptic to the immune system. Recent studies also showed that PS influences FVIII epitope presentation by DCs and subsequently lowers T-cell activation and proliferation (27). Here, we investigated the ability of the head-group moiety of PS i.e. OPLS, to elicit similar beneficial effects (Fig. 2). Naïve DCs from HA mice were pre-exposed to free FVIII *in vitro*. This allowed for effective uptake, processing and presentation of FVIII immune epitopes by DCs. Co-culture of these DCs with splenic CD4⁺ T-cells isolated from free FVIII immunized animals led to a strong T-cell proliferative response (43 ± 12 , Mean SI $\pm$ SEM). In comparison, T-cells obtained from animals immunized with FVIII-OPLS showed significantly reduced proliferative capacity (21 ± 3 , Mean SI $\pm$ SEM). Further, T-cells isolated from FVIII-PChg immunized animals proliferated to similar extent as compared to free FVIII immunized group (49 ± 8 ; Mean SI $\pm$ SEM). It is to be noted that since OPLS

was administered only to the animals and not exposed to DCs therefore; it was not present in the co-culture media. Thus, the observed reduction in T-cell stimulation index is a result of *in vivo* effect of OPLS on the altered generation of FVIII-primed T-cell clones, which fail to recognize native FVIII epitopes presented by DCs, and subsequently undergo inefficient and reduced clonal expansion. Thus, the data suggest that *in vivo* administration of FVIII-OPLS can potentially result in reduction in CD4⁺ T-cell clonal generation and lead to decrease in FVIII immune response.

Effect of OPLS on FVIII pharmacokinetics

OPLS interacts with the lipid binding region (LBR) of C2 domain of FVIII. Further, it has been recognized that LBR is involved in FVIII interactions with vWF and Low density lipoprotein receptor related protein (LRP). Interaction with vWF enhances the plasma survival of FVIII, whereas, LRP mediates FVIII clearance. Both these associations are critical to overall PK of FVIII. Thus, binding of OPLS to the LBR may influence the PK of FVIII. Hence, we compared the PK profiles of free FVIII and FVIII-OPLS complex in naïve HA mice (Fig. 3). Plotting of mean FVIII activity versus time data suggest that free FVIII and FVIII-OPLS exhibit non-linear kinetics. No apparent difference was observed between PK profiles of the two groups. Estimation of basic PK parameters was determined using Non-compartmental analysis and the results are presented in Table 1. FVIII-OPLS showed minimal improvement in area under the plasma activity curve (AUAC) and mean residence time (MRT) compared to free FVIII; however there was no difference in FVIII half-life and clearance between the two groups. Thus, complexation of OPLS preserve the PK profile of FVIII in naïve HA mice, but at the same time leads to reduction in immune response and generation of T-cell clones.

4. DISCUSSION

OPLS exhibits relatively a weaker FVIII binding affinity [apparent K_D of 5.71 μ M (26)] compared to vWF [K_D < 0.5 nM (34)]. Therefore, upon systemic administration, FVIII would dissociate from OPLS and rapidly associate with vWF. A large excess of OPLS was used relative to FVIII to ensure that large fraction of FVIII is bound to OPLS during administration and then transferred to vWF and thus continuously shielding the immunogenic C2 domain. Hence, the observed reduction in anti-FVIII neutralizing antibody ($p < 0.05$) immune response could most likely be a result of OPLS effect at the initial stages of FVIII delivery. Previous reports have shown that OPLS can reduce FVIII aggregation and can shield FVIII immune epitopes from scavenging APC at the site of injection. Further, OPLS complexation could result in reduced injection site inflammation. Together, these factors may culminate in the observed *in vivo* reduction of FVIII-OPLS immune response. FVIII primary immune response is governed by (i) endocytosis by APC, (ii) APC – T-cell interaction, (iii) T-cell – B-cell interaction and (iv) B-cell conversion in to antibody secreting cells. Interference at any of these steps can possibly lead to lowering of FVIII immune response. Naïve DCs pre-exposed to free FVIII efficiently present FVIII epitopes on their surface and express other co-stimulatory molecules that are required for efficient activation of T-cells (27). Upon *in vitro* co-culturing of free FVIII-immunized T-cells with FVIII pre-exposed DCs, the T-cells interact with DCs and recognize the presented immune epitopes.

Further, the co-stimulatory molecules expressed on DCs interact with their corresponding receptors on T-cells. In addition, there is secretion of pro-inflammatory cytokines in the micro-environment. All these signals direct the T-cells to get activated and result in generation of T-cell proliferation. However, the observed reduction in T-cell proliferation in the FVIII-OPLS group compared to the free FVIII group ($p < 0.05$) suggests that T-cells may not have been primed against all the immune epitopes of FVIII *in vivo*, due to shielding of FVIII epitopes by OPLS. Alternatively, complexation of FVIII with OPLS could alter FVIII peptide-fragmentation by APC *in vivo*, resulting in the presentation of neo-epitopes to circulating T-cells. Thus, these T-cells will be primed against neo-epitopes rather than epitopes of native FVIII and following *in vitro* co-culture with free FVIII pre-exposed DCs that present native FVIII immune epitopes, the neo-epitope primed T-cells may have reduced affinity to interact with these DCs. PS is known to have regulatory effects on dendritic cells (35) and T-lymphocytes. Moreover, association of FVIII with PS lipid has shown to induce a state of hypo-responsiveness in HA mice (36). Thus, it is possible that OPLS may have similar direct effects of its own on these cells *in vivo*. Previous studies with PS and OPLS exposed to DCs have also shown that there is an altered cytokine secretion profile in the DC – T-cell co-culture, and it is directed towards immune-regulation rather than pro-inflammation (27). All these factors could result in the observed reduction in T-cell clonal expansion upon FVIII-OPLS immunization. However, further studies are required to investigate the effects of OPLS on DCs and T-cells individually to elucidate the underlying mechanism.

Nevertheless, the relative ease of OPLS complexation with FVIII, followed by improved FVIII *in vitro* stability (26) and *in vivo* activity, along with retention of FVIII PK and reduced immunogenicity constitutes OPLS as an appealing excipient in the development of next generation and/or bio-similar FVIII products.

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ABBREVIATIONS

FVIII	Factor VIII
HA	Hemophilia A
OPLS	O-Phospho-L-Serine
PChg	Phosphocholine chloride calcium salt tetrahydrate
vWF	von Willebrand Factor
APC	Antigen Presenting Cell

DC	Dendritic cell
i.v.	Intravenous
Nab	Neutralizing antibody
PK	Pharmacokinetics
aPTT	Activated Partial Thromboplastin Time
TGF-β	Transforming Growth Factor-beta
IL	Interleukin

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Figure 1.

FVIII immune response in hemophilic mice upon immunization of free FVIII or FVIII-OPLS / PChg: (A) Anti-FVIII neutralizing antibody (Nab) titers on the sixth week after four consecutive weekly administration of the FVIII preparations. (B) Anti-FVIII total antibody titers on the sixth week following four weekly administrations of FVIII preparations. The data was analyzed by one-way ANOVA followed by Tukey's post-hoc test. * $P < 0.05$ was considered as a statistically significant difference.

Figure 2.

In vitro splenic CD4⁺ T-cell proliferation study: The proliferation was measured as counts per minute (cpm) using ³H-Thymidine incorporation technique. Ratio of average cpm in the samples to the average cpm in the negative control was considered as the stimulation index (SI) for that sample. The data was analyzed by one-way ANOVA followed by Tukey's post-hoc test. * $P < 0.05$ was considered as statistically significant difference.

Figure 3. Pharmacokinetic profiles following administration of 400 IU/kg of free FVIII (open triangles) or 400 IU/kg of FVIII-OPLS (open circles) in naïve HA mice. Plot of mean FVIII activity (IU) v/s time (hr) is shown. Limit of detection was 0.005 IU and is indicated with a horizontal line.

Table 1

Non-compartmental analysis to estimate pharmacokinetic parameters using Phoenix WinNonlin 6.2 (Pharsight Inc., Cary, NC) following intravenous administration of 400 IU/kg of free FVIII or 400 IU/kg of FVIII-OPLS complex in HA mice.

Parameters	Units	Free FVIII (400 IU/kg)	FVIII-OPLS (400 IU/kg)
Apparent half-life ($t_{1/2}$)	hr	2.16	2.19
AUAC _{0-t}	IU*hr/ml	59.0	70.3
Clearance (CL)	ml/hr	6.77	5.69
Mean Residence Time (MRT)	hr	8.08	9.53