University of Alberta

Design, synthesis, pharmacokinetics and pharmacodynamics of glucosamine related compounds for the treatment of arthritis

by

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To my beloved country

Iran

THANK YOU GOD FOR GIVING ME SO MUCH MORE THAN I DESERVE

THANK YOU GOD FOR ALWAYS BEING THERE, EVEN WHEN I FORGET YOU

THANK YOU GOD FOR LETTING ME EXPERIENCE JOY WHILE BEING ABLE TO GLORIFY YOUR NAME

AND THANK YOU GOD FOR MAKING ME, ME

ABSTRACT

There are several animal studies reporting strong disease-modifying effects and anti-inflammatory properties of glucosamine (GlcN). The anti-inflammatory properties of GlcN have suggested its use to treat inflammatory diseases such as adjuvant arthritis (AA). In this study, we showed that administration of GlcN at a dose of 300 mg/kg/day prevented arthritis and improved the signs and symptoms after their emergence. In addition, GlcN restored the down-regulating effect of AA on cardiac proteins and response to verapamil. However, clinical trials in humans are flawed; while some studies suggested effectiveness, others were inconclusive, with their results ranging from strongly effective to negligible or no benefit to the patients. The oral bioavailability (BA) of GlcN is limited and this is, at least in part, behind the controversy in the effectiveness of GlcN. Hence, the development of a GlcN pro-drug with improved BA should render beneficial effect in controlling inflammatory conditions.

Fifteen peptide GlcN derivatives were synthesized consisting of eight esters and seven amides. Their stability was assessed at elevated temperature, high and low pH, and exposure to intestinal and liver homogenates. In addition, their permeability through rat jejunum sacks was evaluated. All of the ester and amide conjugates exhibited favorable thermal and chemical stability. Only a few di-peptide esters exhibited reasonable stability in the intestine and rapid degradation in the liver homogenates. Furthermore, only Gly-Val-COO-GlcN (GVG) exhibited a significant increase in gut permeability relative to GlcN. The mechanism for membrane permeation is believed to occur via the *peptide*

transporter 1 (*PepT1*), because a competition assay with the *PepT1* substrate, Gly-Sar, blocked GVG gut permeability. Furthermore GVG was examined for its BA and efficacy to prevent AA. The stability of the GVG was also tested after incubation with rat feces. GVG showed significantly higher plasma concentrations and urinary excretion than GlcN (≈3-fold increase). GVG showed a favorable stability in rat feces. Adjuvant arthritis was completely prevented with doses greater than 20 mg/kg/day, with GVG being 3-fold more potent than GlcN. In conclusion, GVG appears to be a potent anti-inflammatory compound due to its favorable properties to deliver GlcN into the systemic circulation.

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LIST OF ABBREVIATION AND SYMBOLS

AA	Adjuvant arthritis
ABCB1	ATP-binding cassette sub-family B member 1
ACE	Angiotensin-converting enzyme
ACN	Acetonitrile
ACPAs	Anti–citrullinated protein auto-antibodies
ADAM	l-Aminoadmantan
AI	Arthritis index
Ala	Alanine
ANOVA	Analysis of variance
Anti-MCV	Anti–modified citrullinated vimentin
APR	Acute-phase response
ASP	Aspartic acid
ATP	Adenosine triphosphate
AUC	Area under the curve
AUEC	Area under the effect curve
BA	Bioavailability
BOP	Benzotriazol-1-yl-oxy-tris-(dimethylamino) phosphonium hexafluorophosphate
Bsep	Bile salts export pump
CD243	Cluster of differentiation 243
Cmax	Maximum plasma concentration
COX	Cyclooxygenase
CRP	C-reactive protein
CV%	Coefficient of variation
CYP	Cytochrome P450
DCC	Dicyclohexyl-carbodiimide
DCM	Dichloromethane
DIPEA	Diisopropylethylamine
DMARDs	Disease-modifying antirheumatic drugs
DMF	Dimethylformamide
DPBS	Dulbecco's phosphate buffered saline
Fmoc	9-fluorenyl methoxycarbonyl
FruC-6-P	fructose-6-phosphate
GAGs	Glycosaminoglycans
GAIT	Glucosamine/chondroitin Arthritis Intervention Trial
GFAT	Glucosamine: fructose-6-P amidotransferase
GlcN	Glucosamine
GlcN-6-P	GlcN-6-phosphate

GlcNAc	N-Acetyl-glucosamine
GlcN-P-N-AcTF	Glucosamine-phosphate N-acetyltransferase
gluNAc-6-P	N-acetyl-glucosamine-6-P
GLUT	Glucose transporters
Gly	Glycine
GNPDA	Glucosamine-6-phosphate deaminase
GVG	Gly-Val-COO-GlcN
HBP	Hexosamine biosynthetic pathway
HCl	hydrochloride
HOBT	Hydroxybenzotriazole
HOBT.H_2O	Hydroxybenzotriazole monohydrate
HPLC	High performance liquid chromatography
IL	Interleukin
IGF-I	Insulin-like growth factor 1
IS	Internal standard
LiOH	lithium hydroxide
MA	Mannosamine
MAPK	Mitogen-activated protein kinase
MBHA	p-Methoxybenzhydrylamine
MDR1	Multidrug resistance protein 1
MFS	Major facilitator super family
MI	Myocardial infarction
mL	Milliliter
MMPs	Matrix metalloproteinase
mRNA	Messenger ribonucleic acid
Mrp	Multidrug resistance-associated protein
MSM	methylsulfonylmethane
NADPH	Nicotinamide adenine dinucleotide phosphate-oxidase
NF-kB	Nuclear factor-kappa B
NMM	N-methylmorpholine
NMR	Nuclear magnetic resonance
NO	Nitric oxide
NOS	Nitric oxide synthases
NSAIDs	Non-steroidal anti-inflammatory drugs
OA	Osteoarthritis
Oat	Organic anion transporter
Oct	Organic cation transporter
O-GlcNAc	O-linked-N-acetylglucosamine
OTC	Over-the-counter
PAM	4-hydroxymethyl-phenylacetamidomethyl
PD	Pharmacodynamic

PEG2	Prostaglandin E2
PepT	*peptide transporter*
PGE2	Prostaglandin E2
Pgp	P-glycoprotein
Phe	Phenylalanine
PK	Pharmacokinetics
PPAR	peroxisome proliferator-activated receptor
PRRs	Pattern recognition receptors
RA	Rheumatoid arthritis
RF	Rheumatoid factor
RyR2	Ryanodine-2 receptor
SD	Sprague–Dawley
SD	Standard deviation
SLC	Solute carrier proteins
SPPS	Solid-phase peptide synthesis
t-Boc	Tert-butoxycarbonyl
TEA	Ttriethylamine
TFA	Trifluoroacetic acid
Th1	T-helper 1
Th2	T-helper 2
TM	Transmembrane
TNF-α	Tumor necrosis factors alpha
Trp	Tryptophan
Try	Tyrosine
UDP-GlcNAc	Uridine diphosphate-N-acetylglucosamine
Val	Valine
WOMAC	Ontario and McMaster Universities Osteoarthritis index
µg	Microgram
µL	Microlitre
µM	Micromolar

CHAPTER 1

1.1. Introduction

1.1.1. Glucosamine

1.1.1.1. Structure, biochemistry and biological roles

Monosaccharides containing an amino group are called amino-monosaccharides (aminosugars). Glucosamine (GlcN), 2-amino-2-deoxy-D-glucose, is one of the naturally occurring 6-carbon amino-monosaccharides in the body. It is abundant in the shells of shellfish, animal bones and bone marrow. Marketed GlcN is usually prepared by a simple hydrolysis reaction involving chitin. In this reaction, chitin is deacetylated and depolymerized by either sulfuric acid or hydrochloric acid solutions to yield glucosamine sulfate or glucosamine hydrochloride, respectively (Figure 1.1). GlcN sulfate preparations are very hygroscopic and appear to be unstable unless crystalized with KCl (Mojarrad et al., 2007).

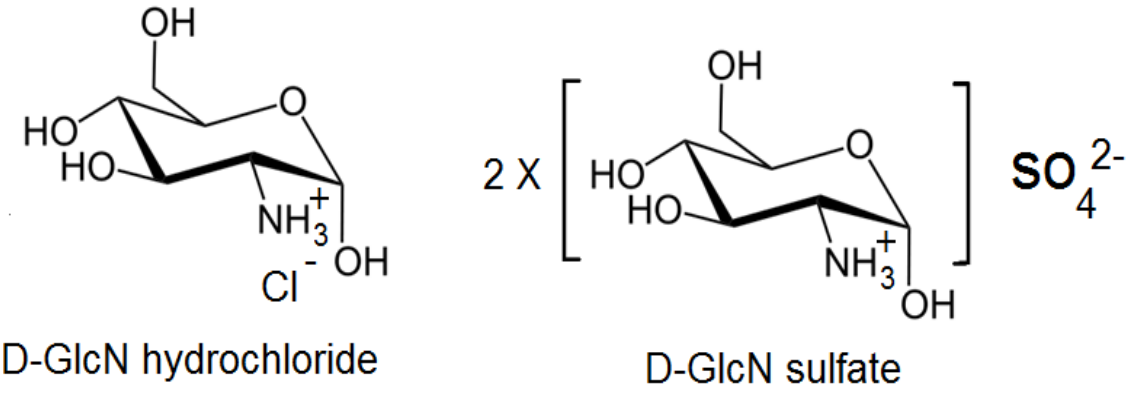

Figure 1.1. Chemical structures of glucosamine hydrochloride and glucosamine sulfate.

Exogenous glucosamine influx into the intestinal cells is suggested to be mediated by a number of facilitative transporters including GLUT1, 2 and 4,

which are sodium independent and normally used to facilitate glucose absorption. GLUT2 demonstrates 20-fold greater affinity for GlcN than glucose, although with lower transporting capacity (Uldry et al., 2002).

Endogenous GlcN is synthesized in the body from glucose through a metabolic pathway known as the hexosamine biosynthetic pathway (HBP). This pathway will be detailed in section 1.1.2.

GlcN is a major precursor in the biochemical synthesis of mucopolysaccharides (such as chondroitin, heparin, and hyaluronic acid), glycoprotein, and glycolipids, which are components of almost all human tissues. It is also an essential part of the structure of the linear polysaccharides chitin and chitosan. Mucopolysaccharides (glycosaminoglycans, GAGs) are large complexes of negatively-charged carbohydrate chains that are present in connective tissues, ligaments, and extracellular matrix of the cartilage (Anderson et al., 2005). GlcN is the essential component of O-linked and N-linked GAGs and is considered as the main building block for cartilage formation. It is a key element to maintain the flexibility, strength, and elasticity of cartilage tissues and serves as a chondro-protective agent. It is assumed that administration of GlcN might be beneficial as a cartilage building block in order to regenerate cartilage and restore normal joint function (Naito et al., 2010).

1.1.1.2. Biochemical pathways

GlcN is synthesized in the body from glucose through a highly regulated metabolic pathway known as the hexosamine biosynthetic pathway (HBP) (Figure

1.2). In the endogenous pathway, glucosamine:fructose-6-P amidotransferase (GFAT) converts fructose-6-phosphate (FruC-6-P) and glutamine to GlcN-6-phosphate (GlcN-6-P). Glucosamine-6-phosphate deaminase (GNPDA) can readily convert GlcN-6-P back to FruC-6-P, which can be further used as an energy source. GlcN-6-P is then acetylated to N-acetyl-glucosamine-6-P (gluNAc-6-P) by glucosamine-phosphate N-acetyltransferase (GlcN-P-N-AcTF). At this step, UDP-N-acetyl-glucosamine pyrophosphorylase converts N-acetyl-glucosamine-6-P (glcNAc-6-P) to uridine diphosphate-N-acetylglucosamine (UDP-GlcNAc). UDP-GlcNAc, which is an essential precursor of all macromolecules containing amino sugars, then enters into several metabolic cascades in order to form mucopolysaccharides. Exogenous glucosamine is first phosphorylated to glucosamine-6-phosphate (GlucN-6-P) by a hexokinase and then enters into the same above mentioned pathway. GlcN transportation from extracellular tissue into cells is through glucose transporters, which is stimulated by insulin (Anderson et al., 2005).

UDP-GlcNAc is also an important component of a dynamic cycle of addition and removal of O-linked-N-acetylglucosamine (O-GlcNAc), which plays an important role in regulating cell growth, gene expression, and the structural integrity of the cytoskeleton (Love and Hanover, 2005). Normally in humans, between 2–5% of FruC-6-P (Milewski, 2002) and 3% of cellular glucose (Anderson et al., 2005) enter the HBP. In humans, the amount of GlcN that is endogenously synthesized ranges between 4 and 20 g/day (Vosseller et al., 2002).

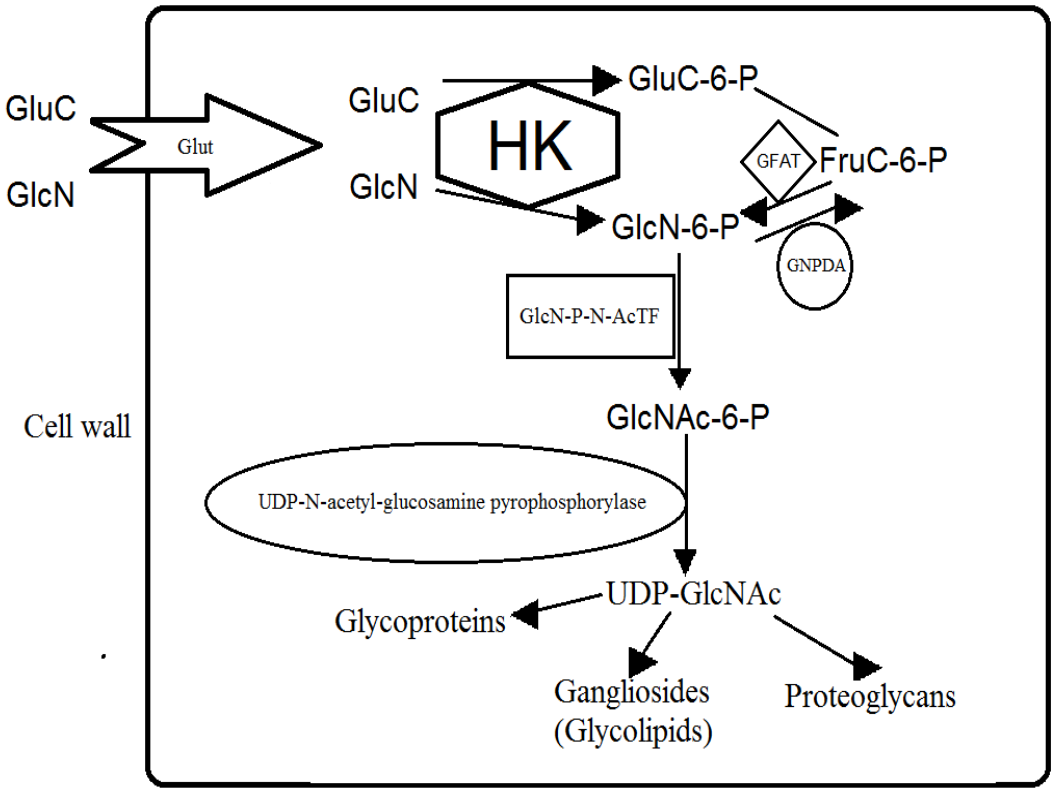

Figure 1.2. A schematic diagram of the hexosamine biosynthetic pathway (HBP). Abbreviations: Glucose (GluC), glucosamine (GlcN), fructose-6-P amidotransferase (GFAT), fructose-6-phosphate (FruC-6-P), glucosamine-6-phosphate (GlcN-6-P), glucosamine-6-phosphate deaminase (GNPDA), N-acetyl-glucosamine-6-P (gluNAc-6-P), glucosamine-phosphate N-acetyltransferase (GlcN-P-N-AcTF), uridine diphosphate-N-acetylglucosamine (UDP-GlcNAc).

1.1.1.3. GlcN as a dietary supplement

GlcN is not a normal component of the daily diet. It is widely used as a nutraceutical for management of osteoarthritis (OA) symptoms. Commercially available GlcN formulations contain the dextrorotatory isomers of the sulfate, hydrochloride (HCl), N-acetyl or chlorhydrate salts. The sulfate and HCl forms

of GlcN usually differ in their purity, sodium content, bioactive glucosamine, and equivalent dosages. N-Acetyl-glucosamine is also used in treatment of OA, albeit to a lesser extent than the sulfate or HCl salts. Unlike GlcN sulfate and HCl, N-acetyl-glucosamine does not appear to have facilitated intestinal transport. It is digested by intestinal bacteria and eventually binds to lectins in the gut, and is excreted in the feces as a lectin-glucosamine complex (Kanzaki et al., 2012; Nagaoka et al., 2012). Since physiological levels of the sulfate ion can affect the GAGs synthesis in the articular tissues (van der Kraan et al., 1990), there is a possibility that the sulfate salts may have superior efficacy over the HCl salt or N-acetyl-glucosamine, which lack the sulfate group (Herrero-Beaumont et al., 2007; Noack et al., 1994).

In some preparations, GlcN is combined with chondroitin sulfate and/or methylsulfonylmethane (MSM). Chondroitin sulfate (CS) is a GAG that is able to maintain viscosity in joints, stimulate cartilage repair and inhibit enzymatic degradation of the cartilage. The Glucosamine/chondroitin Arthritis Intervention Trial (GAIT) reported a trend of superior efficacy in pain relief in patients with moderate to severe OA who received the combination of GlcN HCl and CS over those who received each compound alone (Clegg et al., 2006). However, chondroitin sulfate is a relatively large molecule and poorly absorbed (Herrero-Beaumont et al., 2007). GlcN preparations are mainly administered orally in the form of tablets, caplets, capsules, or powder (Herrero-Beaumont et al., 2007); however, intra-articular and transdermal preparations are also available (Han et al., 2010; Herrero-Beaumont et al., 2007; Kanwischer et al., 2005).

1.1.1.4. GlcN controversy

GlcN is broadly used in the treatment of OA (Vrublevska et al., 2007), with its effect reported to range from negligible (Rozendaal et al., 2008) to as much as those reported for the non-steroidal anti-inflammatory drugs (NSAIDs) (Muller-Fassbender et al., 1994). Hence, the benefit of GlcN in OA is a subject of debate (Aghazadeh-Habashi and Jamali, 2011). There are several animal studies that indicate the effectiveness of GlcN as a chondroprotective agent in animal models of OA (Naito et al., 2010; Oegema et al., 2002; Tiraloche et al., 2005; Wang et al., 2007) and adjuvant arthritis (AA) (Hua et al., 2005). It has been shown that a dose of 300 mg/kg prevents emergence of AA in rats and significantly reduces nitric oxide and prostaglandin E2 levels in plasma (Hua et al., 2005). In humans, GlcN formulations that yield plasma concentrations as high as 1.3 µg/mL appear to be effective in treating human OA (Aghazadeh-Habashi and Jamali, 2011). However, despite numerous animal studies in favor of beneficial effects for GlcN in the treatment of arthritis, randomized human clinical trials and subsequent meta-analysis and systemic reviews are not conclusive (Aghazadeh-Habashi and Jamali, 2011).

A part of this controversy can be attributed to the limited bioavailability (BA) of GlcN (Aghazadeh-Habashi and Jamali, 2011), which prevents the achievement of therapeutic levels in plasma with the usual administered dose in humans (1500 mg/day). Indeed, administration of high doses of GlcN in an adjuvant model of arthritis in rats can prevent the emergence of inflammation and ameliorate the disease signs in early stages (Hua et al., 2005). In addition, GlcN

was not a regulated compound in the past; hence, the quality of the products which were used in clinical trials, is questionable (Oke et al., 2006; Russell et al., 2002). The dosing regimens of GlcN are empirical because of insufficient pharmacological information. Recently a dose-effect study using a pharmaceutical grade GlcN formulation revealed that the minimum effective dose to prevent AA is 40 mg/kg/day in a rat model of AA (Agahzadeh-Habashi et al., 2013), which is several fold greater than the human dose. Moreover, OA progression is usually assessed by joint space width measurements that require a full extension of the knee or based on the patient's response to the questionnaires asking about their pain severity, functional activity and stiffness according to the Western Ontario and McMaster Universities Osteoarthritis index (WOMAC), or the Lequesne index. Obviously the results of such studies are highly subject to the patient's opinion and pain tolerance and associated with high variability (Miller and Clegg, 2011).

1.1.1.5. Active ingredient inconsistency of commercially available GlcN products

In many countries including Canada, GlcN preparations are nutraceutical supplements and hence, are not subject to regular lab tests. Because GlcN sulfate is a very hygroscopic and unstable molecule, varying amounts of sodium or potassium chloride are added during the formulation process in order to stabilize it; hence, the quality of the available products is questionable. According to Russell et al., among fifteen different formulations of GlcN sulfate that were

tested in their study, only one preparation contained the claimed labeled amount of the GlcN and the rest of the products contained much less of the active ingredient. They also revealed that the amount of glucosamine varied from 59 to 138% of the mg content claimed on the label. This statement raises a concern about the validity of the clinical trials that have been done in the past. It is also recommended that if GlcN is used as a therapeutic agent, the exact amount of GlcN should be clearly stated on the label (Russell et al., 2002) .

1.1.1.6. Controversy over the efficacy of the different available salts of GlcN

There is a general belief that the pharmacological effect of the sulfate salt is superior to that of the HCl salt. However most of the clinical studies that reported a greater efficacy of the sulfate salts were sponsored by the sulfate salt suppliers (Herrero-Beaumont et al., 2007; Noack et al., 1994; Rovati et al., 2012). Since both salts are expected to be completely converted in the stomach (pH 1-2) to the free base, there should not be a difference in the efficacy of the salts; if there is any difference, it should be due to differences in the claimed amount of GlcN used in both formulations. To confirm the absence of a natural difference between GlcN sulfate and HCl, Aghazadeh et al. conducted a cross-over bioequivalent study in the rat. GlcN sulfate was extracted from the tablets (Dona®, Rotta Pharmaceutics) and GlcN HCl powder (Sigma-Aldrich Canada, LTD, Oakville, ON) were prepared in PEG 400 and administered orally at an equivalent dose of 100 mg/kg GlcN base to the animals. No significant differences in the pharmacokinetic parameters were observed between the tested groups. These

results were further confirmed by a bioequivalence study on humans after administering the same compounds to four healthy volunteers. It was observed that there were no significant differences in the percentage of the dose excreted in urine over 13 h post dosing, which reflects equivalent body exposure of the two salts (Aghazadeh-Habashi and Jamali, 2011).

Since GAGs synthesis by human articular tissues is very sensitive to any changes in the physiological levels of the sulfate ion (van der Kraan et al., 1990), there is a possibility that the sulfate ions may contribute to the greater observed efficacy of the sulfate salts over the HCl salt. In addition, GlcN enhances the intestinal absorption of the sulfate, which may increase the production of sulfated GAGs (Hoffer et al., 2001). However, the role of the sulfate ion in the GlcN pharmacological effect needs further investigation.

1.1.1.7. GlcN as a structure-modifying and anti-inflammatory agent: possible mechanisms

Glucosamine has been shown to be effective in cartilage metabolic responses. There are several examples of such metabolic activities. For instance, when GlcN is added to cultured human chondrocytes from OA cartilage, a dose-dependent increase in proteoglycan synthesis occurs (Bassleer et al., 1998). Additionally, GlcN is able to affect human chondrocyte gene expression, causing a twofold increase in steady-state levels of perlecan and aggrecan mRNA, and increases in the synthesis of cartilage-specific type II collagen in human fetal chondrocytes (Deal and Moskowitz, 1999).

GlcN has been used for the treatment of several inflammatory conditions in animals such as AA in rats (Hua et al., 2005) and mice (Azuma et al., 2012), and atopic dermatitis like skin lesions in mice (Kim et al., 2011). In addition, GlcN has been reported to exert neuro-protective effects by suppression of inflammatory processes (Hwang et al., 2010). The suggested mechanism for exerting its pharmacologic effects is mainly through decreasing serum tumor necrosis factor alpha (TNF-α), interleukin 6 (IL-6), and IL-1-induced gene expression by inhibiting the cytokine intracellular signaling cascade in general and nuclear factor-kappa B (NF-kB) activation in particular (Rovati et al., 2012; Sakaguchi et al., 2003). Additionally, GlcN has been shown to suppress IL-1-β-induced production of IL-8, nitric oxide (NO), and prostaglandin E2 (PGE2) and inhibit phosphorylation of p38 mitogen-activated protein kinase (MAPK) in synoviocytes (Hua et al., 2007). Moreover, a dose of 300 mg/kg prevents emergence of AA in rats and significantly reduces NO and PGE2 levels in plasma (Hua et al., 2005). GlcN exerts its anti-inflammatory actions, at least in part, by suppressing neutrophil functions such as superoxide generation, phagocytosis, granule enzyme release and chemotaxis (Hua et al., 2002). It has been also suggested that some of the anti-inflammatory action of GlcN may be due to stimulation of biosynthesis of GAGs, which in turn may stabilize cell membranes of macrophages and inhibit lysosomal enzymes (Verbruggen et al., 1998).

1.1.1.8. Clinical and research uses

1.1.1.8.a. Osteoarthritis

Osteoarthritis (OA) is one of the leading causes of disability in humans worldwide. It is characterized by degeneration of cartilage and its underlying bone within a joint and is accompanied with bony overgrowth. It may affect any joint, but it is most common in the weight-bearing joints such as hip and knee. In OA, cartilage wears away, which leads the bones rubbing together. Pain, swelling, and joint stiffness are the most common symptoms of OA, which can cause a disability state in the patients. It is reported that approximately 7.1 million people in the US suffer from OA and this number is growing as the population continues to age. There is no known cure for OA at the present time and current treatments only focus on symptom relief and improvement the patient's overall function (Martin et al., 2012).

GlcN alone or in combination with chondroitin sulfate (CS) and/or methylsulfonylmethane (MSM), is widely used for the management of OA. It is estimated that over 5% of the general population in the US use GlcN. It is also reported that up to 9% of elderly men and 7% of elderly women use GlcN on a regular basis (Simon et al., 2011).

Under normal conditions, cartilage integrity is maintained by a cytokine-mediated anabolic and catabolic process. In OA patients, over-expression of catabolic enzymes such as aggrecanase and matrix metalloproteinase (MMPs), shift the process toward catabolism, which in turn results in cartilage damage. Consequently, synovial membrane over-produces inflammatory cytokines such as

IL-1β and TNF-α, which initiate a cascade of inflammatory reactions (Kim et al., 2007). Inflammation causes the joints to swell, causing pain and stiffness. As the inflammatory conditions progress, further catabolic enzymes are produced and more articular cartilages are degraded. GlcN is believed to slow down the progression of OA by promoting the production of GAGs and helping to alter cartilage turnover in OA patients, which leads to stopping the breakdown of the cartilage (Petersen et al., 2010). Moreover, GlcN has been reported to suppress inflammatory processes mainly through decreasing the IL-1 induced gene expression by inhibiting the cytokine intracellular signaling cascade and nuclear factor-kappa B (NF-kB) activation (Hwang et al., 2010). The recommended oral dose of GlcN in management of OA is 1500 mg/day taken once or divided into three equal doses.

However, the beneficial effects of GlcN in OA is a subject of debate (Aghazadeh-Habashi and Jamali, 2011) and its effect is reported to range from negligible (Rozendaal et al., 2008) to as much as those reported for the nonsteroidal anti-inflammatory drugs (NSAIDs) (Muller-Fassbender et al., 1994). In 2008, The American Academy of Orthopaedic Surgeons published a guideline in which the use of GlcN and chondroitin sulfate was not recommended for patients with symptomatic OA of the knee (AAOS, 2008). On the other hand, the results of Glucosamine/chondroitin Arthritis Intervention Trial (GAIT), the largest randomized multi-center clinical study on GlcN in the US, revealed that glucosamine exerted a beneficial effect in reduction of the Western Ontario and

McMaster University Osteoarthritis (WOMAC) Index over a 24 month period (Sawitzke et al., 2010).

Nevertheless, there are several other trials that showed GlcN preparations were superior to placebo in the treatment of pain and improvement of the functional impairment in symptomatic OA patients (Towheed et al., 2005).

1.1.1.8.b. Rheumatoid arthritis

Rheumatoid arthritis (RA) is a chronic, systemic, and inflammatory disease that mainly affects the joints and their surrounding tissues but it may also cause inflammation in other organs**.** RA has a population prevalence of 0.5% to 1% based on descriptive epidemiology studies, with women affected twice as often as men. Risk factors associated with RA include genetics, infectious diseases, estrogen, smoking, and formal education. RA increases the risk of developing depression, gastrointestinal, respiratory, infectious, and hematologic diseases in patients (Gabriel, 2001). RA patients seem to have an increased risk for myocardial damage compared to control subjects (Koivuniemi et al., 2012).

Several inflammatory cascades are involved in RA pathogenesis, which all lead towards synovial inflammation and cause articular cartilage degradation and damage to the underlying bone (Scott et al., 2010). Synovial and cartilage cells are the dominant cells that can be affected by RA. There are two major types of synovial cells, namely fibroblast-like and macrophage-like synoviocytes. Macrophage-like synoviocytes are the major cells involved in over production of pro-inflammatory cytokines (Muller-Ladner et al., 1996). Several pro-

inflammatory cytokines are believed to play a crucial role in RA. Tumour necrosis factor (TNF) is known as one of the major inflammatory cytokines. Over expression of TNF can cause both synovial inflammation and joint destruction. TNF overproduction has several causes, including interactions between T and B lymphocytes, synovial-like fibroblasts, and macrophages. This process will activate a cascade, which leads to overproduction of many cytokines such as IL6, which in turn causes further inflammation and joint destruction (Choy et al., 2002).

RA is considered an autoimmune disease. Rheumatoid factor (RF) is the classic serologic measure in diagnosis of RA. IgM and IgA RFs are key pathogenic markers directed against the Fc fragment of IgG. Several other auto-antibodies have been described in recent years, including anti–citrullinated protein auto-antibodies (ACPAs), anti-CCP (anti–CCP-3), and the anti–modified citrullinated vimentin (anti-MCV) autoantibody, which has led to the development of various new tests for diagnosis of RA with higher specificity and sensitivity (van der Linden et al., 2009) .

Currently there is no cure for RA and the treatment is based on alleviating the pain and symptoms. Management of RA includes non-drug measures (such as span exercise, joint protection, foot care, and psychological support) and drug intervention. NSAIDs are usually used to reduce the pain and stiffness. However, NSAIDs have lost their conventional role as first-line treatment because of concerns about their limited efficacy and gastrointestinal and cardiovascular side effects (Schaffer et al., 2006; Scott et al., 2007). Disease-modifying anti-

rheumatic drugs (DMARDs) are the mainstay treatment for RA. They have diverse mechanisms of actions to reduce joint swelling and pain, decrease acute-phase markers, limit progressive joint damage, and improve patients' function (Donahue et al., 2008).

Methotrexate (MTX) is the most prescribed DMARD in management of RA. Sulfasalazine and leflunomide are also widely used. Hydroxychloroquine and chloroquine have DMARD-like properties. Gold (rINN sodium aurothiomalate) and cyclosporine are other DMARDs with limited use because of their toxic effects. DMARDs are sometimes combined, and several combinations of DMARDs have proven to be efficient in management of RA. Adverse effects of DMARDs include those that are considered minor (such as stomach upset and nausea), and severe (such as hepatotoxicity, blood dyscrasias, and interstitial lung disease). Monitoring of adverse effects is very important and requires pretreatment screening and subsequent safety recording of blood counts and liver function tests (Scott et al., 2010). Biological agents, such as TNF inhibitors have been proven to be highly effective in the treatment of RA. Biological agents are usually combined with methotrexate (Atzeni et al., 2012). There are several new biological drugs under investigation, including the agents that target proximal effects on the immune response and growth factors for T-cell subsets (such as IL-17). Moreover, inhibitors of the kinases JAK and SYK have provided promising data in management of RA (Kyttaris, 2012). Increased risk of infection is the main concern raised about biological agents. There is also some evidence that these agents may increase the risk of demyelination and cancer (such as

lymphoma) in RA patients (Strangfeld et al., 2010). Oral or intramuscular glucocorticoids are also administered during flare-ups of the disease as a short term treatment until other agents (such as DMARDs) with slower onset of action start to work. In addition, intra-articular glucocorticoids are highly effective for local treatment of individual joints (Goossens et al., 2000). Although use of steroids in this way is considered to be low risk, a correlation between use of these agents and myocardial infarction (MI) has been recently established (Scott et al., 2010).

Currently, there is no clinical data available on how effective GlcN is against RA in humans. However, GlcN has been shown to be effective in prevention (Hua et al., 2005) and treatment (Yamagishi et al., 2012) of AA in rats, a widely used RA animal model. Due to its anti-inflammatory properties and safety record, GlcN should be considered as a potential alternative to other anti-inflammatory drugs.

1.1.1.8.c. Adjuvant arthritis (AA)

Adjuvant arthritis (AA) is one of the experimental animal models which resembles human RA in many features and therefore is one of the most widely used models for studying the anti-inflammatory properties of anti-arthritic compounds (Rosenthale and Capetola, 1982).

AA is usually induced with inoculation of animals with *Mycobacterium butyricum* at the base of the tail. *Mycobacterium butyricum* is usually suspended in an oil, although in some strains of rats it can be induced with oily adjuvants in

the absence of *Mycobacteria*. AA produces a profound systemic inflammation resulting in severe joint swelling and remodeling. Unlike the collagen-induced arthritis models, the AA model achieves sustained serum elevations of certain cytokines. The model is dependent upon a T-cell component. The model is quite robust and can be completed in about 30 days. Animals develop arthritis within 10-14 days following the adjuvant injection. Swelling develops in all limbs (hind and fore paws), which persists for 30 days (van Eden et al., 2001). There are several methods to monitor swelling and clinical scores in AA. AA is usually monitored by recording various signs during the experiment including increased paw thickness, reduced weight gain, erythema and scaling of the right paw, formation of eye nodes and calculation of arthritis index (AI) (Piquette-Miller and Jamali, 1995).

Hua et al. (2005) reported that GlcN at a dose of 300 mg/kg prevents emergence of AA in rats and significantly reduceS nitric oxide and prostaglandin E2 levels in plasma. In addition, synovial hyperplasia, pannus formation with cartilage erosion and severe leukocytic cell infiltration (mononuclear cells and neutrophils) in the knee joints were suppressed in AA rats. Although this dose seems to be high compared with a regular dose for osteoarthritis patients (1.5 g/day, approximately 25 mg/kg), this study revealed that administration of GlcN at therapeutic levels was able to suppress the progression of AA in the experimental animals (Hua et al., 2005). In another study, Setnikar et al. reported that oral administration of GlcN (50~800 mg/kg/day), had a suppressive effect on AA in rats; the AI decreased significantly compared to the control group and the

decrease of body weight was repressed by GlcN (Setnikar et al., 1991). Moreover, GlcN and N-acetylglucosamine (GlcNAc) have been reported to suppress the progression of AA in mice. GlcN suppressed the progression of arthritis in SKG/jcl mice, which are human RA mouse models. GlcNAc also showed suppressive effects on experimental RA in mouse models. However compared to GlcN, GlcNAc has been reported to have a different anti-inflammatory mechanism. In the GlcN group, TNF-α and IL-6 concentrations were significantly decreased compared to the control group, whereas in the GlcNAc group, serum IL-10, transforming growth factor β-1, and IL-2 concentrations were significantly elevated compared to the control group (Azuma et al., 2012).

1.1.1.8.d. Others

GlcN is reported to serve as a neuroprotective agent by suppression of inflammatory processes. GlcN exerts the neuroprotective effect even when administered 3 h after reperfusion, indicating that GlcN acts via a mechanism related to delayed damage processes in the post-ischemic brain (Hwang et al., 2010). Moreover, GlcN showed promising effects in the treatment of atopic dermatitis like skin lesions in mice through immunomodulatory effect (Kim et al., 2011). GlcN at dose of 300 mg/kg prevents emergence of AA in the rats and significantly reduces nitric oxide and prostaglandin E2 levels in plasma (Hua et al., 2005). It has been also shown that activation of the HBP with GlcN may induce cardio-protection and improve the tolerance of the isolated perfused heart to ischemia/reperfusion (Liu et al., 2006). It is been reported that intravenous

administration of GlcN improves organ function after traumahemorrhage and reduces circulatory inflammatory cytokines (Yang et al., 2006). Since GlcN has anti-inflammatory properties, it can be used in management of systemic inflammatory complications such as atherosclerosis, in which GlcN significantly reduces the atherosclerotic lesions in aortic root (Duan et al., 2005) and inflammatory bowel diseases (such as ulcerative colitis, Crohn's disease, interstitial cystitis and Reiter's syndrome) (Russell, 1999). GlcN is highly promising as an alternative for current medications used in cancer therapy, as previously noticed by Quastel et al (Quastel and Cantero, 1953). D-glucosamine, but not GlcNAc, inhibited the growth of subline M-10 of Walker 256 carcinosarcoma in Charles River CD rats *in vitro* with minimal side effects on the normal host cells (Bekesi and Winzler, 1970). Recent findings confirmed the cytotoxic activity of GlcN in different experimental cancerous cell lines by inducing cell cycle arrest and stimulating apoptosis through inhibition of signal transducer and activator of transcription 3 (STAT3) signaling (Chesnokov et al., 2009). Additionally, regular administration of GlcN has been shown to decrease the risk of lung cancer (Brasky et al., 2011). However, the antitumor activity of GlcN is still under investigation.

1.1.1.9. GlcN bioavailability

GlcN is reported to have a low oral bioavailability. The absolute oral bioavailability of GlcN was determined to be 12%, 2.5%, and 19%, in dogs, horses and rats respectively (Adebowale et al., 2002; Aghazadeh-Habashi et al.,

2002; Du et al., 2004). Using radiolabeled GlcN, the absolute oral bioavailability of the compound in humans was found to be 44%; however, this value cannot be considered as the true oral bioavailability of GlcN since it did not differentiate between GlcN and its metabolic products (Setnikar and Rovati, 2001). Several factors may be involved in the loss of drugs in the gut including chemical degradation, poor absorption, metabolism and/or uptake by the gut microflora (Ibrahim et al., 2012).

Recently a complete pharmacokinetic characterization of GlcN has been conducted in rats (Ibrahim et al., 2012). Table 1 depicts GlcN pharmacokinetic parameters after different routes of administration. It was revealed that GlcN was rapidly and completely absorbed after 2 different i.p. doses (F = 1.00 as compared with i.v. doses). On the other hand, following oral doses, F ranged from 0.05% to 0.06%, which was dose-independent. This indicates that gut rather than liver is involved in the pre-systemic loss of GlcN in the intestinal mucosa.

Table 1.1. GlcN pharmacokinetic parameters (mean ± SD) after different routes of administration.

	Routes of Administration			
Parameter	i.v. (10 mg/kg)	i.p. (10 mg/kg)	i.p. (50 mg/kg)	p.o. (200 mg/kg)
n	5	3	5	5
T_{max} (h)		0.19 ± 0.10	0.22 ± 0.07	0.80 ± 0.48
C_{max} (μg/mL)		12.37 ± 1.54	51.38 ± 7.07	5.27 ± 2.36
AUC_{0-6} [(μg·h)/mL]	7.81 ± 1.38	7.86 ± 1.70	38.24 ± 2.0	9.03 ± 2.63
$t_{\frac{1}{2}}$ (h)[a]	0.21 ± 0.04	0.31 ± 0.05	0.29 ± 0.06	2.84 ± 1.21
F		1.00 ± 0.22	0.98 ± 0.05	0.06 ± 0.02[b]

i.p., intraperitoneally; i.v., intravenously; p.o., orally.
[a]For i.v. and i.p. doses 0–1.5 h data were used.
[b]$p < 0.05$ as compared with other routes.

GLUT2 is a type of glucose transporter, which is abundant at the basolateral membrane of the gut and is considered to be the main transporter in mediating the transportation of glucose, galactose and fructose from enterocytes to the blood stream. Since GLUT2 appears to be involved in transporting of GlcN in the gut, the possibility of saturation in the absorption process and non-linear pharmacokinetics should be considered, especially at high doses as previously reported (Persiani et al., 2005) . However, it has been shown that the area under the curve (AUC) values of GlcN were linear within the range of 200–600 mg/kg (Table 2). These findings suggest that there are likely two parallel processes involve, namely a capacity limited mechanism that can be saturated with low doses and a linear process that is operative following higher doses (Ibrahim et al., 2012). Further human data are needed to clarify these observations. The

percentage urinary excretion of GlcN was constant over the examined dosage range (Table 2). Approximately 1% of the administered oral dose was detectable in urine.

Table 1.2. Pharmacokinetic parameters (mean ± SD) following oral administration of different doses of GlcN.

	Dose (mg/kg)		
Parameter	200	400	600
n	5	6	6
T_{max} (h)	0.80 ± 0.48	0.79 ± 0.62	1.13 ± 0.67
C_{max} (μg/mL)	5.27 ± 2.36	8.38 ± 4.39	11.35 ± 6.75
AUC_{0-6} [(μg·h)/mL]	9.03 ± 2.63	16.12 ± 8.88	26.32 ± 14.82
$t_{\frac{1}{2}}$ (h)	2.84 ± 1.21	1.36 ± 0.51	1.04 ± 0.40
% of dose in urine (0–6 h)	1.2 ± 0.5	1.13 ± 0.2	1.13 ± 0.4
F	0.06 ± 0.02	0.05 ± 0.03	0.06 ± 0.03

In vitro studies on the everted rat segments demonstrated a linear relationship between GlcN concentration in the incubation media and the accumulation rate from mucosal to serosal fluid, which indicated that GlcN intestinal absorption is linear and not capacity limited. However these findings did not rule out the possibility of the process saturation at supra-therapeutic doses. The involvement of gut microflora in low bioavailability of GlcN has been also investigated. After eradication of the intestinal flora with a standard combination of antibiotics, it was observed that the percentage of the dose that was excreted in

the urine was significantly increased, which indicated that gut microflora were able to efficiently clear GlcN (Ibrahim et al., 2012). Overall, it can be concluded that the low GlcN bioavailability appears to be, at least in part, dependent on a transport-facilitated absorption, loss through the first-pass metabolism (mainly by gut but not liver), and degradation by the gut microflora (Ibrahim et al., 2012).

1.1.1.10. GlcN safety and side effects

Oral GlcN is usually well tolerated by animals and humans. Oral administration of GlcN to different species revealed that the estimated LD_{50} for oral GlcN is as follows: for rats, >5000 mg/kg; for mice, >8000 mg/kg; and for rabbits, >8000 mg/kg. In general, daily doses of 194–2700 mg/kg administered over a period of 12–365 days were not associated with any significant adverse effects in animals. In humans, usual doses of GlcN are well tolerated for periods of up to three years. In addition, doses of up to 3200 mg/day showed no adverse effects on blood chemistry, hematologic parameters, urinalysis, occult blood in feces, or cardiovascular parameters and were well tolerated by the study subjects. In some cases, reported symptoms or side effects were even significantly less frequent with GlcN compared to placebo (Anderson et al., 2005).

Despite a high safety record of GlcN within normal ranges, there are few studies in which GlcN has been reported to show side effects. For instance, GlcN administration has been considered to have the potential for affecting sugar metabolism by inducing insulin resistance in animals (Rossetti et al., 1995) and in humans (Monauni et al., 2000). However, Tannis et al. observed that any change

in the blood glucose level was not significant after a 12-week treatment with GlcN sulfate, suggesting a lack of any harmful effects of the compound on glucose metabolism. In addition, no abnormalities of any other laboratory tests were observed and no treatment-related adverse events were experienced throughout the follow-up period (Tannis et al., 2004). Since the cellular concentration of GlcN with usual doses in humans is estimated to be 100-fold less than required tissue levels to affect glucose metabolism, no adverse effects on glucose homeostasis should be expected when GlcN is administered in therapeutic ranges (Anderson et al., 2005).

There is also a case report where a patient has shown a symptomatic hepatotoxicity upon taking over-the-counter (OTC) GlcN. Several of her liver enzymes were elevated, which returned to normal levels four weeks after discontinuing of GlcN. However, the authors stated that despite the extensive use of glucosamine supplements, the possibility of significant elevation of liver enzymes is rare and the mechanism of hepatotoxicity is unknown (Ebrahim et al., 2012).

In terms of drug/drug interactions, concurrent GlcN administration with warfarin has been reported to have the ability to increase warfarin's effect, which in turn leads to increased risk of bleeding (Knudsen and Sokol, 2008). In addition, some *in vitro* studies suggested that GlcN may induce resistance toward some anticancer drugs, including etoposide, doxorubicin, and teniposide through a decrease in topoisomerase II (topo II) levels; however there are no clinical studies supporting these observations (Yun et al., 1995).

Based on existing data on efficacy and safety assessments of GlcN, it can be concluded that GlcN supplements can be safely administered.

1.1.2. Inflammation

1.1.2.1. Signs, cellular component, molecular mechanisms, and inflammatory mediators

Inflammation has been described as the host's response to infection and injury provided that the injury is not of such a degree as to destroy the host's structure and vitality (Hanafy et al., 2012). Inflammation is usually associated with five major signs including redness, swelling, heat, pain, and loss of function (Punchard et al., 2004). Inflammation is characterized by two phases: *i)* acute phase in which blood flow and vascular permeability is increased, which is associated with the accumulation of fluid, leukocytes, and inflammatory mediators such as cytokines; and *ii)* the sub-acute/chronic phase, which is characterized by the development of specific humoral and cellular immune responses to the pathogen(s) (Feghali and Wright, 1997).

Inflammation is regulated by several extracellular mediators and regulators, including cytokines, growth factors, eicosanoids (prostaglandins, leukotrienes, etc.), complement system and peptides. In general, activation of endothelial cells, adhesion and migration of leukocytes and activation of these leukocytes to become immune effector cells are the crucial steps in emergence of inflammation. There are two major signaling pathways for activation of endothelial cells including NF-□B activation and the MAP kinases pathway.

Activation of endothelial cells induces the expression of a family of cell adhesion molecules called selectins, which in turn mediate the initial rolling of leukocytes at the beginning of the adhesion and transmigration steps. Although major cells of the immune system are involved in inflammatory conditions, leukocyte recruitment is an essential step in any inflammatory condition. The migration of leukocytes in response to inflammatory stimuli and activated endothelial cells requires directional processes within the cells and polarized interactions with the monolayer and the extracellular space. Monocytes are able to sense the cellular environment by low density focal adhesions within their filopodia and lamellipodia. Recognition of a pathogen through the pattern recognition receptors (PRRs) leads to activation of immune cells and release of inflammatory mediators. These inflammatory mediators are responsible for exudation of plasma proteins and fluid into the tissue and chemotactic migration of leukocytes (mainly neutrophil) into the tissue (extravasation) (Feghali and Wright, 1997;Sunderkotter et al., 2003). Overproduction of inflammatory mediators (such as cytokines, C-reactive protein (CRP) and nitric oxide (NO)) by innate (macrophages, monocytes, neutrophils) and adaptive (T-lymphocytes) immune cells results in inflammation.

Cytokines are a diverse group of soluble messenger proteins involved in the activation, growth, control and repair of cells and regulation of the immune system, which act within the same cell (autocrine), nearby (paracrine) or at distant sites (endocrine) (Delves and Roitt, 2000). Pro-inflammatory cytokines such as TNF-α, IL-1, IL-6, and C-reactive protein (CRP) are usually found in many

inflammatory conditions such as RA patients (Ling et al., 2009). Among all the above mentioned cytokines, TNF-α plays as a central pro-inflammatory mediator in many inflammatory conditions such as RA. Increased concentrations of TNF-α result in elevation of IL-1, IL-6, TNF-α, GM-CSF **(**granulocyte-macrophage colony-stimulating factor), and chemokines including IL-8, RANTES (Regulated on Activation, Normal T cell Expressed and Secreted), chemokine C-X-C motif ligand 1 (CXCL1), macrophage inflammatory proteins (MIP) -1α and -1β, and monocyte chemotactic protein-1 (MCP-1), which in turn lead to emergence of the disease. TNF-α is the major regulator of IL-1 and other pro-inflammatory cytokines; inhibition of TNF-α results in inhibition of other pro-inflammatory cytokines (Feldmann et al., 1996; Feldmann et al., 2001). Sequential expression of cytokine cascades occurs in many inflammatory conditions such as rheumatoid arthritis, in which IL-1, IL-6, IL-8, and granulocyte macrophage-colony stimulating factor are expressed downstream of TNF-α (Feldmann et al., 2001). Anti-inflammatory cytokines such as IL-4 and IL-10 generally counteract the cellular activation and production of pro-inflammatory cytokines (Singh et al., 1999). A group of inflammatory cytokines such as IL-1, TNF-alpha, IL-6, IL-11, IL-8 and other chemokines (including G-CSF, and GM-CSF) are involved in acute inflammation, whereas others are involved in chronic inflammatory conditions. The latter group has been further divided into two sub-groups including the cytokines mediating humoral responses such as IL-4, IL-5, IL-6, IL-7, and IL-13 and those mediating cellular responses such as IL-1, IL-2, IL-3, IL-4, IL-7, IL-9, IL-10, IL-12, interferons, transforming growth factor-β,

and TNF-α and -β. On the other hand, some cytokines, such as IL-1, may contribute to both acute and chronic inflammation (Feghali and Wright, 1997). T-lymphocytes secrete various pro- and anti-inflammatory cytokines depending on the population of activated cells. CD4+ T cells usually are involved in adaptive immune responses in reaction to foreign antigens and can be divided into two distinct subpopulations including T-helper 1 (Th1) and T-helper 2 (Th2), based on the unique cytokine they produce. Th1 cells mainly produce IL-2, IFN-γ and TNF-α. These cytokines induce cellular immune responses and activate macrophages. On the other hand, the Th2 phenotype predominantly secretes IL-4, IL-5, IL-10 and IL-13, which are important in aiding B cell activation and antibody production. As a common rule, Th1 cytokines suppress Th2 and vice-versa; hence a balance of Th1/Th2 activity is desired in order to maintain normal physiological conditions (Kulmatycki and Jamali, 2005)

Chemokines receptors such as CXCR1 (or interleukin 8 receptor alpha, IL8RA), CXCR2 (or interleukin 8 receptor beta, IL8RB), C-C chemokine receptor (CCR) type 2 and 3 are also involved in leukocyte migration during inflammation (Lukacs et al., 1999).

NO is a soluble gas that mediates several normal physiological processes such as vasodilation and neurotransmission; however, NO over-production may lead to inflammatory conditions. NO is normally synthesized from L-arginine in the body and the reaction is catalyzed by a group of enzymes called nitric oxide synthases (NOS). There are three major forms of NOS including, inducible NOS (iNOS), endothelial NOS (eNOS), and neuronal NOS (nNOS). The latter two

forms are called constitutive forms. Unlike constitutive forms, iNOS activity is induced by pro-inflammatory cytokines (e.g., IFN-γ, IL-1β and TNF-α) and is involved in inflammatory disorders (Alderton et al., 2001; Groeneveld et al., 1997).

PGE2 (prostaglandin E2) is also one of important inflammatory mediators in inflammatory diseases. PGE2 has been shown to play a crucial role in emergence of inflammation in RA patients (Hua et al., 2005).

As mentioned above, inflammation is tightly controlled by several extracellular mediators, regulators, and down-regulating control mechanisms; the impairment of the down-regulating control mechanisms, ineffective elimination of pathogens or misjudgment of self-antigens can lead to chronic inflammation or autoimmune diseases (Sunderkotter et al., 2003).

1.1.2.2. Effect of inflammation on metabolizing enzymes, transporters, receptors, and plasma proteins

Inflammation is known to cause alteration of the expression of several metabolizing enzymes, receptors, plasma proteins and transporters (Cressman et al., 2012; Hanafy et al., 2012). Inflammation-mediated alterations of cytochrome P450 and related biotransformation have been previously reported (Ling and Jamali, 2009; Morgan et al., 2008; Renton, 2001; Renton, 2004). In most reported cases individual cytochrome P450 forms are down-regulated at the level of gene transcription, which in turn leads to decrease in the corresponding mRNA, protein and enzyme activity. For example, Ling et al. reported that total microsomal

cytochrome P450 as well as contents of CYP3A1/2 and CYP1A1/2 isoenzymes were significantly down-regulated in a rat model of pre-adjuvant arthritis compared with control animals. The loss in drug metabolism is predominantly attributed to the production of cytokines, which ultimately modify specific transcription factors (Ling and Jamali, 2009).

Inflammation can alter the expression of several important drug transporters including P-glycoprotein (Pgp). Pgp (also known as multidrug resistance protein 1 (MDR1), ATP-binding cassette sub-family B member 1 (ABCB1), or cluster of differentiation 243 (CD243) is a glycoprotein which transports a wide variety of substrates across extra- and intracellular membranes. Pgp is particularly important in hepatic-biliary drug clearance. Initial studies into the impact of inflammation on this drug transporter has been investigated demonstrated that turpentine-induced acute-phase response (APR) in rats was associated with a 50–70% reduction in the in vivo hepatic expression and activity of Pgp within 24 to 48 hours after treatment. The reduction of Pgp expression and activity was attributed to suppression of MDR 1a/and 1b gene transcription (Piquette-Miller et al., 1998). Recently, Hanafy et al. characterized the effect of inflammation on selected molecular targets and transporters in a rat model of AA. They reported that the constitutive expression of drug transporters, adrenergic receptors, voltage-gated ion channels and cyclooxygenase (COX) 1 and 2 genes as well as their up/or down regulation during AA were tissue-specific. In the liver, organic cation transporter 1 (oct1), organic anion transporter p4a1 (oatp4a1) and multidrug resistance-associated protein 1 (mrp1) gene expression were

significantly reduced. There was also a general trend towards drug transporter down-regulation except for MDR-associated protein 3 (mrp3) and MDR 1a (mdr1a). The changes, however, were not statistically significant. There was no difference in the liver of AA and control animals with respect to COX-1, COX-2, ion channels and adrenergic receptor gene expression. In the kidney, AA resulted in a dramatic down-regulation of oatp2b1, mrp6 and bile salts export pump (bsep) gene expression. On the other hand, oatp4a1 demonstrated a trend towards up-regulation. Voltage-gated sodium channel (Nav1.2) gene expression was significantly induced (up to four-fold) in inflamed rats. There was a general trend towards renal cytokine gene up-regulation in inflamed animals. However, only the elevation of IFN-γ was statistically significant. There was no difference between AA and control animals with respect to COX-1, COX-2, other ion channels, and adrenergic receptor gene expression in the kidney. In the hearts of inflamed animals, oatp4a1 and mdr1a were reported to be significantly increased compared to control animals. Mrp3 was not detectable in inflamed animals. On the other hand COX-2, oatp1b2, oat2 and oat3 were detected only in inflamed rats. There was no significant difference between AA and control animals with respect to ion channels and adrenergic receptor gene expression. AA resulted in significant inhibition of expression of the cardiac COX-1 gene (Hanafy et al., 2012). These findings are not restricted to the transporters and proteins that are mentioned above. There are several studies in which alteration of other transporters has been evaluated during inflammatory conditions. For instance, it has been reported that DSS-induced rat colitis did not alter *peptide transporter 1* (*PepT1*) substrate

bioavailability despite certain modifications in the mRNA expression. However there is no report in the literature about the effect of AA on expression of *PepT1* protein; this remains to be further investigated (Radeva et al., 2007). Furthermore, Phillips et al revealed that glucose transporter 1 (GLUT1) was increased in chondrocytes stimulated with several growth factors and cytokines but GLUT 3 was only up-regulated by insulin-like growth factor 1 (IGF-I) (Phillips et al., 2005). On the other hand expression of GLUT 2 was not affected by pro-inflammatory mediators (Garnett et al., 2012).

Inflammatory conditions such as AA have been found to be associated with altered expression of drug receptors including beta-adrenergic, potassium , and calcium channel receptors secondary to over-expression of pro-inflammatory cytokines and/or NO (Hanafy et al., 2008; Kulmatycki et al., 2001; Sattari et al., 2003).

As an example, Western blotting experiments on the functional Cav1.2 subunit of cardiac L-type calcium channels revealed that the low molecular weight Cav1.2 subunit (190 kDa) was significantly reduced in inflamed animals, which was associated with the observed altered binding of the calcium channel blocker to the target protein and reduced potency of verapamil (Hanafy et al., 2008). Plasma proteins including alpha1 acid glycoprotein (an acute phase plasma protein that binds to cationic drugs) have been reported to be elevated during inflammatory conditions, which in turn can alter the clearance of highly cleared drugs such as verapamil (Piafsky et al., 1978).

1.1.2.3. Effects of inflammation and anti-inflammatory drugs on inflammation-induced alteration of PK/PD of drugs

It has been shown that inflammatory disorders, such as RA (Mayo et al., 2000) and Crohn's disease (Sanaee et al., 2011) as well as conditions including obesity (Hanafy et al., 2009) and experimental inflammation (Sattari et al., 2003) are associated with increased plasma concentrations but reduced response to some commonly used calcium channel blockers (such as verapamil), β-adrenoceptor blocking agents, and anti-arthritic drugs (Hanafy et al., 2008; Kulmatycki and Jamali, 2005; Schneider et al., 1981). Possible explanations for increased concentrations of these drugs are attributed to higher protein binding due to increases in α1-acid glycoproteins during the acute phase response, and/or reduced metabolism due to down regulation of cytochrome P450 isozymes (Kulmatycki and Jamali, 2005). Reduced drug-receptor binding secondary to a down-regulation of the target proteins expression has been assumed to be the possible explanation for the reduced response (Hanafy et al., 2008). The conditions are restored when the inflammation is controlled due to remission (Ling et al., 2009) or administration of drugs with anti-inflammatory properties such as pravastatin (Clements and Jamali, 2007) and valsartan (Hanafy et al., 2008). However, inflammation does not reduce the response to all cardiovascular drugs. For example, Daneshtalab et al. reported that the potency of valsartan, an angiotensin II receptor type I blocker, was not reduced by RA. Indeed, for valsartan, a trend towards increased potency has been observed (Daneshtalab et al., 2004).

1.1.3. Peptide transporters

1.1.3.1. Overview

The *peptide transporters* (*PepT*) 1/2 belong to the peptide transporter (PTR) family. The *PepT1*/2 are integral membrane proteins from the solute carrier proteins (SLC15A1/2) family, which are responsible for the cellular uptake of di- and tri-peptides in the body. In general, PTR family proteins function in a proton-dependent manner; movement of protons provides an inwardly directed electrochemical proton gradient, which in turn transports peptides against a substrate gradient. *PepT1* was initially discovered as the carrier responsible for the uptake of di- and tri-peptides in the small intestine, and *PepT2* as the main transporter responsible for peptide uptake in the kidney. In the last decade several members of this family have been cloned, either using classical cloning strategies or *in silico* techniques (i.e. by screening for the conserved domains) (Rubio-Aliaga and Daniel, 2008). Human *PepT1* and *PepT2* are located on chromosomes 13 and 3, respectively (Liang et al., 1995). The mammalian peptide transporter proteins *PepT1* and *PepT2* have 700–730 amino acid residues, and both are believed to have twelve trans-membrane domains and a large extracellular loop between the ninth and tenth trans-membrane domains (Daniel et al., 2006). The human peptide transporters exist as different genetic variants. In general, *PepT1* shows a lower level of genetic variability compared to *pept2*. Two main *PepT2* variants are hPEPT2*1 and hPEPT2*2. The first variant was shown to have a three-fold higher affinity for a model di-peptide than the other genetic variant in a

heterologous expression system. Moreover, both variants differ in their pH sensitivity (Anderle et al., 2006; Pinsonneault et al., 2004).

1.1.3.2. *PepT* family structure; relationship between the protein regions with their function

Structurally the *PepT* family belongs to the major facilitator super family (MFS) of secondary active transporters that typically contain 12, but sometimes 14, trans-membrane (TM) helices (Pao et al., 1998). By investigating the crystal structures that have been obtained from several MFS transporters, it appears that there is a common fold in the structure of the transporters, which consists of two 6-transmembrane bundles. These bundles assemble together in the membrane to form a 'V'-shaped transporter with a central substrate-binding site, which is formed between the two bundles. The *PepT* family adopts the canonical MFS fold between helices H1 to H6 in order to form the N-terminal bundle and helices H7 to H12 in order to form the C-terminal bundle. These bundles represent an inward facing conformation for the *PepT* family (Figure 1.3). The two bundles adopt similar structures and superimpose with a root mean square deviation (r.m.s.d.) of 2.7 A˚ over 153 Ca atoms. The substrate-binding site is located at the apex of an elongated hydrophilic cavity, which opens outwards from the interior towards the intracellular side of the membrane (Solcan et al., 2012).

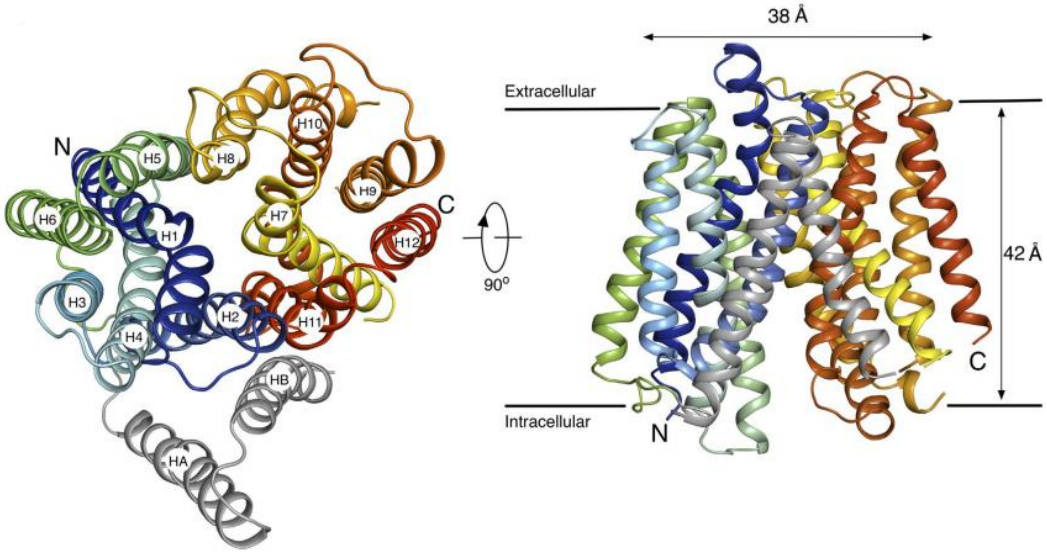

Figure 1.3. *PepT* structure reveals an inward open conformation. Overall structure of *PepT* viewed from the extracellular side of the molecule. The 12-trans membrane MFS fold is colored blue to red with helices HA and HB colored grey. The helices are labeled. The right-hand image shows a view in the plane of membrane with approximate dimensions of the molecule.

Peptide residues that seem crucial for transport or controlling transport characteristics such as substrate affinity and overall transport rate have been identified using site-directed mutagenesis approaches. In the first trans-membrane domain, changing Y12 to alanine reduced the transport capacity by 25%. In the second trans-membrane domain changing Y64 (a highly conserved residue) to alanine almost completely abolished the transport capacity, while a change to phenylalanine reduced substrate affinity. In the third trans-membrane domain, mutation of tyrosine residue Y91 to either a phenylalanine, alanine or

cysteine, resulted in reduced transport rates by 70–80%. Y167, which is highly conserved in all *PepT* family members in the PTR2 domain, has been shown in several studies to be essential for transport function. Changing W294 in the seventh trans-membrane domain to an alanine residue, increased affinity to a given substrate, but on the other hand reduced the V_{max}. Replacing E595 by an alanine residue abolished transporter function. Histidine residues that have been suggested to play a role in the pH dependency of the proteins have been also systematically studied. While H111, H121 and H261 still remain less well defined in their role in overall transporter function, H57 in the second trans- membrane domain has been proven in several studies to be a core residue for *Pept1* activity (Bolger et al., 1998; Chen et al., 2000; Fei et al., 1994; Kulkarni et al., 2003; Links et al., 2007; Meredith and Price, 2006; Yeung et al., 1998). The large extracellular loop between trans-membrane domain 9 and 10 seems to be not essential for transport function as it is not present in some species. However, the comparison of bacterial and mammalian transporters suggested that the functionally important amino acid residues may have to be revisited; further structural information is required in order to enable scientists to obtain a better understanding between protein architecture and function (Panitsas et al., 2006).

1.1.3.3. Regulation of peptide transporters

Alteration in sodium-proton exchanger expression, food intake, hormones, pathological conditions and drugs may alter peptide transporter expression levels and function. Since *PepT* function relies on the presence of a proton-motive force,

any alteration in sodium-proton exchanger NHE3 expression or function may result in impaired peptide transporter function and thereby reduce capacity for absorption of di- and tri-peptides or other substrates (Wada et al., 2005).

Food intake can also alter the expression of peptide transporter mRNAs and/or protein levels as well as their functions. For example, when rats were fed a high-protein diet, an increase in *PepT1* mRNA levels from 1.5- to two-fold in the small intestine was observed that was accompanied with an increase in the uptake of a model di-peptide. This increase was most likely due to an increase in mRNA stability and activation of the *PepT1* promoter by di-peptides and amino acids (Erickson et al., 1995; Shiraga et al., 1999). Gene expression of Ptr-2, a PTR family member, in yeast appeared to be up-regulated by selected di-peptides through activation of an ubiquitin-dependent proteolytic pathway (Turner et al., 2000). Prolonged periods of starvation also seem to increase *PepT1* expression substantially and time dependently, which is mediated by the peroxisome proliferator-activated receptor (PPAR) (Shimakura et al., 2006).

Hormones play an important role in the regulation of peptide transporter activity. It has been observed that hyperthyroidism decreases *PepT1* expression and activity in the small intestine in rats. This effect is most likely due to a direct or indirect effect of thyroid hormone on the transporter mRNA stability and/or transcription rate of *PepT1* (Ashida et al., 2004). Moreover, hypothyroidism increases *PepT1* and *PepT2* expression and protein levels in the rat's kidney, which could be normalized by thyroid hormone administration (Lu and Klaassen, 2006). A short-term exposure to leptin has been shown to increase *PepT1*-

mediated transport activity through an increase in *PepT1* translocation rate (Buyse et al., 2001), whereas in the absence of leptin in the ob/ob knockout mice, *PepT1* expression level and activity decreased (Hindlet et al., 2007). Insulin has been shown to elevate *PepT1*-mediated transport in the intestinal cells. This phenomenon is probably due to recruitment of *PepT1* protein from intracellular stores to the membrane surface (Thamotharan et al., 1999). Long-term exposure to basolateral epidermal growth factor (EGF) suppressed *PepT1*- mediated uptake in Caco-2 cells (Nielsen et al., 2001), and decreased *PepT*2 transport capacity and expression in the renal cell line, SKPT0193 (Bravo et al., 2004).

Disease conditions may lead to alteration of *PepT* expression and activity. For instance, inflammatory bowel diseases resulted in elevation of *PepT1* protein expression compared to healthy colon tissues (Merlin et al., 2001). Cytokines, including interferon-ɣ and tumor necrosis factor-α increase *PepT1* specific substrate uptake in mouse proximal and distal colon, but not in the small intestine. Since neither TNF-α nor IFN-ɣ increase *PepT1* mRNA expression in any segment of the intestine, this regulation is thought to be mediated by post-translational modifications (Vavricka et al., 2006).

PepT1 can be regulated by drugs, such as pentazocine, 5-fluorouracil, tacrolimus and cyclosporine A. Pentazocine, a sigma-1 receptor ligand, appeares to increase the uptake capacity of *PepT1*, most likely through up-regulation of gene expression (Fujita et al., 1999). The transport activity and mRNA expression of *PepT1* were slightly increased by 5-fluorouracil in the gastric cancer cell line

MKN45 (Inoue et al., 2005), whereas, tacrolimus and cyclosporine A reduced *PepT1*-mediated uptake in Caco-2 cells (Motohashi et al., 2001).

1.1.3.4. Substrate template for *PepT1*

Di- and tri-peptides, various peptide-like drugs such as β-lactam antibiotics, and non-peptidic compounds such as valacyclovir are taken up into intestinal epithelial cells by *PepT1* (Terada et al., 2000).

By considering different combinations of potential binding features in 3-D space, a template for *PepT1* substrates has been suggested (Figure 1.4). This model provides an indication of whether a substrate can be identified and transported by the transporter. This template has the following features as illustrated in Figure 1-4; *i)* a strong binding site for an N-terminal NH3 group; *ii)* a side chain R_1 (preference for the stereochemistry is L to accommodate R_1); *ii)* an extended planar backbone from the N-terminal Cα atom to R_2; *iv)* a hydrogen bond to the carbonyl group of the first peptide bond; *v)* alkylation of N_2 is permitted; *vi)* specific orientation of three groups at the second residue (usually L); *vii)* a hydrophobic pocket, which has a strong directional vector; *viii)* a carboxylate binding site; *ix)* available space for the side-chain R_3 (charged residues are high-affinity substrates of *PepT*) ; *x)* a second carboxylate binding site, with stereochemical consequences on the adjacent chiral center (usually L).
In this model, four essential binding sites are identified including the sites 1, 4,
7, and 8/10. On the other hand the 3-D layout of the template is defined by the other stereochemical and conformational features. In general, high-affinity

substrates can generally adopt the appropriate 3-D conformation easily at a low energy cost. It is the aggregate effect of all of these features, which governs whether a substrate has high, medium, or low affinity for *PepT1* (Bailey et al., 2000; Bailey et al., 2006; Rubio-Aliaga and Daniel, 2008).

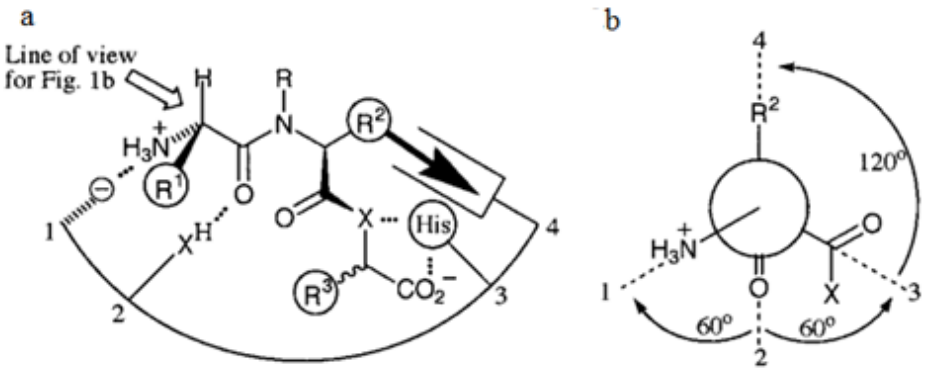

Figure 1.4. a) Substrate template for binding to *PepT1*. Free di-peptides terminate with O^- at X, whilst tri-peptides are extended by X=NH; possible complementarity to *PepT1* is indicated for the 4 main binding features; plain bonds (—) lie close to the plane of the paper. b) The orientation of the four main binding features, viewed as indicated in a.

1.1.3.5. Exploiting intestinal *PepT1* in order to enhance oral bioavailability of poorly absorbed drugs

One of the strategies to reduce the required dose of the drugs in order to achieve the desired bioavailability (hence, dose: efficacy ratio), is to synthesize prodrugs.

For polar drugs, prodrug design to target the membrane transporters such as *PepT1* has received particular attention in recent decades (Han and Amidon, 2000; Yu et al., 1996). *PepT1* is an ideal target in order to enhance bioavailability of poorly absorbed drugs due to its high level of expression in the intestinal epithelium, high capacity and broad substrate specificity. Several hydrophilic compounds seem to be absorbed efficiently via this path. Peptide transporters are involved in the absorption of a broad range of therapeutic agents, including ACE inhibitors, beta-lactam antibiotics, renin inhibitors, bestatin and *valacyclovir,* a valine ester prodrug of acyclovir. In addition to valacyclovir, there are several other examples in which researchers have attempted to improve oral absorption of various poorly absorbed drugs via attachment to amino acids or di-peptides in order to resemble *PepT1* substrates, mostly through ester bonds (Anand et al., 2004; Purifoy et al., 1993; Szczech, 1996; Weller et al., 1993)

In general, the activation of the prodrug after hydrolyzing by plasma or liver esterases does not involve toxicity issues because the only activation byproduct is an amino acid (or a dipeptide) and the parent drug (Anand et al., 2004; Krylov et al., 2013).

1.1.4. Peptide synthesis

Peptides are important organic compounds in the fields of health care and nutrition. Peptide synthesis is the production of peptides, in which multiple amino acids are linked via amide bonds (peptide bonds). Several approaches are now available for peptide production including chemical and enzymatic synthesis. In

general, the choice of synthesis depends on the objective of the synthesis. The chemical route appears to be a better option than the enzymatic methods in most cases. Moreover it has been considered as a fundamental tool for understanding the structure-function relationship in proteins and peptides, the discovery of novel therapeutic and diagnosis agents and the production of synthetic vaccines (Noya et al., 2003). Chemical synthesis of peptides is generally achieved through in-solution (Bourguet et al., 2010; Sakakibara, 1999) or solid phase (Coin et al., 2007) synthesis. Peptides are synthesized by coupling the carboxyl group or C-terminus of one amino acid to the amino group or N-terminus of another. Because of the possibility of unintended reactions, amino group protection is usually necessary. Chemical peptide synthesis usually starts at the C-terminal end of the peptide and ends at the N-terminus. This is the opposite of protein biosynthesis, which starts at the N-terminal end. In-solution peptide synthesis is the classical approach in peptide synthesis, which has been replaced by solid phase synthesis in the past few decades, especially in research labs; however it retains its importance in large-scale production of peptides for industrial purposes. Solid phase peptide synthesis (SPPS) is the process of elongation of a peptidic chain anchored to a solid matrix by successive additions of amino acids through an amide bond formation between the carboxyl group of the one amino acid and the amino group of another amino acid. This process will be continued until the peptide of the desired sequence and length is synthesized (Nilsson et al., 2005).

Different protection strategies may be used in peptide synthesis based on the requirements that a protecting group should meet in each process, particularly

with respect to the requirement of the preservation of other functionalities (Goodman et al., 2001). Most of the peptide syntheses are conducted based on two main schemes of protection, which are known as t-Boc/Bzl and Fmoc/tBu strategies. In the t-Boc/Bzl approach, the t-Boc (tert-butoxycarbonyl) group is used in order to protect the amino group and a benzyl or cyclohexyl is used for the side chain protection of several amino acids. On the other hand, in Fmoc/tBu strategy, the Fmoc (9-fluorenyl methoxycarbonyl) group is used for the protection of the amino group and the tert-butyl group is utilized for the protection of the side chains of several amino acids (Albericio, 2000).

Coupling reagents activate the carboxyl groups of the amino acids for coupling reaction. Nowadays, the most common coupling reagents are uronium and phosphonium salts and hydroxybenzotriazole (HOBT) because of the high reactivity, high coupling yield and higher specificity as compared to the conventional reagents, such as DCC/HOBT (dicyclohexyl-carbodiimide/1-hydroxybenzotriazol) and symmetric anhydrides. Chaotropic salts (CuLi, $NaClO_4$, KSCN) and mixtures of solvents (such as N,N-dimethylformamide, trifluoroethanol, dimethylacetamide and N-methylpyrrolidone) are used to improve the efficiency of coupling reactions (Miranda and Alewood, 1999).

1.1.4.1. In-solution peptide synthesis

This approach has been usually utilized for the synthesis of small peptides composed by only a few amino acid residues. The main advantage of this method is the isolation of the intermediate products, which in turn enables researchers to

purify the intermediate products after each step of synthesis. The intermediate products may further be deprotected and attached to other amino acids to obtain larger peptides of the desired sequence. This technique is highly flexible with respect to the chemistry of coupling and the combination of the peptidic blocks. New strategies for in-solution synthesis have been developed in order to design of the functional groups for the side chains and condensation of fragments for the synthesis of large molecules (Nilsson et al., 2005; Nishiuchi et al., 1998)

1.1.4.2. Solid phase peptide synthesis

Solid phase peptide synthesis (SPPS) was introduced by Merrifield in 1963. This method is the process of synthesizing a peptide chain, which is anchored to a solid matrix (resin). Amide bond formation is achieved by activating the carboxyl group of the incoming amino acid, which reacts with the amino group of the amino acid previously bound to the resin, until the peptide of the desired sequence and length is synthesized (Nilsson et al., 2005). This method has several advantages over the classical in-solution approach including convenience, acceleration of the overall process and the ability to achieve good yields of purified products. This allows the development of a large number of peptides with different sequences in short period of time. The synthesis method (Fmoc or t-Boc strategies), the nature of the solid carrier, the coupling reagents, and the procedure of cleavage of the peptide from the resin are the most relevant variables in SPPS. In a classic solid phase peptide synthesis, the N-protected C-terminal aminoacid residue is anchored via its carboxyl group to a resin. The protecting group is then

removed by treatment with trifluoroacetic acid (TFA) (for the t-Boc protecting group) or with piperidine (for the Fmoc protecting group). The next protected amino acid is coupled to the already synthesized peptide chain bound to the polymeric matrix; these coupling-deprotection cycles are repeated until the desired amino acid sequence is synthesized. Finally, the peptide is cleaved from the resin and side chain protecting groups are removed to yield the peptide with either a free acid or amide depending on the chemical nature of the functional group in the solid matrix. The cleavage reagent must be able to remove the protecting groups of the side chains of the amino acids at the time of cleavage (Amblard et al., 2006).

Resins that are used in SPSS should meet several requirements including appropriate and uniform size, being mechanically robust and easily filterable, chemically inert and chemically stable under the conditions of synthesis and highly accessible to the solvents allowing the penetration of the reagents and the enlargement of the peptide chain within its microstructure. They must not interact physically or chemically with the peptide chain and should be capable of being functionalized by a starting group. Several polymeric supports are now available. Some examples are the p-methoxybenzhydrylamine (MBHA), 4-hydroxymethyl-phenylacetamidomethyl (PAM) and hydroxymethyl functionalized resins used for t-Boc/Bzl, and the 4-(2',4'-dimethoxyphenyl-aminomethyl)-phenoxymethyl-polystyrene (Rink), diphenyldiazomethane and 2-chlorotrityl chloride functionalized resins used for Fmoc/tBu (Amblard et al., 2006; Pipkorn et al., 2013).

1.1.4.3. 2-chlorotrityl chloride resin

2-Chlorotrityl chloride resin is an incredibly versatile, acid labile resin that is vastly used in SPPS. The 2-chlorotrityl is often loaded at 0.45-0.65 mmol/gram for high-throughput synthesis of biologically active molecules. 2-chlorotrityl resin fulfills the requirements to obtain peptide synthesis in both high yield and purity. In classical method, Fmoc amino acids (t-butyl side chain protection) are reacting with the polystyrene-functionalized 2-chlorotrityl chloride using a DIPEA (diisopropylethylamine)/dichloromethane protocol followed by a DIPEA/methanol capping procedure. Eventually cleavage of the functional groups from the resin is generally achieved using 1-95% trifluoroacetic acid (TFA) in dichloromethane (DCM), depending on the nature of the process (Barlos et al., 1991; Hoekstra, 2001).

Amines have also been attached to 2-chlorotrityl chloride resin in good yield. Its ability to be successfully used for the immobilization of amines as well as the carboxyl group is one of the prominent advantages of this resin. It also has negligible perturbation of the attached chain/group due to formation of aldehyde, as seen with benzyl alcohol-type resins. In addition high loading capacity and ease of acid-mediated cleavage are other advantages of this resin (Hoekstra, 2001).

1.2. The study rationale and objectives

1.2.1. Study rationale

Inflammatory diseases such as RA (Mayo et al., 2000) and Crohn's disease (Sanaee et al., 2011) as well as conditions including obesity (Abernethy and Schwartz, 1988; Hanafy et al., 2009) and experimental inflammation such as AA (Sattari et al., 2003) are associated with increased plasma concentrations but reduced response to some commonly used cardiovascular drugs such as verapamil. This is mainly due to a reduced drug-receptor binding secondary to a down-regulation of the expression of target proteins expression (Hanafy et al., 2008). The condition is restored when the inflammation is controlled due to remission (Ling et al., 2009) or administration of drugs with anti-inflammatory properties such as pravastatin (Clements and Jamali, 2007) and valsartan (Hanafy et al., 2008).

Glucosamine (GlcN) is an amino-sugar with anti-inflammatory properties. There are several animal studies reporting strong disease-modifying effects and anti-inflammatory properties of GlcN (Hua et al., 2007; Hwang et al., 2010; Kim et al., 2011). The anti-inflammatory properties of GlcN have suggested its use in treatment of inflammatory conditions such as AA. Considering the high safety record of GlcN (Anderson et al., 2005), it should be an ideal drug in controlling inflammatory conditions.

GlcN bioavailability is limited, which prevents the achievement of therapeutic levels in plasma given the usual administered dosage in humans (1500 mg/day) (Aghazadeh-Habashi and Jamali, 2011). Indeed, administration of high

doses of GlcN in adjuvant model of arthritis in rats can prevent the emergence of inflammation and ameliorate the disease signs in early stages (Hua et al., 2005). Hence, a glucosamine pro-drug with a relatively high bioavailability that yields high plasma concentrations will be effective against inflammatory conditions such as AA.

1.2.2. Objectives

1.2.2.1. First objective

1.2.2.1.a. Hypothesis

GlcN has the ability to control inflammatory conditions such as AA.

1.2.2.1.b. Objective:

- To investigate whether GlcN is able to restore the reported down-regulation of calcium channels (Hanafy et al., 2008) and β-adrenergic (Guirguis and Jamali, 2003) target proteins caused by inflammation and consequently restores the diminished response to verapamil by controlling inflammation.

- To explore whether GlcN, in addition to its reported preventative effect on AA (Hua et al., 2005), reduces the symptoms of AA after the emergence of the disease.

- To investigate the effect of AA on pharmacokinetics of GlcN.

1.2.2.2. Second objective

1.2.2.2.a. Hypothesis

A GlcN pro-drug with relatively high GlcN bioavailability will be effective against AA and other bone and joint abnormalities.

1.2.2.2.b. Objective

- To synthesize GlcN derivatives with improved pharmaceutical superiorities and enhanced gut permeability.
- To conduct a series of tests to assess the stability of the newly synthesized prodrugs in different physical and chemical conditions and biological homogenates.
- To determine the bioavailability of selected derivatives.
- To study the efficacy of the candidate pro-drug(s) in prevention of AA.

Chapter 2

[1]Glucosamine and adjuvant arthritis: A pharmacokinetic and pharmacodynamic study

2.1. Introduction

Glucosamine (GlcN), a naturally occurring amino-monosaccharide, is a major precursor in the biochemical synthesis of glycosaminoglycans that are present in connective tissues (Hua et al., 2005). It is a key element to maintain the flexibility, strength, and elasticity of cartilage tissues and serves as a chondroprotective agent in animal models of osteoarthritis (OA) (Naito et al., 2010; Oegema et al., 2002; Tiraloche et al., 2005; Wang et al., 2007; Wen et al., 2010) and AA (Hua et al., 2005). In addition, GlcN is reported to exert neuro-protective effects by suppression of inflammatory processes (Hwang et al., 2010) and to have promising effects in the treatment of atopic dermatitis like skin lesions in mice (Kim et al., 2011).

GlcN is broadly used in the treatment of OA (Vrublevska et al., 2007), with its effect reported to range from negligible (Rozendaal et al., 2008) to as much as those reported for non-steroidal anti-inflammatory drugs (NSAIDs) (Muller-Fassbender et al., 1994). Hence, the beneficial effect of GlcN in OA is a subject of debate (Aghazadeh-Habashi and Jamali, 2011). Nevertheless, GlcN at a dose of 300 mg/kg prevents emergence of AA in rats and significantly reduces NO and prostaglandin E2 levels in plasma (Hua et al., 2005). In humans, GlcN

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formulations that yield plasma concentrations as high as 1.3 µg/ mL appear to be effective in treating human OA (Aghazadeh-Habashi and Jamali, 2011).

The aim of this study was to further investigate the anti-inflammatory effect of GlcN using two indicators: *(i)* to explore whether GlcN, in addition to its reported preventative effect (Hua et al., 2005), reduces the symptoms of AA after the emergence of the disease, and *(ii)* to investigate if GlcN restores the reported down-regulation of calcium channel and β-adrenergic target proteins caused by inflammation. Diseases such as RA (Mayo et al., 2000) and Crohn's disease (Sanaee et al., 2011) as well as conditions including obesity (Abernethy and Schwartz, 1988;Hanafy et al., 2009) and experimental inflammation (Sattari et al., 2003) are associated with increased plasma concentrations, but reduced response to some commonly used cardiovascular drugs such as verapamil, which is due to a reduced drug-receptor binding secondary to a down regulation of the expression of target proteins (Hanafy et al., 2008). The condition is restored when the inflammation is controlled due to remission (Ling et al., 2009) or administration of drugs with anti-inflammatory properties such as pravastatin (Clements and Jamali, 2007) and valsartan (Hanafy et al., 2008). Considering the high safety record of GlcN (Anderson et al., 2005), it would be an ideal drug in restoring the response to cardiovascular medications. We also studied the effect of inflammation on the pharmacokinetics (PK) of GlcN. GlcN is reported to have a low oral bioavailability due to its loss through first-pass metabolism (Setnikar and Rovati, 2001). Inflammation is known to cause an elevation of plasma

concentrations of drugs with intermediate and high first-pass metabolism (Emami et al., 1998).

2.2. Materials and methods

2.2.1. Chemicals

D-(+)-Glucosamine (GlcN), (±)-verapamil hydrochloride, aspergillus nitrate reductase, lactic dehydrogenase, FAD, NADPH, Tris base, Tris hydrochloride, sodium nitrite, sodium nitrate, pyruvic acid, phosphoric acid, naphthalene ethylene diamine, sulphanilamide and protease inhibitor cocktail were purchased from Sigma-Aldrich (St. Louis, MO, USA). Squalene was purchased from Kodak (Rochester, NY, USA). *Mycobacterium butyricum* was purchased from Difco (Detroit, MI, USA). Polyethylene glycol 400 was purchased from Wiler (London, Ontario, Canada).

2.2.2. Animals and treatments

The experimental protocol was approved by the Health Sciences Animal Policy and Welfare Committee of the University of Alberta. The study was performed on male Sprague–Dawley (SD) rats (250–280 g) which were acclimatized in a controlled temperature room with a 12:12 h dark/light cycle. Animals were randomly assigned to control and inflamed groups. Control groups were further divided into Control GlcN and Control-Placebo subgroups. Inflamed groups were divided into four subgroups: Inflamed-Prevention, Inflamed-Placebo, Inflamed–Treated–Continued, and Inflamed–Treated–Discontinued. At the end of the

experiment each group consisted of six animals. On day one, 0.2 ml of *M. butyricum* in squalene (50 mg/mL) or physiological saline was injected to the inflamed and control groups, respectively. The Inflamed-Prevention and Control-GlcN groups were administered GlcN HCl/water (300 mg/kg/day, orally) commencing on day zero, while the Control-Placebo and Inflamed-Placebo groups received water. The treated groups received the same dose of GlcN but only after developing the early signs of AA. After improvement, GlcN administration continued in the Inflamed–Treated–Continued group, but was stopped in the Inflamed–Treated–Discontinued rats. AA was monitored by recording various signs during the experiment including increased paw thickness, reduced weight gain, erythema and scaling of the right paw, and formation of eye nodes. On day 18, AI was calculated according to the method previously described (Piquette-Miller and Jamali, 1995). A maximum score of 14 could be assigned to each animal as follows: On a 0–4 basis hind paws were scored where zero was no virtual signs of arthritis, one was involvement of a single joint, two was involvement of more than one joint and/or ankle, three was involvement of several joints and ankle with moderate swelling, and four was involvement of several joints and ankle with severe swelling. Each forepaw was scored on a 0–3 basis scoring system where 0 was none; one was involvement of single joint, two was involvement of more than one joint and/or wrist, and three was involvement of wrist and joints with moderate-to-severe swelling. The paw thickness was measured using a caliper (Mitutoyo Canada Inc., Toronto, ON) with a sensitivity of 25 µm. An increased paw thickness of almost 500 µm was considered an early

sign of AA emergence and a return to almost 200 µm was considered improvement.

2.2.3. Serum nitrite analysis

Using a previously described method (Grisham et al., 1995), serum nitrite was measured. Briefly, nitrate was reduced to nitrite by incubating 100 µl of samples or standard solutions with 10 µl of Aspergillus nitrate reductase (10 U ml^{-1}), 25 µl of 0.1 mM FAD, 50 ml NADPH (1mM), 25ml of 1M HEPES (pH 7.4) and 290 µl of deionized water for 30 min at 37º C. This was followed by adding 5 µl of lactate dehydrogenase (1500 U ml^{-1}) and 50 µl of 100 mM pyruvic acid and re-incubation for another 10 min at 37º C. Subsequently, 1 ml of Griess reagent (0.2% naphthalene ethylene diamine and 2% sulphanilamide in 5% phosphoric acid) was added and incubated for 10 min at room temperature. The absorbance of the developed color was measured at 543 nm using a V_{max} Molecular Devices plate reader (BioTek Instruments Inc., Winooski, VT, USA). Standard curves were linear over the concentration range of 3–200 µM ($r^2 \geq 0.99$, coefficient of variation $\leq 10\%$).

2.2.4. Effect of inflammation and GlcN treatment on the cardiac target proteins and verapamil pharmacodynamics

The cardiovascular response to verapamil (PR-interval) was measured in all groups described under ‘‘Animals and treatments’’. To measure the PR-interval, ECG leads were implanted s.c. on day 14 for the placebo, control and prevention

groups and on day 18 for the treated groups that coincided with the reversal of the AA signs in response to treatment in the Inflamed–Treated–Continued group. A baseline ECG was measured for each rat after recovery from the operation. This was followed by a single oral dose of 25 mg/kg verapamil and the measurement of average PR-interval at 0, 20, 40, 60, 80, 100, 120, 140, 180, 240 min.

2.2.5. Target protein expression in the heart

Heart samples were prepared using a previously reported method (Sattari et al., 2003) with minor modifications. After each set of the experiments, animals were anesthetized and their blood was removed by cardiac puncture. The thoracic cavity of the rats were opened and heart was excised and washed in ice-cold Tris buffer (0.05 M; pH = 7.4). It was then placed in liquid nitrogen for instant freezing and kept in -80º C. On the day of experiment, hearts were thawed, weighed, and cut into small pieces. 10 mL of a mixture of ice-cold Tris buffer/protease inhibitor cocktail (19:1) was added to each heart followed by homogenization using a Brinkmann homogenizer (Kinematica AG, Littau-Lucerne, Switzerland) for 30 s. The crude homogenate was then centrifuged at 5000 g at 4º C for 10 min to disrupt nuclei and cytoskeleton particles. The supernatants were divided into aliquots of 100 ml for Western blotting. The protein contents of the samples were determined using a commercially available protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA). Briefly, 25 ml of alkaline copper tartarate solution was added to 5 mL of the samples, followed by adding a 250 ml of 10% Folin reagent. The absorbance of the samples was

measured by spectrophotometer at 570 nm using a Vmax Molecular Devices plate reader (Bio-Tek Instruments Inc.). Standard curves were linear over the concentration range between 0.2 and 1.44 mg/ml. ($r^2 \geq 0.99$, coefficient of variation $\leq 10\%$).

The density of L-type calcium channel, β-1 adrenergic receptor and ryanodine-2 receptor (RyR2) proteins were measured by Western blotting approach. Briefly, the cardiac samples were boiled at 100º C and 50 µL of each samples was loaded on a precast 4–20% Tris–HCl gel (Bio-Rad laboratories) and SDS-gel electrophoretic separation was conducted for1 h at 200 V. The separated proteins were transferred onto a nitrocellulose membrane and incubated overnight in a nonspecific binding blocking solution (2% bovine serum albumin, 5% skim milk and 0.05% Tween 20 in Tris-buffered saline). After washing the membranes with the washing buffer, the primary antibodies were added. For detecting the L-type calcium channels, the primary antibody was the polyclonal rabbit anti-calcium channel – Cav1.2 subunit (Millipore Corporation, MA, USA).

Polyclonal rabbit anti-β-1 adrenergic receptor (Thermo scientific, Rockford, IL, USA) and monoclonal mouse anti-ryanodine receptor (Abcam Inc., Cambridge, MA, USA) were the primary antibodies for detection of β1-adrenoreceptor and RyR2, respectively. A dilution of 1:1000 of polyclonal rabbit anti-β actin (Abcam Inc., Cambridge, MA, USA) was used for detecting the beta actin (β actin). Following addition of the primary antibodies, the secondary antibodies (1:7500 dilution of horseradish peroxidase-conjugated goat anti-rabbit or anti mouse IgG antibody; Bio-Rad Laboratories, ON, Canada) were added to

produce a luminescent derivative of the protein of interest, allowing it to be detected and quantified by chemiluminescence (ECL Western blotting detection reagents; Bio-Rad Laboratories, ON, Canada). The density of the bands was determined using Image J software (National Institutes of Health, Bethesda, MD, USA).

2.2.6. Effect of inflammation and GlcN treatment on verapamil pharmacokinetics

All animals were cannulated in their right jugular vein according to a previously described method (Dagenais and Jamali, 2005). Briefly, rats were anesthetized with a mixture of halothane/oxygen. A polyethylene cannula (Dow Corning Corp., Midland, MI, USA) tipped with 2 cm of silastic tubing (Becton Dickinson, Sparks, MD, USA) was inserted into the right jugular vein. A 3 day wash-out period was allowed between pharmacodynamic and pharmacokinetic studies so that for placebo, control and prevention groups the blood samples were collected on day 17, whereas in the treated groups blood sampling took place on day 21. All the animals were administered a single oral dose of 25 mg/kg verapamil and serial blood samples were collected at 0, 15, 30, 45, 60, 90, 120, 180 and 240 min and assayed for verapamil enantiomers in plasma using a previously reported method (Shibukawa and Wainer, 1992) with some modification (Mehvar and Reynolds, 1996). Standard curves were linear over the concentration range of 0.01–10 µg/mL ($r^2 \geq 0.99$, coefficient of variation $\leq 10\%$).

2.2.7. Effect of AA on glucosamine pharmacokinetics

A different set of animals was assigned to study the pharmacokinetic characteristics of GlcN in control and inflamed rats. Animals were randomly divided into two groups (Inflamed or Control, n = 6). AA was developed in the inflamed animals as described under ‘‘Animals and treatments’’. On day 14, all the animals were cannulated in the right jugular vein and after an overnight recovery period a dose of GlcN.HCl (300 mg/kg GlcN equivalent) was administered and serial blood samples were collected at 0, 15, 30, 45, 60, 90, 120, 180, 240 min for the measurement of GlcN in plasma as previously reported (Jamali and Ibrahim, 2010). Standard curves were linear over the concentration range of 0.05–20 μg/mL ($r^2 \geq 0.99$, coefficient of variation ≤ 10%).

Urine was collected over a period of 24 h to determine the percent of intact GlcN excreted in the urine.

2.2.8. Data treatment and statistical analysis

Data are presented as mean ± SD. For the effect of AA on GlcN pharmacokinetic a two-tailed Student t-test was used. Differences among the six study groups in area under the concentration–time curve from 0 to 4 h (AUC0–4 h), maximum plasma concentration (C_{max}), and area under the effect time curve (AUEC) were determined utilizing one-way ANOVA, followed by the Bonferroni post-test. Statistical analyses were carried out using Prism software (GraphPad Software Inc., San Diego, CA, USA) at $p < 0.05$.

2.3. Results

2.3.1. Emergence of AA and effect of GlcN on the disease development

As expected, rats that received *M. butyricum* but were not dosed with GlcN commencing on the day of adjuvant injection (Inflamed-Placebo, Inflamed–Treated–Continued, and Inflamed–Treated–Discontinued) exhibited significant increased paw thickness in 8–10 days post-adjuvant injection (Fig.2.1a). Rats that were administered GlcN from day one (Inflamed-Prevention group), on the other hand, did not demonstrate significant increases in paw thickness despite the adjuvant injection (Fig.2.1a). The Inflamed–Treated–Continued group developed significant increased paw thickness but subsequent daily GlcN doses significantly improved this condition in 4–5 days. The GlcN treatment was discontinued in the Inflamed-Treated–Discontinued group upon significant improvement in paw thickness. This resulted in the return of the disease presented as paw thickness in Fig.2.1b. The emergence of AA was associated with increased AI score and occurrence of eye nodes as well as erythema and scaling of the right paw (Table 2.1). No sign of arthritis was observed in control groups or in the Inflamed-Prevention group (i.e., AI = 0). All Inflamed-Placebo animals developed AA signs; on day 18 post adjuvant injection, the average AI score was 8.5 for this group showing severe development of AA. GlcN decreased the intensity of the effects of inflammation in both treated groups. Inflammation slowed down body weight gain. GlcN had a significant effect on preventing the slowdown of the body weight gain in the animals who received *M. butyricum* (Fig.2.2).

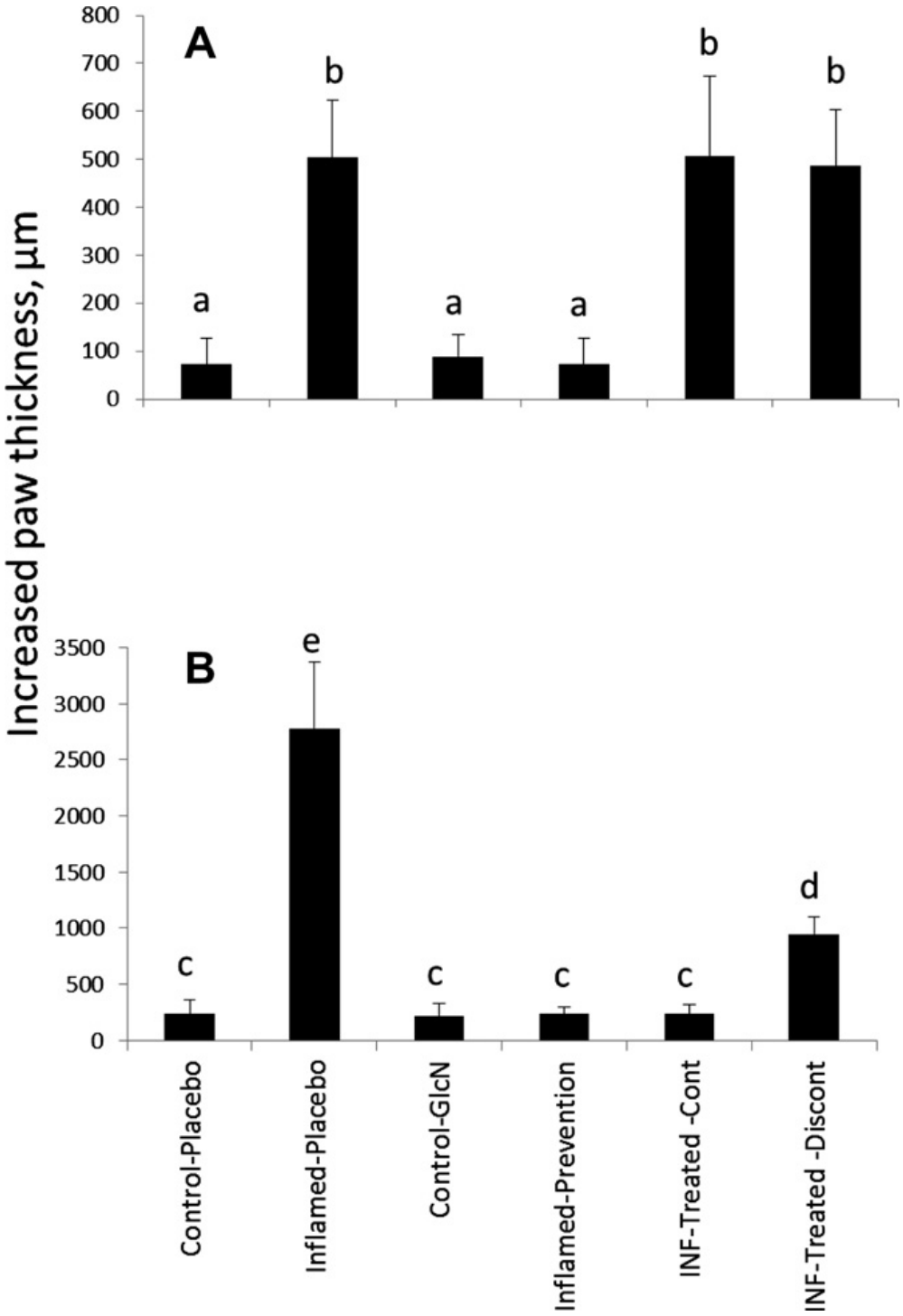


Fig.2.1. Average increase in paw thickness after developing early signs of AA (day 8–10) (A) and at the end of the experiment (B). Error bars represent standard deviation. The same character indicates no significant difference between means at $p < 0.05$; n = 6/group.

Table 2.1. Signs of adjuvant arthritis on day18 post-adjuvant injection. The number of animals in each group (n = 6) that developed (+) or did not develop (-) eye nodes and erythema and scaling of the right paw. Arthritic index is shown in the last column.

	Formation of nodes around the eyes	Erythema and scaling of the right paw	Arthritic index (AI) ± SD
Inflamed-Placebo	++ ++ ++	++ ++ ++	8.5 ± 2.2
Control-Placebo	– – – – – –	– – – – – –	0
Inflamed-Prevention	– – – – – –	– – – – – –	0
Control-GlcN	– – – – – –	– – – – – –	0
Inflamed–Treated–Continued	– – – – – –	+– – – – –	2.3 ± 1.6
Inflamed–Treated–Discontinued	– ++ + – –	– + + + + +	5.5 ± 4.2

Each sign represent one animal.

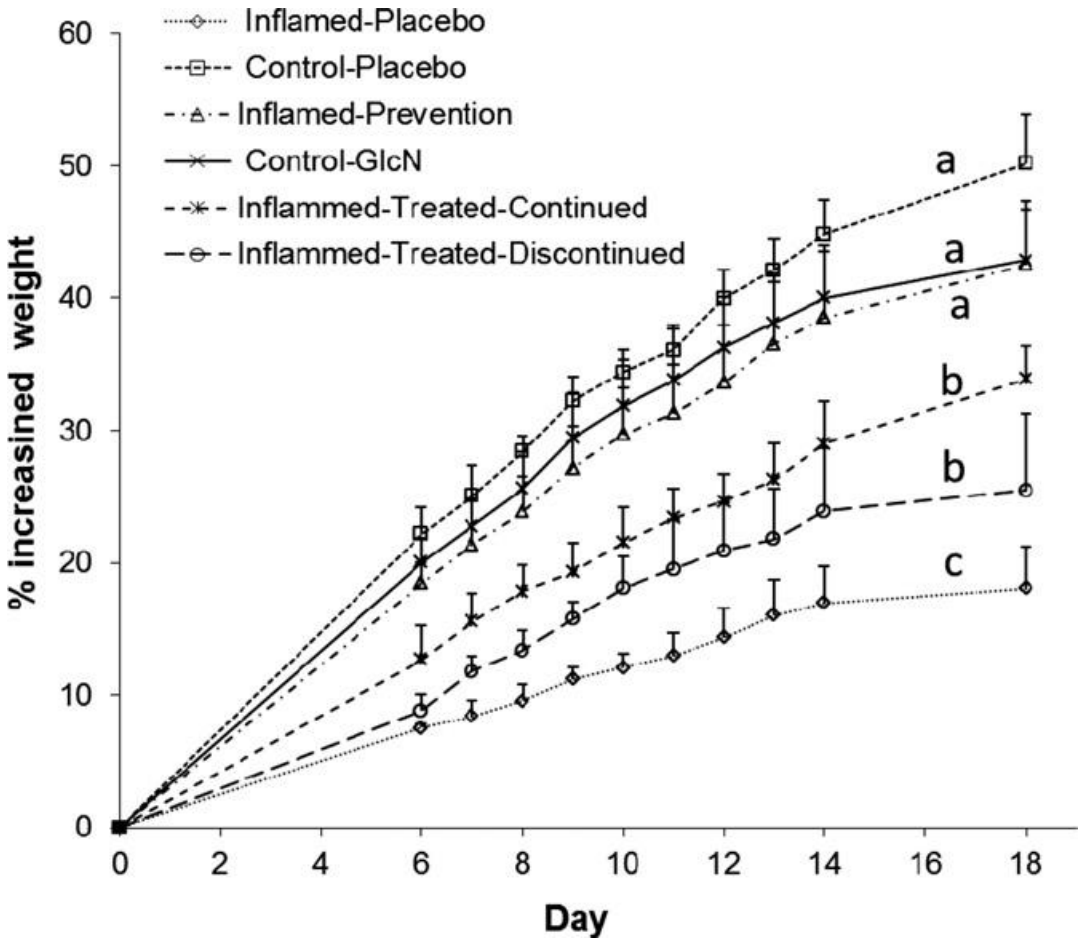


Fig. 2.2. The average percent weight from baseline (n=6). Error bars represent standard deviation. The same character indicates no significant difference between means at $p < 0.05$.

2.3.2. Serum nitrite concentration

Inflammation resulted in increased serum nitrite levels. Prevention of AA with GlcN resulted in serum nitrite concentrations equal to those of healthy control animals (Fig.2.3). Although continued treatment with GlcN significantly reduced the nitrite level as compared with the Inflamed-Placebo group, it did not normalize nitrite levels to the control level. The serum nitrite concentrations in Inflamed–Treated-Discontinued group were approximately the same as those in the Inflamed-Placebo group (Fig.2.3).

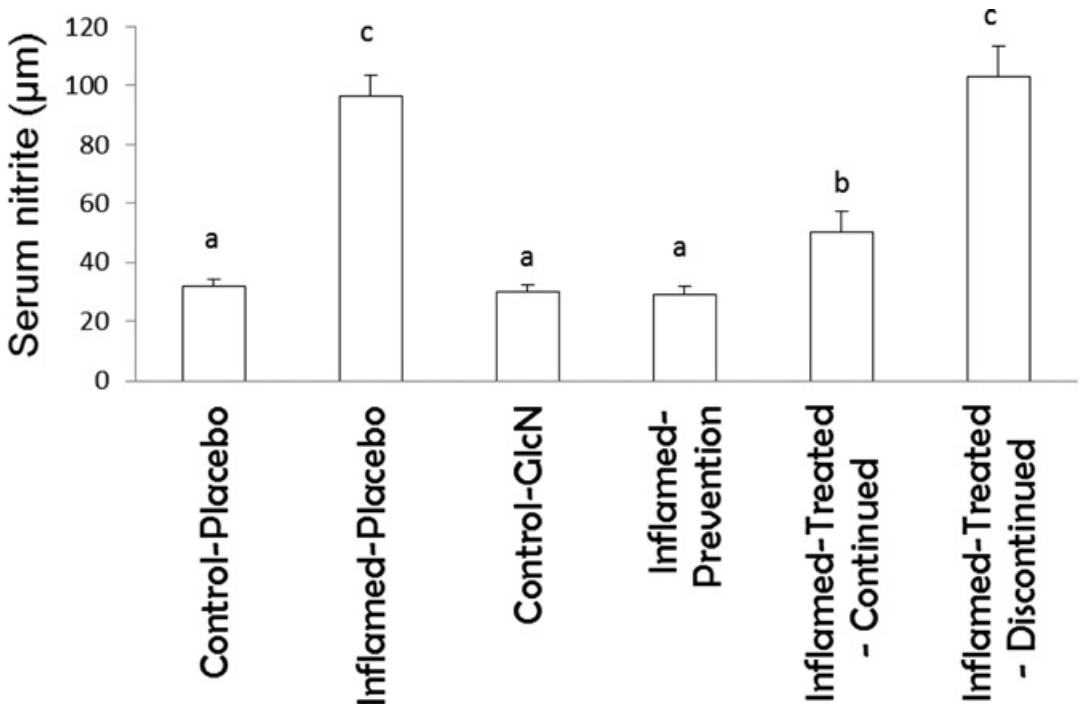


Fig.2.3. The effect of inflammation, prevention, and treatment with GlcN on serum nitrite concentrations (n = 6); error bars represent standard deviation. The same character indicates no significant difference between means at $p < 0.05$.

2.3.3. Effect of inflammation and GlcN treatment on the cardiac target proteins and verapamil pharmacodynamics

Verapamil prolonged PR-interval in all groups, with a peak effect of around 60 min post-dose (Fig.2.4a). Inflammation led to a significant reduction in response to verapamil in terms of PR-interval prolongation. Inflamed animals showed 78% reduction in the corresponding area under the effect curve (AUEC) compared to the control group (Fig.2.4b). Prevention and continued treatment with GlcN restored the diminished effect of verapamil in inflamed rats. AUEC values for the Inflamed Treated–Discontinued group was not significantly different from those for the inflamed group that did not receive GlcN (Inflamed-Placebo).

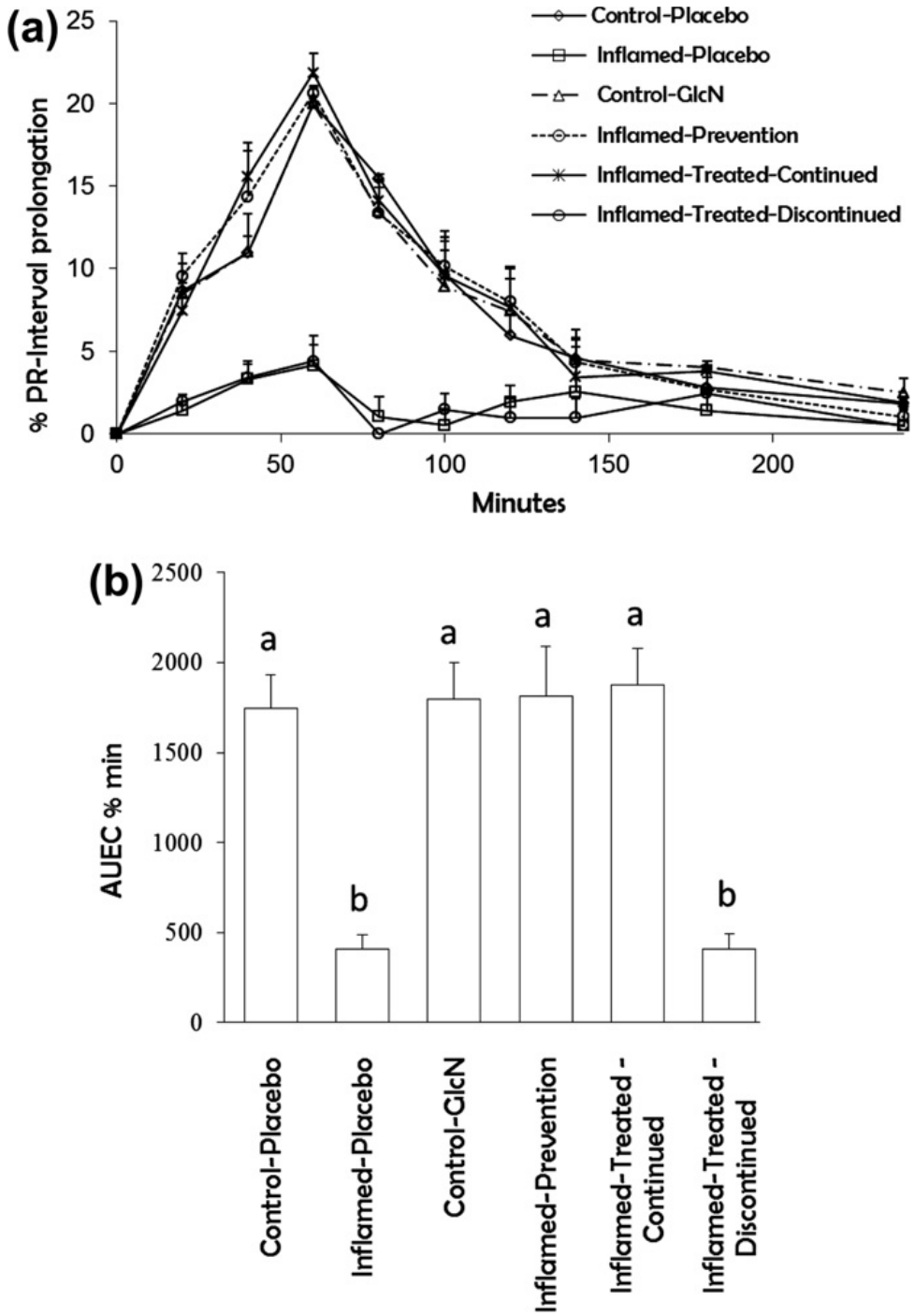


Fig. 2.4. (a) The effect of inflammation, prevention with GlcN, continued GlcN treatment and discontinuation of GlcN administration on PR-interval prolongation after a single oral dose of 25 mg/kg verapamil. (b) AUEC values generated from the effect–time profiles. The same character indicates no significant difference between means at $p < 0.05$; n = 6/group; error bars represent standard deviation.

2.3.4. Target protein expression

As depicted in Fig.2.5, inflammation significantly down-regulated the expression of the low molecular weight cardiac Cav1.2 subunit (190 KDa) by 63% and β1 adrenoreceptor by 46% as compared with the control groups. Prevention and continued treatment with GlcN maintained the density of the proteins at the control level. The expression of these proteins in the Inflamed–Treated–Discontinued group was approximately the same as in the Inflamed-Placebo animals (Fig.2.5a and b). There was also a trend toward a reduced expression of the high molecular weight subunit (210 KDa) in the inflamed animals that did not reach significance (Fig.2.5a). The expression of the cardiac RYR2 was influenced by neither inflammation nor the treatment (Fig.2.5c).

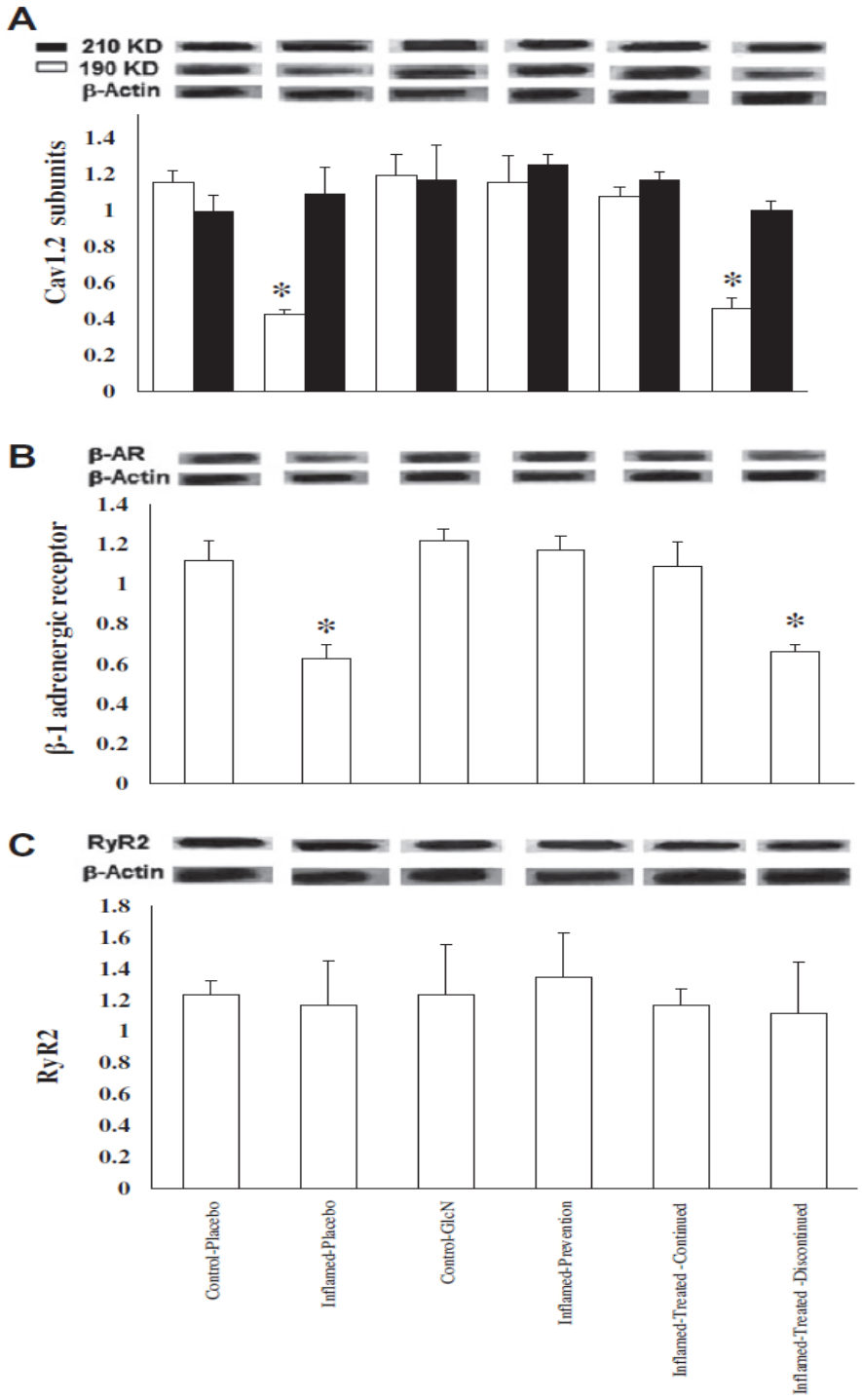


Fig.2.5. The effect of inflammation, prevention and treatment with GlcN on the levels of three cardiac proteins relative to β-actin content presented as both the average density and the response bands. (A) Cav1.2 subunits (190 KDa and 210 KDa), (B) β-1 adrenergic receptor and (C) ryanodine receptor 2. *Significantly different from the groups with no asterisk; $p < 0.05$; error bars represent standard deviation. (n=6)

2.3.5. Effect of inflammation and GlcN treatment on verapamil pharmacokinetics

As depicted in Table 2.2 and Fig. 2.6, inflammation resulted in increased plasma concentrations of verapamil as compared with those in control rats. Inflamed-Placebo rats had substantially and significantly greater AUC values (Table 2.2). Prevention of inflammation with GlcN resulted in plasma S- and R-verapamil concentrations being approximately super-imposable to those of healthy controls. After the emergence of AA, continued treatment with GlcN (i.e. Inflamed–Treated–Continued group) significantly reduced AUC but did not bring the level down to those of the healthy control rats. The AUC values for the Inflamed–Treated-Discontinued group were close to those of Inflamed-Placebo.

Table 2.2, Effect of inflammation, prevention and treatment with GlcN on the pharmacokinetics of verapamil enantiomers.

Group	Inflamed-Placebo	Control-Placebo	Inflamed-Prevention	Control-GlcN	Inflamed–Treated–Continued	Inflamed–Treated–Discontinued
C_{max}, μg/mL						
R	1.54 ± 0.21^{c}	0.39 ± 0.03^{a}	0.37 ± 0.08^{a}	0.35 ± 0.2^{a}	0.88 ± 0.37^{b}	1.59 ± 0.26^{c}
S	5.15 ± 0.12^{c}	1.42 ± 0.06^{a}	1.41 ± 0.07^{a}	1.45 ± 0.6^{a}	3.33 ± 0.33^{b}	5.26 ± 0.67^{c}
AUC_{0-4}, μg h/mL						
R	4.42 ± 0.35^{c}	0.90 ± 0.07^{a}	0.87 ± 0.06^{a}	0.89 ± 0.07^{a}	1.91 ± 0.40^{b}	4.49 ± 0.42^{c}
S	14.94 ± 0.68^{c}	3.37 ± 0.13^{a}	3.32 ± 0.18^{a}	3.44 ± 0.13^{a}	10.15 ± 0.63^{b}	15.08 ± 0.80^{c}

A single oral dose of 25 mg/kg verapamil was given to the animals and the PK characteristics were measured. Data are shown as mean (± SD). Different superscript letters indicate significant differences between means in a row ($P < 0.05$); similar superscript letters denote no significant differences between means in a row.

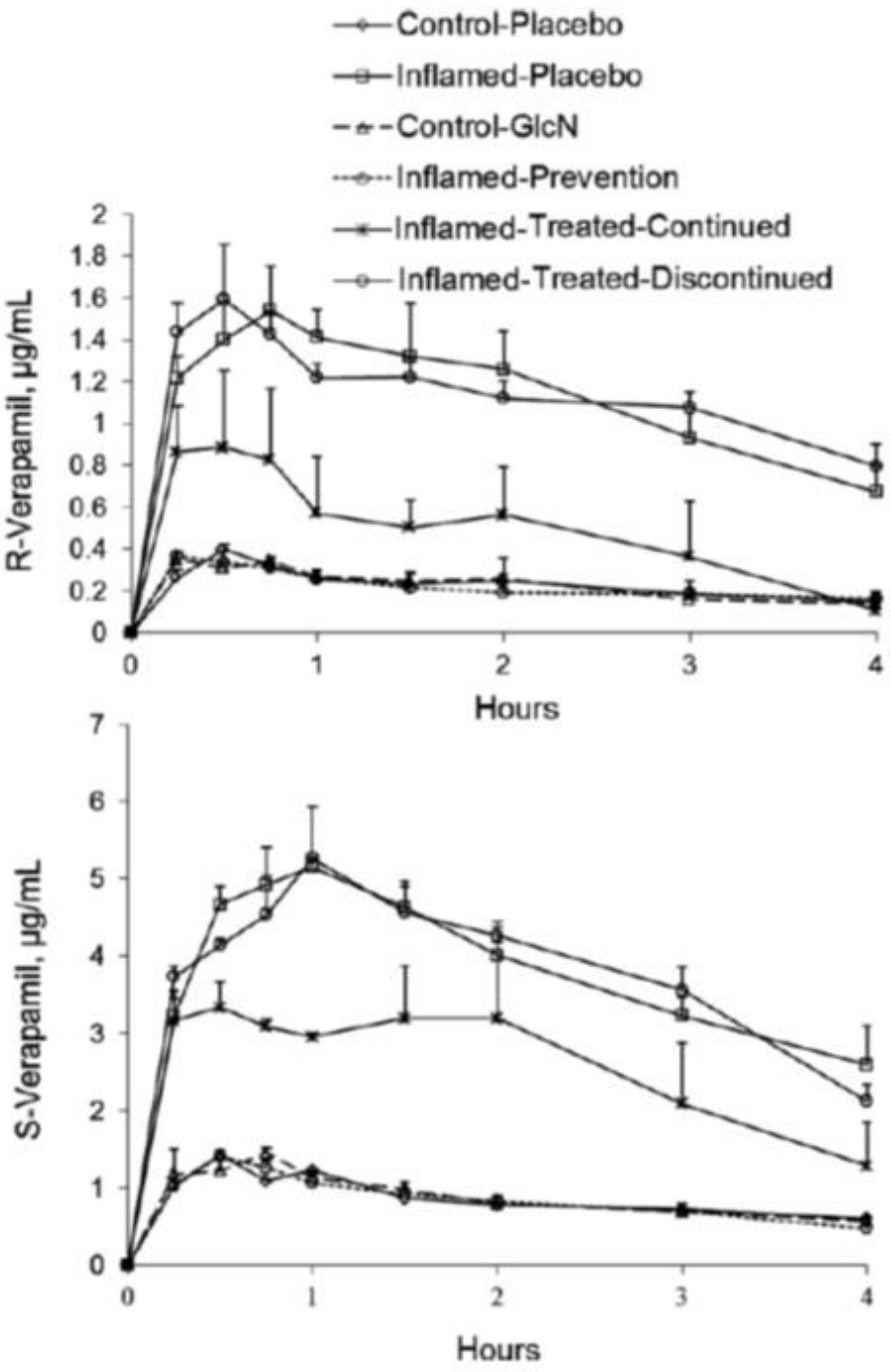


Fig. 2.6. The effect of inflammation, prevention, and treatment with GlcN on plasma R- and S-verapamil concentration–time profile (n = 6) following a single 25 mg/kg oral dose of racemic verapamil.

2.3.6. Effect of AA on GlcN pharmacokineticS

AA did not significantly affect the pharmacokientics of single 300 mg/kg doses of GlcN as no significant differences were noticed between the groups in C_{max}, AUC_{0-4} and the percent of dose excreted in the urine in 24 h (Fig.2.7 and Table 2.3).

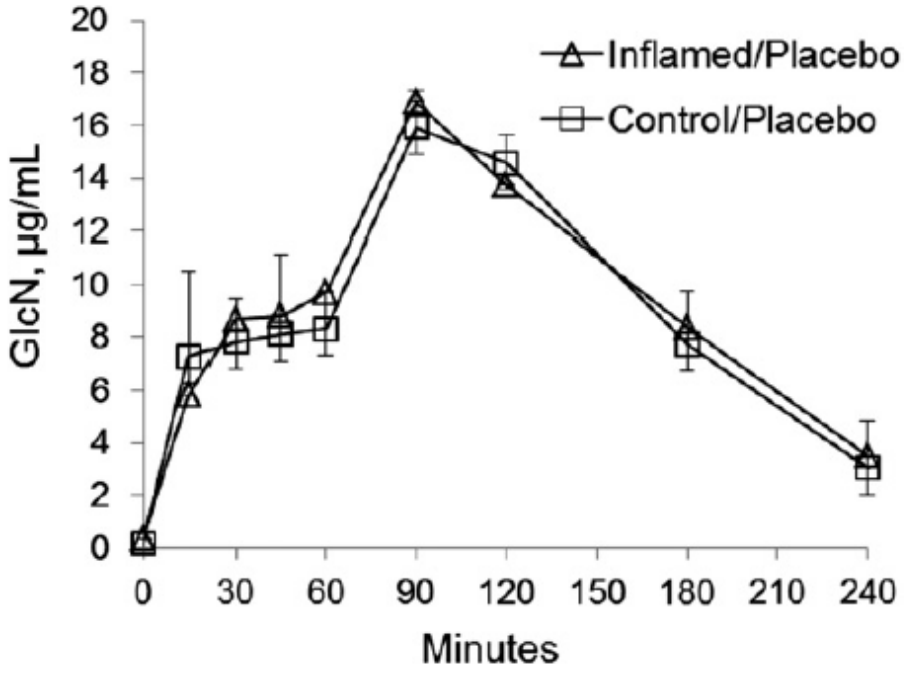


Fig.2.7. Mean plasma concentration versus time profiles of glucosamine in control and inflamed rats following oral administration of single 300 mg/kg doses.

Table 2.3. PK characteristics of GlcN in control and inflamed rats.

Group	Inflamed/Placebo	Control/Placebo
C_{max} (µg/mL)	16.9 ± 1.4	15.5 ± 1.9
T_{max} (min)	90	90
AUC_{0-4} (µg h/mL)	37.8 ± 1.9	36.5 ± 1.8
% of Dose excreted in the $urine_{0-24}$	2.2 ± 0.3	2.2 ± 0.3

2.4. Discussion

Our data confirm the previous observation that GlcN prevents the emergence of AA (Hua et al., 2005). More importantly, we are reporting, for the first time, that GlcN is also effective in reducing signs of AA after its emergence (Fig.2.1). These beneficial effects of GlcN were associated with decreased levels of the pro-inflammatory biomarker, serum nitrite (Fig.2.3) and enhanced body weight gain (Fig. 2.2). The mechanism for the anti-inflammatory effect of GlcN is not quite clear but it has been attributed to its ability to suppress neutrophil functions (Hua et al., 2005), to inhibit NF-kB activation (Hwang et al., 2010), to suppress the maturation of naïve CD4+ T cells to Th2 cells (Kim et al., 2011), and to induce tissue TGF β1 and connective tissue growth factor (Ali et al., 2011).

We examined the beneficial effects of GlcN following a dosage regimen of 300 mg/kg/day that yields a plasma peak concentration of approximately 16 µg/mL (Fig.2.7) that is substantially higher than those recorded for humans following the commonly used dosage of 1500 mg/day (<3.4 µg/mL) (Aghazadeh-Habashi and Jamali, 2011). This, however, does not imply that lower doses are ineffective in the treatment of rat AA. Indeed, doses as low as 40 mg/kg/day that corresponds to GlcN plasma mean peak concentration of 1.32 ± 0.24 µg/mL start demonstrating preventative effects on emergence of AA in the rat (Agahzadeh-Habashi et al., 2013). Whether these low preventative doses are effective in the treatment of existing disease remains to be studied.

It is known that Inflammatory diseases such as RA (Mayo et al., 2000) and Crohn's disease (Sanaee et al., 2011) as well as conditions including obesity

(Abernethy and Schwartz, 1988; Hanafy et al., 2009) and experimental inflammation such as AA (Sattari et al., 2003) can result in serious cardiovascular complications (Gerli and Goodson, 2005; Kapetanovic et al., 2011) and down regulation of the calcium channel and β-adrenergic target proteins, hence, reduced response to some pharmacotherapeutic approaches (Kulmatycki and Jamali, 2005). Our present data confirm the adverse effect of inflammation on these target proteins and reduced response to verapamil, a calcium channel blocker. Moreover we have, for the first time, demonstrated that GlcN restores these conditions by both preventing and controlling the disease. Our group has previously reported that a control of the severity of the inflammation by various means results in restoration of the target proteins expression (Clements and Jamali, 2007; Hanafy et al., 2008). One of the most common approaches in curtailing inflammation is the use of NSAIDs. These drugs, however, have their own cardiovascular side effects that limit their use (Davies and Jamali, 2004; Harirforoosh and Jamali, 2009). Our present data on the beneficial effects of GlcN may suggest a safe (Anderson et al., 2005; Sawitzke et al., 2010) alternative to NSAIDs in the treatment of inflammatory conditions. In addition, we have demonstrated that, unlike the case with NSAIDs, treating healthy rats with GlcN does not result in any alteration of the examined cardiovascular factors.

Glucosamine either as a preventive measure or as a treatment tool completely restored the diminished response to verapamil as well as the down regulation of the 190 kDa Cav1.2 subunit protein. Western blotting of the Cav1.2 subunit of L-type calcium channels results in two bands corresponding to a low (190 kDa) and

high (210 kDa) proteins. It has been reported that AA down-regulates the 190 kDa protein with no significant effect on the 210 kDa protein (Hanafy et al., 2008). In addition, GlcN restored (Fig.2.5) the down-regulating effect of inflammation on β-adrenoreceptor target protein.

RyR2 of the sarcoplasmic reticulum play an important role in excitation/contraction coupling in myocytes (Farrell et al., 2004). Voltage-dependent L-type calcium channels allow an influx of extracellular Ca^{2+} into the cell upon depolarization that triggers RyR2 activation. They cause release of Ca^{2+} from sarcoplasmic reticulum in a process known as Ca^{2+}-induced Ca^{2+} release. The released Ca^{2+} binds to myofilaments and induces contraction (Farrell et al., 2004). An interruption of this, is expected to result in cardiovascular complications including reduced response to calcium channel blockers (Valdivia et al., 1990). Our data revealed that RyR2 is not down regulated during inflammation or by GlcN.

The down-regulating effect of inflammation is not limited to the cardiac target receptor proteins as it also results in reduced expression of the norepinephrine transporters parallel to the same effect on the β-adrenoreceptor target protein (Clements and Jamali, 2009). This may, at least in part, explain the observed reduced receptor protein expression. Inflammation also down-regulates the cardiac angiotensin converting enzyme-2 (ACE-2) that has been suggested to be involved in the renin–angiotensin system as a cardio-protective protein. The balance between angiotensin-converting-enzyme (ACE) and ACE-2 is important for the regulation of blood pressure and electrolyte homeostasis (Hanafy et al.,

2009). It is also known that inflammation reduces the efficiency of the hepatic drug-metabolizing enzymes (Morgan et al., 2008), resulting in increased plasma concentrations of drugs such as verapamil that are efficiently metabolized before reaching the systemic circulation. The present data suggest that both prevention and control of AA by GlcN results in rebuilding of this metabolic pathway, hence normalizing, at least in part, the pharmacokinetics of verapamil (Fig.2.6).

Similar to verapamil, GlcN is a drug with substantial presystemic clearance, and hence has a low oral bioavailability (>20%) in both humans (Setnikar et al., 1984) and rats (Aghazadeh-Habashi et al., 2002). However, unlike verapamil that demonstrates substantial reduced clearance under inflammatory conditions, GlcN pharmacokinetics were not influenced by AA (Fig.2.7). This is likely due to the fact that for GlcN, the first-pass loss upon oral administration is attributed to its clearance in the gut rather than the liver (Aghazadeh-Habashi et al., 2002). It appears that the mechanism involved in the gut clearance of GlcN is not significantly influenced by inflammation.

In conclusion, in addition to its preventative effect GlcN controls the symptoms of AA after its emergence. It also restores the down-regulation of the calcium channel, β-adrenergic and pharmacokinetics of verapamil, suggestive of restoration of the drug's metabolic pathway. These actions of GlcN are indicative of its anti-inflammatory properties.

Chapter 3

A Novel Peptide Prodrug of Glucosamine with Enhanced Gut Permeability

3.1. Introduction

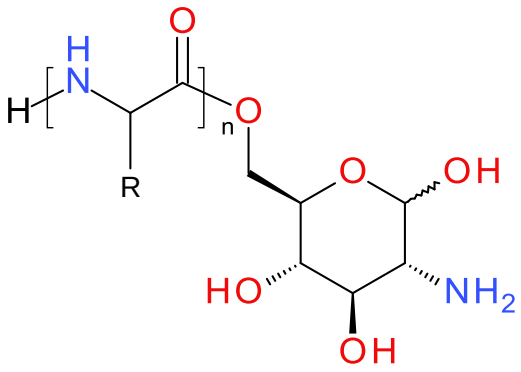
Glucosamine (GlcN), a naturally occurring amino sugar with chondroprotective (Oegema et al., 2002) and anti-inflammatory effects (Gilzad-Kohan and Jamali, 2012; Hua et al., 2005), is commonly administered to treat OA (Sherman et al., 2012). Although there are several animal studies indicating the potential anti-inflammatory and disease-modifying effects of GlcN (Gilzad-Kohan and Jamali, 2012), human clinical trials are controversial (Aghazadeh-Habashi and Jamali, 2011). A part of this controversy can be attributed to the limited bioavailability of GlcN (Aghazadeh-Habashi and Jamali, 2011), which prevents the achievement of therapeutic levels in plasma given the usual administered dosage in humans (1500 mg/day). Indeed, administration of high doses of GlcN in an adjuvant model of arthritis in rats can prevent the emergence of inflammation and ameliorate the disease signs in early stages (Gilzad-Kohan and Jamali, 2012; Hua et al., 2005). In addition, GlcN was not a regulated compound in the past; hence, the quality of the products that were used in clinical trials is questionable (Oke et al., 2006; Russell et al., 2002). The dosing regimens of GlcN are empirical because of insufficient pharmacologic information. Recently a dose-effect study using a pharmaceutical grade glucosamine formulation revealed that the minimum effective dose to prevent AA is 40 mg/kg/day in a rat model of adjuvant arthritis (Agahzadeh-Habashi et al., 2013), which yields much greater concentrations in

plasma than the commonly used human doses (Jackson et al., 2010). In fact only animal (Gilzad-Kohan and Jamali, 2012; Hua et al., 2005) and human (Persiani et al., 2007) trials that have been carried out with relatively high doses or were associated with high plasma glucosamine concentrations have been reported to be effective in controlling inflammatory conditions. GlcN sulfate appears to be unstable unless crystalized with KCl (Russell et al., 2002). Hence, the mere size of the available tablets deters patients from taking more than 500 mg three times a day to benefit from the treatment.

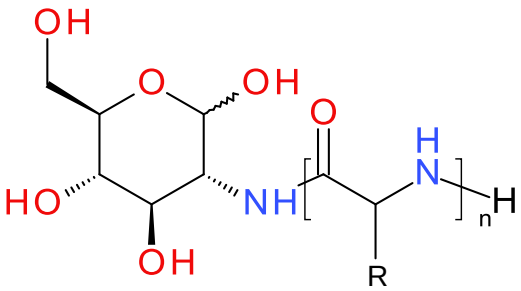
One of the strategies to reduce the required dose of the drugs in order to achieve the desired bioavailability (hence, dose: efficacy ratio), is to synthesize prodrugs. For polar drugs, prodrug design to target the membrane transporters such as *peptide transporter 1* (*PepT1*) has received particular attention in recent decades (Han and Amidon, 2000; Yu et al., 1996). Several hydrophilic compounds seem to be absorbed efficiently via this path. Peptide transporters are involved in the absorption of a broad range of therapeutic agents, including ACE inhibitors, beta-lactam antibiotics, renin inhibitors, bestatin and *valacyclovir*, a valine ester prodrug of acyclovir (Anand et al., 2004; Purifoy et al., 1993; Szczech, 1996; Weller et al., 1993).

In this study, 15 peptide GlcN derivatives were synthesized consisting of 8 esters and 7 amides (Figure 3.1). In general, the choice of synthesis depended on the objective of the synthesis (Merrifield, 1997). The peptide-GlcN ester derivatives were synthesized using a stepwise synthesis method on 2-chlorotrityl chloride resin, whereas the peptide-GlcN amide derivatives were synthesized in

solution by direct coupling of the protected amino acid or dipeptide to GlcN. We hypothesized that there exists a GlcN prodrug that possesses a relatively high bioavailability. Our goal was to synthesize a physicochemically stable prodrug with high stability and permeability through the gut, followed by a rapid release of GlcN in the liver. We used the everted jejunum (Pang, 2003) to test the gut permeability.



Peptide-GlcN ester



Peptide-GlcN amide

Ester derivatives
Val-COO-GlcN
Trp-COO-GlcN
Tyr-COO-GlcN
Phe-COO-GlcN
Phe-Phe-COO-GlcN
Val-Val-COO-GlcN
Val-Gly-COO-GlcN
Gly-Val-COO-GlcN

Amide derivatives
Val-CONH-GlcN
Phe-CONH-GlcN
Trp-Asp-CON-GlcN
Trp-Asp-CONH-GlcN
Als-Asp-CONH-GlcN
Val-Val-CONH-GlcN
Phe-Phe-CONH-GlcN

Figure 3.1. General chemical structures and names of peptide-GlcN ester and amide derivatives studied herein. L-amino acids and D-GlcN were used.

3.2. Experimental section

3.2.1. Materials

Fmoc- and Boc-protected L-amino acids**,** 2-chlorotrityl chloride resin, BOP (benzotriazol-1-yl-oxy-tris-(dimethylamino) phosphonium hexafluorophosphate), and HOBt.H_2O (hydroxybenzotriazole monohydrate) were purchased from Novabiochem (Merck KGaA, Darmstadt, Germany). Fmoc-Gly-Val and Fmoc-Val-Gly were purchased from Bachem (Bachem Americas, Inc., Torrance, USA). D-Glucosamine HCl, Glycylsarcosine (Gly-Sar), NMM (N-methylmorpholine), mannosamine HCl (MA), amantadine HCl (1-aminoadmantane HCl, ADAM), TEA (triethylamine), TFA (trifluoroacetic acid), DPBS (dulbecco's phosphate buffered saline*), Diaion HP-20* resin*, silica gel,* and Fmoc-Cl (9-fluorenylmethoxycarbonyl chloride) were purchased from Sigma-Aldrich Canada, Ltd, (Oakville, ON, Canada). HPLC grade acetonitrile (ACN), water, dichloromethane (DCM) and dimethylformamide (DMF) were obtained from Caledon Laboratories Ltd (ON, Canada). All other chemicals and solvents were commercial products of analytical or HPLC grades.

3.2.2. Animals

The experimental protocol was approved by the Health Sciences Animal Policy and Welfare Committee of the University of Alberta. Adult male Sprague–Dawley (SD) rats (250–280 g), which were acclimatized in a controlled temperature room with a 12:12 h dark/light cycle were used.

3.2.3. Synthesis and Characterization

Of several functional groups of GlcN, either the primary alcohol or the amine were selected to synthesize several mono-amino acid and di-peptide GlcN derivatives (Figure 3.1).The chemical structures of the 15 synthesized compounds are shown in Figure S1 (supporting information).

The peptide-GlcN ester derivatives were synthesized using a stepwise synthesis method on a 0.5 mmol scale of 2-chlorotrityl chloride resin (1.2 % DVB cross-linked) based on the standard SPPS approach as previously described (Soliman et al., 2011), with some modifications (Figure 3.2A). Briefly, GlcN (2 eq) was dissolved in a DMF/TEA (5/1, 10 mL) mixture and added to the pre-swelled resin in DCM. Coupling between the resin and the amine group of the GlcN was achieved by stirring the mixture overnight at room temperature. The resin was then drained to remove all the reagents and solvents, and was washed with DMF and DCM. In the next step, the Fmoc- or Boc-protected amino acid or di-peptide (2 eq) was activated by the addition of BOP (2 eq), HOBt (2 eq), and NMM (2 eq) in DMF (6 mL). The activated amino acid or di-peptide was then added to the resin and the mixture was stirred for several hours at room temperature. The primary alcohol group of GlcN reacted with the activated carboxylate group of the amino acid (or di-peptide). In order to synthesize the di-peptide derivatives (when the di-peptide was not commercially available), the last step was repeated by adding the next mono-amino acid. After draining and washing, the loaded resin was treated with 20% piperidine to remove the Fmoc from the terminal amino group prior to final cleavage. The Boc removal from the

terminal amino group was achieved during the final cleavage step. The final cleavage of the product from the resin was achieved by treating the resin with TFA in DCM (1/1, 6 mL). The resulting solution was dried under vacuum, and then washed with cold ether (4º C) in order to obtain the final product.

The peptide-GlcN amide derivatives were synthesized using the in-solution synthesis approach by direct coupling of the protected amino acid or dipeptide to GlcN (Figure 3.2B). In brief, GlcN (1 eq) was dissolved in a DMF/TEA (5/1, 10 mL) mixture, followed by addition of a pre-activated Boc-amino acid or Boc-di-peptide (1 eq) as described above in order to form an amide bound between the amine group of the GlcN and the carboxylate group of the amino acids or di-peptides. The resulting mixture was stirred overnight and water was added to precipitate the Boc-peptide-GlcN amide conjugate. The conjugate was then dried by evaporation under vacuum and treated with TFA in DCM (1/1, 6 mL) to remove the Boc-protecting group. The sample was then evaporated to dryness. The product was washed with cold ether (4º C) to yield the final amide GlcN derivative.

During the synthesis process (either solid phase or in-solution synthesis), the progress of the reactions was monitored by thin layer chromatography (TLC), and formations of the desired derivatives were confirmed by mass spectrometry. If a chromophore or fluorophore group existed in the structure of a prodrug, the compound was also identified by a direct HPLC method. In the direct method, the compound was directly injected into the HPLC system and the peak was detected based on fluorescence or UV detection. Mobile phase compositions and assay

conditions for the direct HPLC methods were listed in Table S1. HPLC chromatograms of the eight prodrugs with chromophore or fluorophore groups, which could be also identified with the direct HPLC method, are depicted in Figure S6. For the prodrugs that lacked a chromophore or fluorophore group, an indirect assay was used in which the compound was hydrolyzed and converted to GlcN using LiOH followed by analyzing of GlcN by a previously described method (Jamali and Ibrahim, 2010).

The mono-amino acid and di-peptide GlcN derivatives were purified using *Diaion HP-20* (Korda et al., 2006) or *silica gel* (Talluri et al., 2008) column chromatography. A stepwise procedure was followed for purification of the compound. Glass columns (100 mL) were filled with *Diaion HP-20* resin or *silica gel*. *Diaion HP-20* resin was fully hydrated before the purification procedure. The column was loaded with an aqueous solution (when using *Diaion HP-20* resin) or a mixture of hexane and ethanol (when using *silica gel*). The solution (200 mL) was passed through the column followed by 200 mL of water or hexane for *Diaion HP-20* resin or *silica gel*, respectively (no leakage of the peptide was observed up to this point). Finally the loaded conjugates were eluted from the resin using an optimum mixture of ACN/and or methanol/water (from *Diaion HP-20* resin) or hexane/ethanol (from *silica gel*) until the TLC test indicated that all desired material had been eluted. Throughout the process, a rate of 2 bed volumes per hour was maintained. Finally, the solvents were evaporated to yield the final purified GlcN prodrugs. Purified compounds were characterized using mass spectrometry, and mass balance study. In addition, the direct HPLC methods

(Table S1) were used for compounds with a chromophore or fluorophore group in their structure.

The structure of a representative ester derivative (Gly-Val-COO-GlcN.HCl) was further confirmed by **^{1}H** and **^{13}C** NMR spectroscopy (Figures S4 and S5). The GVG.TFA salt was converted to GVG.HCl by treatment with 0.5 M HCl for 35 minutes. The product GVG.HCl exists as an inseparable mixture of α- and β-isomers. Some protons of the same number, as shown in the structure (Figures S4), often appear at different ppm. The presence of isomers is an intrinsic property of the D-glucosamine sugar moiety.

^{1}H NMR [CD_3OD, 400 MHz]: [D_2O, 400 MHz]: δ 5.40 (d, 1H, J = 2.9 Hz, H1α), 4.95 (d, 1H, J = 7.5 Hz, H1β), 4.30-4.65 (m, 3H, H5, H6a and H7), 4.06-4.14 (m, 1H, H6b), 3.90 (s, 2H, H10), 3.66-3.85 (m, 1H, H4), 3.45-3.65(m, 1H, H3). 3.00 and 3.30 (m, 1H, H2), 2.20-2.40 (m, 1H, H8), 0.99 (d, 6H, J = 8.6 Hz, H9). ^{13}C NMR [D2O, 150 MHz]: δ 172.8 (C12), 167.2 (C7), 92.7 and 89.1 (C1α and C1β), 73.5, 71.7 and 69.5 (C3, C4 and C5), 63.8 (C6), 58.5 (C8), 56.5 (C2), 40.2 (C13), 30.0 (C9), 18.1 and 17.1 (C10 and C11).

3.2.4. Analytical HPLC assay for GlcN

The HPLC system comprised a Shimadzu Prominence system (Mandel Scientific, Guelph, ON Canada) equipped with fluorescence and *photodiode array* detectors and a Phenomenex C18 (100 mm X 4.6 mm, id 3 micron) reversed phase column. The gradient mobile phase consisted of 0.1% acetic acid/ACN at 1 mL/min flow rate. A previously reported assay (Jamali and Ibrahim, 2010) with some

modifications was used to measure GlcN levels in the samples. Briefly, aliquots of the samples (100 µL) were spiked with mannosamine HCl (MA) as the internal standard (IS) and treated with cold ACN (4º C) to precipitate the proteins. They were then centrifuged and their supernatants were derivatized with Fmoc-Cl (8 mM in ACN) in the presence of borate buffer (0.2 M) at 30º C for 30 min. The excess amount of derivatizing agent was removed with ADAM (300 mM in ACN/water 1:1) and 5 µL of the final solution was injected into the HPLC. Standard curves were linear over the concentration range of 0.05 to 20 µg/mL (r2 ≥ 0.99, coefficient of variation ≤10%). Mass balance calculation was achieved by determining the amount of GlcN. As each mole of the compounds consists of one mole of GlcN, by measuring the amount of the GlcN formed in each experiment, the amount of the peptide-GlcN derivatives that was degraded (in the stability tests) or transported (in the gut permeability experiments) could be calculated indirectly.

3.2.5. Thermal and chemical stability studies

Powdered GlcN derivatives were transferred into dry transparent glass vials (50 mL) and placed in a 60° C oven for 48 h. At the end of the experiment, a given portion of the compounds was incubated with LiOH, and the GlcN content was measured. The amount of the degraded compound was determined based on a mass balance calculation. When possible, the amount of intact prodrugs were also measured using the direct HPLC methods in order to further confirm the results obtained from the mass balance calculations.

In order to evaluate the effect of pH on the chemical stability of the GlcN conjugates, the prodrugs were incubated in an aqueous HCl solution (pH 2) or Krebs-Heneseleit bicarbonate buffer (pH 7.4) at 37º C for 2 h followed by the direct or indirect assay described under "Synthesis and characterization".

3.2.6. Intestinal and liver homogenate studies

Liver and intestinal homogenate hydrolysis studies were performed according to a previously described method (Talluri et al., 2008) with some modifications. Briefly, rats were sacrificed under anesthesia and their livers and intestines were excised. After being washed with cold Dulbecco's phosphate-buffered saline (DPBS) (4º C, pH 7.4), specimens were placed in liquid nitrogen for instant freezing and stored at −80° C until further use. On the day of experiment, tissues were thawed and homogenized in 10 mL of chilled (4° C) *DPBS* for about 30 s with a tissue homogenizer (Kinematica AG, Littau-Lucerne, Switzerland) in an ice bath. Subsequently, the homogenates were centrifuged at 12,500 G for 25 min at 4° C to remove cellular debris and the supernatants were collected for hydrolysis studies. The protein contents of the samples were determined using a commercially available protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA). Solutions of the prodrugs (1 mM, 100 uL) were added to 400 μL of the liver or intestinal homogenized supernatants (n=6) and the mixtures were incubated at 37° C in a shaking water bath. Aliquots of 50 μL were withdrawn at 0, 15, 30, and 60 min, and 50 μL of ice-cold ACN was added in order to precipitate the cellular proteins and stop the reaction. Samples were stored at −80°

C until further analysis. Finally, GlcN concentrations were determined in all samples and the degraded amount of the compounds was determined based on a mass balance calculation. In general, GlcN prodrugs decompose to GlcN and the amino acid or di-peptide moiety; by measuring the amount of GlcN released during the experiment, the amount of degraded compounds was calculated indirectly. The direct HPLC methods were also used to confirm the results where applicable. The measured concentrations from the intestinal homogenate study were plotted against the time points on a *semi-logarithmic graph* and the first order rate constants (k_{obs}) and the half-lives ($t_{1/2}$= 0.693/k_{obs}) of the degradation were calculated from the slope of the lines.

3.2.7. Permeability study through everted rat jejunum sacks

The permeability experiment was performed according to a previously described method (Ibrahim et al., 2012) with some modifications. Male SD rats (250-280 g) (n=6) were deprived of food 12 h before the experiment with free access to water. A mid-line incision was made in the abdomen of the anesthetized rats. The intestine was exposed, and six segments (10 cm each) of the jejunum were cut 10 cm after the ligament of Treitz. The segments were immersed immediately into ice cold Krebs-Heneseleit bicarbonate buffer (pH 7.4), and the intestinal contents were removed. Each segment was everted over a glass rod and tied from one side with a silk thread. A silastic catheter (0.58 mm i.d. x 0.965 mm o.d) was inserted into the other side and tied. The segments were filled with 2 ml of the buffer. 30 ml of freshly prepared solution of each prodrug (1.5 mM total) in the buffer was

added to perfusion apparatus vessels (n=6), and a single everted jejunum sack was immersed in each vessel in order to introduce the prodrug solution to the mucosal (outer surface of the everted gut) side. The solutions were oxygenated by a mixture of O_2/CO_2 (95/5) during the experiment, and temperature was kept at 37° C. Serial samples (0.5 mL) were collected from both mucosal and serosal sides at 0, 15, 30, and 60 min. The withdrawn volume was replaced with an equal volume of the Krebs buffer. Samples were kept frozen at -20° C until the day of analysis. On the day of the analysis, samples were thawed, and were divided into two portions. The first portion was incubated with lithium hydroxide (LiOH, 1 N) for 2 h in order to yield the parent drug (GlcN) and the amino acid or di-peptide moiety. The amounts of GlcN transferred into the serosal site and remaining in the mucosal site were measured using the analytical HPLC assay for GlcN; this represented the amount of the prodrugs moved from the mucosal side toward the serosal side. Using the direct HPLC methods, a second portion of the permeability test samples were assayed for intact prodrugs to further confirm the obtained results where possible. Cumulative amounts of prodrugs inside the jejunum sacks (the serosal side) were calculated and compared with the values for GlcN. In addition, percent average ratio of GlcN concentration inside/outside the jejunum sacks (n=6) after 60 min incubation with the GlcN derivatives was calculated and compared to that of GlcN.

In a separate study, for a representative di-peptide GlcN ester derivative (Gly-Val-COO-GlcN), a permeability inhibition test in the presence and absence

of a specific *PepT1* substrate, Gly-Sar, was carried out. Rats' jejunum sacks were pre-incubated either with Gly-Sar (10 mM) or Krebs-Heneseleit bicarbonate buffer for 5 min, and a sufficient amount of the compound (Gly-Val-COO-GlcN) was then added to the solution to make a final concentration of 1.5 mM. Aliquots of the samples (0.5 mL) were collected from both the mucosal and the serosal sides at 60 min. Samples were incubated with LiOH to cleave the ester bond in order to yield the parent drug (GlcN). Finally the GlcN concentrations inside and outside of the jejunum sacks were measured using the analytical HPLC assay for GlcN, and the percent ratio of GlcN concentration inside/outside the jejunum sacks was calculated.

3.2.8. Statistical analysis

Data are presented as mean ±S.D. Differences among the cumulative amount of GlcN (µg) inside the jejunum sacks, and the percent average ratio of GlcN concentration inside/outside the jejunum sacks after 60 min was determined utilizing a one-way ANOVA, followed by the Bonferroni post-test. Statistical analyses were carried out using Prism software (GraphPad Software Inc., San Diego, CA, USA) at $P < 0.05$.

3.3. Results

3.3.1. Ester and amide peptide-GlcN derivatives

Different peptide-GlcN prodrugs were designed to target the *PepT1* for enhancing the bioavailability of the parent drug, GlcN. Fifteen different GlcN conjugates, 8 esters and 7 amides (Figure S1, supporting information), were synthesized and characterized for evaluation as GlcN prodrugs. The synthesis of the ester derivatives was achieved on the 2-chlorotrityl chloride resin, whereas amide derivatives were synthesized in solution as described in the experimental section (Figure 3.2). Crude compounds were purified using *Diaion HP-20* (Korda et al., 2006) or *silica gel* (Talluri et al., 2008) column chromatography to give final pure crystalline solid compounds as trifluoroacetate salts. The identity of each compound was determined using mass spectrometry (Figure S2). As an example, Figure S3 presents the mass spectra of the Gly-Val-COO-GlcN (GVG) conjugate before and after purification using *Diaion HP-20* resin. GVG was further characterized using $^{1}\mathbf{H}$ and $^{13}\mathbf{C}$ NMR spectroscopy (Figure S4 and S5). Eight compounds with either a fluorophore or chromophore group in their structure were characterized using HPLC (or direct HPLC) with purity between 90-99% (Table S1 and Figure S6). The remaining 6 compounds with no fluorophore or chromophore were characterized using an indirect HPLC method, where the release of free GlcN was determined. Unlike GVG, these 6 compounds were not further characterized using NMR as they displayed poor gut permeability.

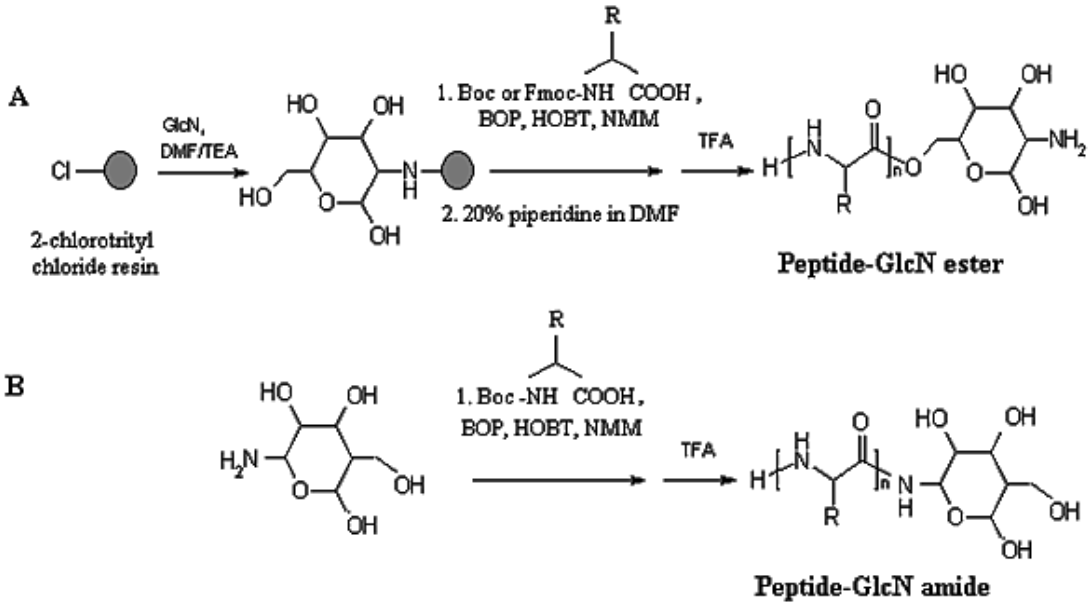


Figure 3.2. General synthetic scheme for A) solid-phase synthesis of peptide-GlcN ester and B) in-solution synthesis of peptide-GlcN amide derivatives.

3.3.2. Stability of the peptide-GlcN derivatives

Thermal stability of the GlcN derivatives was evaluated by placing them in a 60° C oven for 48 h, as described under methods. In order to investigate the pH stability of the prodrugs, they were incubated in an aqueous HCl solution (pH 2) and Krebs-Heneseleit bicarbonate buffer (pH 7.4) at 37º C for 2 hours. The results of the stability tests based on the remaining amounts of the prodrugs at the end of the experiment are listed in Table S2. All synthesized GlcN prodrugs showed acceptable thermal and chemical stability under the test conditions. The amide derivatives were more stable (>98% intact prodrug) compared to the esters (>96% intact peptide-GlcN ester derivatives) under similar conditions.

3.3.3. Peptide-GlcN derivatives in the presence of intestinal or liver homogenate

All peptide GlcN derivatives were exposed to intestinal and liver homogenate in order to evaluate their stability as described under the experimental section. All ester prodrugs hydrolyzed to yield the parent drug, GlcN, in intestinal homogenate. The first order degradation rate constants and stability half-lives of peptide-GlcN derivatives in the rat intestinal homogenate are listed in Table 3.1. The degradation half-lives of the Gly-Val-COO-GlcN, Phe-Phe-COO-GlcN, and Val -Gly-COO-GlcN were calculated as 17.9, 17.3, and 16.1 min respectively, which was the highest in comparison with other ester prodrugs (Table 3.1). None of the amide prodrugs cleaved to yield free GlcN during the experimental period.

Analysis of the samples after incubation of GlcN prodrugs with liver homogenate revealed that all of the ester prodrugs were rapidly cleaved to the parent drug (GlcN) in less than 15 minutes, whereas amide conjugates did not hydrolyze. GlcN was stable in the intestine and liver homogenates and did not degrade during the experiment period; the average recovery rate for GlcN was 98.3±1.3 % after 60 min.

Table 3.1. First order degradation rate constants and stability half-lives (±SD) of peptide-GlcN derivatives in the rat intestinal homogenate (*n*=6)

Peptide-GlcN derivatives	**k_{obs}[a] (min $^{-1}$)**	**$t_{1/2}$ (min)**
Val-COO-GlcN	0.08±0.005	8.1±0.5
Trp-COO-GlcN	0.08±0.017	8.7±2.1
Tyr-COO-GlcN	0.07±0.012	9.4±1.6
Phe-COO-GlcN	0.06±0.009	10.8±1.4
Phe-Phe-COO-GlcN	0.04±0.012	17.3±4.7
Val-Val-COO-GlcN	0.06±0.008	11.7±1.6
Val -Gly-COO-GlcN	0.05±0.008	16.1±2.4
Gly-Val-COO-GlcN	0.04±0.008	17.9±3.8

None of the peptide-GlcN amide derivatives were hydrolyzed by the rat intestinal homogenate. [a] First order rate constant. All values are mean ± SD

3.3.4. Permeability study through everted rat jejunum

All GlcN prodrugs were assessed for their permeability through rats' jejunum sacks as described under the experimental section. As listed in Table 3.2, except one ester derivative, Gly-Val-COO-GlcN, all other prodrugs failed to significantly increase the cumulative amount of GlcN inside the jejunum sacks at all time-points. Two ester conjugates, Phe-Phe-COO-GlcN and Val -Gly-COO-GlcN, also showed a slight increase in the cumulative amount of GlcN inside the jejunum sacks at all time-points, which did not reach statistical significance.

Figure 3.3A shows the percent ratio of GlcN concentration inside/outside the jejunum at the end of the experiments (t=60 min) for the ester derivatives. Only Gly-Val-COO-GlcN showed a significant increase in the ratio compared to GlcN, which in turn indicates a significant increase in gut permeability. There is

also a non-significant trend toward increased ratio for Phe-Phe-COO-GlcN and Val -Gly-COO-GlcN that was in accordance to the trend observed for the cumulative amount detailed in Table 3.2.

Table 3.2. Cumulative amount of GlcN (μg) inside the jejunum (serosal site) (n=6) at different time points, after incubation with 1.5 μM of GlcN and GlcN conjugates

Peptide-GlcN derivatives	Time(min)			
	0	15	30	60
	Cumulative amount of GlcN (μg)			
GlcN	0	11.7±0.7[a]	25.8±1.1[c]	49.6±2.0[e]
Val-COO-GlcN	0	11.1±1.9[a]	27.2±1.4[c]	50.8±1.6[e]
Trp-COO-GlcN	0	11.8±2.0[a]	27.7±3.3[c]	50.2±3.5[e]
Tyr-COO-GlcN	0	11.2±1.5[a]	26.8±3.0[c]	49.2±1.8[e]
Phe-COO-GlcN	0	11.4±1.2[a]	27.3±0.9[c]	49.7±1.1[e]
Phe-Phe-COO-GlcN	0	16.9±4.3[a*]	35.6±7.7[c*]	65.5±15.3[e*]
Val-Val-COO-GlcN	0	12.5±2.0[a]	27.9±1.9[c]	50.3±1.8[e]
Val -Gly-COO-GlcN	0	15.8±3.2[a*]	34.2±6.1[c*]	61.6±11.9[e*]
Gly-Val-COO-GlcN	0	80.1±10.8[b]	197.0±7.8[d]	358.8±14.1[f]
Val-CONH-GlcN	0	NM	NM	NM
Phe-CONH-GlcN	0	NM	NM	NM
Trp -Asp-CONH-α-GlcN	0	NM	NM	NM
Trp -Asp-CONH-β-GlcN	0	NM	NM	NM
Ala-Asp-CONH- β-GlcN	0	NM	NM	NM
Val-Val-CONH-GlcN	0	NM	NM	NM
Phe-Phe-CONH-GlcN	0	28.86±3.9**	68.38±6.7**	129.74±9.5**

Data are shown as mean (±SD). Different superscript letters indicate significant differences between means in a column ($P < 0.05$); similar superscript letters denote no significant differences between means in a column. *Trend toward increased permeability (did not reach statistical significance). **Intact prodrug measured. NM: not measured

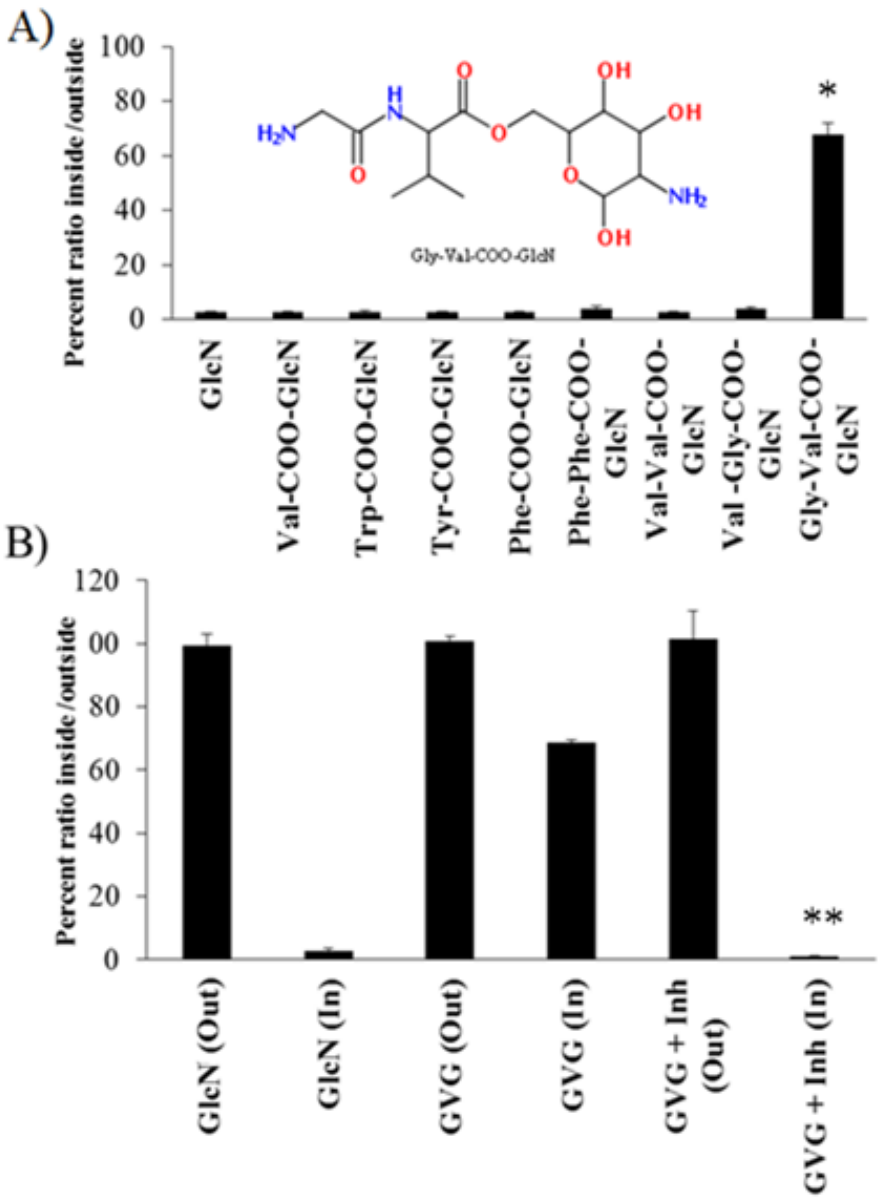


Figure 3.3. Percent average ratio of GlcN concentration (+SD) inside (In)/outside (Out) the jejunum sacks (n=6) after 60 min incubation with (A) peptide-GlcN ester derivatives and (B) GlcN, Gly-Val-COO-GlcN (GVG) in the presence and absence of Gly-Sar (Inh). * means GVG showing significant increase in the ratio compared to GlcN and other peptide-GlcN ester derivatives (P < 0.05). GVG showed a significantly higher concentration of GlcN inside the jejunum sacks compared to GlcN. ** means GVG permeability was inhibited significantly in the presence of Gly-Sar (P <0.05).

As detailed above, only Gly-Val-COO-GlcN prodrug showed a significant increase in both the cumulative amount of GlcN inside the jejunum sacks and the percent ratio of GlcN concentration inside/outside the jejunum sacks at the end of the experiment, which indicates an enhanced permeability across the gut membrane compared to GlcN. To examine if the increased permeability of Gly-Val-COO-GlcN is due to its translocation by *PepT1*, permeability was measured in the presence and the absence of the *PepT1* specific substrate, Gly-Sar. Relative to GlcN, Gly-Val-COO-GlcN increased the concentration of GlcN inside the jejunum sacks (serosal site); in the presence of Gly-Sar, this increase was completely inhibited (Figure 3.3B).

3.4. Discussion

GlcN is an amino-sugar with anti-inflammatory properties (Gilzad-Kohan and Jamali, 2012; Hua et al., 2005). Several animal studies have shown that GlcN can control inflammatory diseases such as AA (Gilzad-Kohan and Jamali, 2012; Hua et al., 2005; Wang et al., 2007; Wen et al., 2010); however, its low bioavailability limits its beneficial therapeutic effects (Aghazadeh-Habashi and Jamali, 2011). Designing prodrugs to target a specific receptor in the gastrointestinal (GI) tract in order to enhance bioavailability is a widely utilized method in drug development (Han and Amidon, 2000; Yu et al., 1996). The prodrugs are usually inactive entities that quickly yield the parent drug as soon as they enter the systemic circulation. Among several intestinal transporters, *peptide transporter 1 (PepT1)* has attracted a great deal of attention in recent years (Han and Amidon, 2000).

PepT1 shows broad substrate specificity and transports di- and tri-peptides. Introducing an amino acid or di-peptide moiety into the molecular structure of parent drugs via an ester or amide bond is a common strategy in this matter (Anand et al., 2004; Weller et al., 1993). New drug conjugates should have a desired physicochemical and intestinal stability. On the other hand they should convert to their parent drug as soon as they cross the GI membrane and enter the systemic circulation. The site of prodrug cleavage might be the plasma or the liver.

We synthesized and characterized several GlcN mono amino-acid and di-peptide ester and amide conjugates in order to find a GlcN prodrug with enhanced gut permeability. We used an in-solution synthesis method to synthesize the amide derivatives (Figure 3.2B). Since the amine group of GlcN is several fold more reactive than the primary and secondary hydroxyl groups (Qi-Wei et al., 2002), several amide prodrugs were synthesized easily in-solution without protecting the secondary hydroxyl groups. The ester derivatives were synthesized using a solid-phase approach (Figure 3.2A). There are several reasons to use the solid-phase approach including convenience, acceleration of the overall process and the ability to achieve good yields of purified products. This allowed the development of a large number of candidate prodrugs. In addition, an in-solution method for synthesis of ester prodrugs requires the protection of the amine groups which, in turn, could add several steps to the process. In the classic solid-phase peptide synthesis, the N-protected C-terminal amino-acid residue is anchored via its carboxyl group to a resin (Amblard et al., 2006). However the synthesis of the

ester derivatives of GlcN required a resin with the ability to anchor the amine group. 2-Chlorotrityl chloride resin is an incredibly versatile, acid-labile resin that can be successfully used for the immobilization of amines. Cleavage of the functional groups is generally achieved using 1-50% TFA in DCM (Hoekstra, 2001). Herein, we synthesized several GlcN ester derivatives with high yields and purity using the stepwise solid phase synthesis on 2-chlorotrityl chloride resin.

One of our primary goals of this study was to synthesize GlcN derivatives with desirable physical stability. GlcN is available on the market as sulfate and HCl salts, with the latter being the salt in the most of the commercially available products for which clinical data are available (Herrero-Beaumont et al., 2007; Noack et al., 1994;Rovati et al., 2012). The bioequivalence and possible identical efficacy of the two salts has been discussed elsewhere (Aghazadeh-Habashi and Jamali, 2011); however, since the sulfate salt is known to lack physical stability (unless formulated as crystalline GlcN sulfate with additional stabilizing salts) (Russell et al., 2002), we aimed to synthesize new GlcN derivatives with enhanced chemical stability. The stable derivatives do not require the addition of other stabilizing salts such as KCl. This approach, in turn, provided the opportunity to reduce the size of the administrable bulk. A reduction in the size of the finished product is of primary importance, particularly for elderly patients. The currently available data regarding the stability of the HCl salt is not conclusive; however, the stability of the finished products can be an issue since GlcN HCl crystals are hygroscopic. Further investigations are needed in order to assess the stability of the HCl salt. Newly synthesized compounds were tested for

their physicochemical stability. All of the synthesized GlcN prodrugs that have been studied here appear to have a desirable chemical stability. Next, pH stability of the newly synthesized compounds was also studied at two different pH, 2 and 7.4, which are corresponding with the normal pH of different segments of the gastro-intestinal track. All the studied compounds exhibited a high stability in the aforementioned pH conditions.

In addition, all the prodrugs were investigated for their GI stability, their ability to be biotransformed into the parent drug in the liver, and their transportability through the everted rat gut. GI stability is another prerequisite for suitable orally administered prodrug candidates, for if they cleave before reaching their site of absorption, they will lose their property to enhance the bioavailability of the parent drug (Anand et al., 2004). Amide and ester derivatives demonstrated varied susceptibility to the hydrolyzing intestinal enzymes. Gly-Val-COO-GlcN, Phe-Phe-COO-GlcN, and Val-Gly-COO-GlcN demonstrated cleavage half-lives of around 16-17 min in the intestinal homogenate, which was the highest stability among all other ester prodrugs. In another study (Anand et al., 2004), a series of acyclovir di-peptide prodrugs were synthesized and tested *in vitro* and *in vivo*. According to the authors, intestinal homogenate hydrolysis studies revealed that a minimum cleavage half-life of 15 min was desirable in order to have a potential candidate with increased bioavailability. Among all the synthesized ester derivatives only Gly-Val-COO-GlcN, Phe-Phe-COO-GlcN, and Val -Gly-COO-GlcN met this requirement. However, none of the amide conjugates showed GI instability. In fact, all of the amide prodrugs were too stable to release GlcN even

when incubated with liver homogenates. In contrast, all of the ester prodrugs were rapidly cleaved to yield GlcN after incubation with liver homogenates in less than 15 min. In this step we conclude that the successful candidate(s) should be an ester conjugate rather than an amide conjugate.

In order to improve bioavailability, in addition to an acceptable stability profile, GlcN prodrugs needed to demonstrate an acceptable transportability via *PepT1*. Previously, the affinity of several di-peptides toward *PepT1* has been studied (Bailey et al., 2006). Many of the studied di-peptide sequences exhibited high affinity toward the transporter; however high affinity may not always be translated into high transporter-mediated transportation after oral administration because the substrates might only bind to the transporter without being translocated (Anand et al., 2004). We utilized the permeability test through the everted rat gut in order to determine the transportability of the GlcN derivatives. Gly-Val-COO-GlcN fulfilled this criterion better than other derivatives, showed a significant increase in the cumulative amount of GlcN inside the jejunum sacks. Likewise, Anand et al. showed that after oral administration, Gly-Val dipeptide ester prodrug of acyclovir appeared to be more efficiently absorbed across the intestinal mucosa compared to other ester derivatives (Anand et al., 2004). We assumed that the increase in the permeability of the Gly-Val-COO-GlcN prodrug was possibly due to the recognition of the conjugate by the *PepT1*. This is most likely due to the fact that this conjugate met the general requirements concerning the structures of the *PepT1* substrates, including possession of a di- or tripeptide skeleton, high affinity toward the transporter, and an appropriate small size

(Bailey et al., 2006). Phe-Phe-COO-GlcN and Val -Gly-COO-GlcN also showed an increase in the cumulative amount crossing through the gut wall; these, however, did not reach statistical significance.

As mentioned above, the increase in the permeability of the Gly-Val-COO-GlcN prodrug was possibly due to the recognition of the conjugate by the *PepT1*. To prove this assumption, we performed a permeability inhibition test with Gly-Sar, a specific *PepT1* substrate. As depicted in Figure 3.3B, Gly-Sar inhibited transport of Gly-Val -COO-GlcN, indicating penetration by way of the *PepT1*.

3.5. Conclusion

The GlcN di-peptide conjugate, Gly-Val-COO-GlcN, demonstrated the most desirable chemical and physical stability and the greatest gut permeability of GlcN as compared to GlcN and the other synthesized derivatives. The uptake of this conjugate was efficiently mediated by *PepT1*, as it was significantly inhibited in the presence of Gly-Sar. We, therefore, suggest Gly-Val-COO-GlcN to be a desired GlcN prodrug in order to increase GlcN bioavailability.

Supporting Information

Additional tables (S1-S2) and figures (S1-S6) related to the characterization of the peptide-GlcN derivatives are available under the supporting information in the appendix.

Chapter 4

[2]The Antiinflammatory Action and Pharmacokinetics of a Novel Di-peptide Aminosugar

4.1. Introduction

Glucosamine (GlcN) is a naturally occurring amino-sugar with anti-inflammatory properties (Gilzad-Kohan and Jamali, 2012; Hua et al., 2005; Hwang et al., 2010). However, the oral bioavailability of GlcN is limited and, since, at least outside Europe, it is not a regulated compound, the quality of the marketed products and also those used in clinical trials are questionable (Aghazadeh-Habashi and Jamali, 2011). The latter issues have been suggested to be, at least in part, behind the controversy in the effectiveness of GlcN. Nevertheless, GlcN is vastly used in the treatment of OA (Ragle and Sawitzke, 2012).

One approach for improving the oral bioavailability of a poorly absorbed polar drug such as GlcN is to synthesize prodrugs that facilitate their translocation across the intestinal wall by the di/tri-peptide oligopeptide transporter (*PepT1*). Recently, we have designed and synthesized several mono- and di-peptide GlcN ester and amide derivatives (Gilzad-Kohan et al., 2013). Among the tested prodrugs, an ester derivative, Gly-Val-COO-GlcN (GVG) showed increased *in vitro* permeability through everted rat gut, which was, at least in part, efficiently mediated by *PepT1*. GVG was relatively stable in the gut, readily crossed the gut wall *in vitro* and was rapidly hydrolyzed to yield the parent drug (GlcN) after

[2] *A version of this chapter has been published. Mohammad H. Gilzad-Kohan, Kamaljit Kaur and Fakhreddin Jamali, J Pharm Pharm Sci,16(2) 279 - 288, 2013*

incubation with liver homogenates; therefore GVG appears to be a promising candidate for further investigation.

The first aim of this study was to investigate the *in vivo* bioavailability of three di-peptide esters of GlcN, namely GVG, phenylalanine-phenylalanine-COO-GlcN (FFG), and valine-glycine-COO-GlcN (VGG), relative to GlcN. For this purpose, we developed a sensitive HPLC assay for simultaneous measurement of GlcN and its ester derivative, GVG, based on fluorescence detection. Both GlcN and GVG lack chromophores; hence, they were derivatized with 9-fluorenylmethoxycarbonyl (Fmoc), a highly fluorescent compound (Einarsson et al., 1983; Jamali and Ibrahim, 2010).

The second aim of this study was to assess GVG stability in the rat feces. It has been previously shown that GlcN can be degraded by feces, which in turn affects its bioavailability (Ibrahim et al., 2012).

The third aim of this study was to explore the efficacy of GVG in prevention of experimental inflammation in a rat model of AA, which has been used for decades to test anti-rheumatoid drugs (Rosenthale and Capetola, 1982). There are several studies indicating that GlcN at high doses, which is associated with high plasma concentrations, is effective in prevention and controlling AA symptoms (Gilzad-Kohan and Jamali, 2012; Hua et al., 2005), hence, we hypothesized that a GlcN prodrug (e.g. GVG) with a relatively high bioavailability offers a novel drug for the treatment of inflammatory conditions such as AA. The new GlcN derivative can produce therapeutic GlcN levels with smaller daily doses compared to GlcN, which in turn increases patient's

compliance as the available product is excessively large; a typical 500 mg GlcN tablet weighs 1.4 g.

Finally, we investigated the possible effect of inflammation on the pharmacokinetics (PK) of GVG. Inflammation is known to cause alteration of the expression of several transporters (Cressman et al., 2012). It has been reported that DSS-induced rat colitis does not alter *PepT1* substrate bioavailability despite certain modifications in the mRNA expression (Radeva et al., 2007).

4.2. Materials and methods

4.2.1. Chemicals

The examined dipeptides (GVG, FFG and VGG) were synthesized in our lab based on a previously described method (Gilzad-Kohan et al., 2013). D-(+)-GlcN, 9-fluorenylmethoxycarbonyl chloride (Fmoc-CL), mannosamine HCl, 1-aminoadmantane HCl (amantadine), boric acid, sodium hydroxide, lactic dehydrogenase, *aspergillus* nitrate reductase , FAD, NADPH, Tris base, Tris hydrochloride, sodium nitrite, sodium nitrate, pyruvic acid, phosphoric acid, naphthalene ethylene diamine, and sulphanilamide were purchased from Sigma-Aldrich (St. Louis, MO, USA). *Mycobacterium butyricum* was purchased from Difco (Detroit, MI, USA). Squalene was purchased from Kodak (Rochester, NY, USA). HPLC grade acetonitrile and water were purchased from Caledon Laboratories Ltd, (ON, Canada). All other chemicals and solvents were commercial products of analytical or HPLC grades.

4.2.2. Animals

The experimental protocol was approved by the University of Alberta Animal Care Committee. Male Sprague–Dawley rats (250–280 g) were acclimatized in a temperature-controlled room with a 12:12 h dark/light cycle and fed with standard rodent chow food containing crude protein 23%, crude fat 4.5%, crude fiber 6%, ash 8%, and mineral 2.5%.

4.2.3. Simultaneous HPLC assay for GlcN and GVG

Plasma concentrations and/or urinary excretion of GlcN were measured and used as a measure of bioavailability for all tested compounds. In addition, intact GVG was measured to test its presence in various biological samples. The HPLC system consisted of a Shimadzu Prominence HPLC system (Mandel Scientific, Guelph, ON Canada) equipped with a RF-10AxL fluorescence detector, a LC-20AT pump, a DGU-20A5 degasser, a SIL-20A auto-sampler, a CTO-20AC column oven, a CBM-20A communication bus module and a Phenomenex C18 (100 mm x 4.6 mm, id 3 µm) reversed phase column guarded with a security guard cartridge C18 (4 mm x3 mm) column. The gradient mobile phase consisted of 0.1% acetic acid in HPLC-grade water (A) and acetonitrile (B) which was run at 1 mL/min; the gradient system commenced and continued for 17 min with 24% B followed by an increase of B to 90% in 1 min. The composition was maintained for 10 min and decreased back to 24% B in 1 min. Column oven temperature was set at 37º C. The detection was carried out at excitation and emission wavelengths

of 256 nm and 315 nm, respectively. The sample run time was 38 min. The peak height was used for all calculations.

An adequate amount of GlcN.HCl or GVG.HCl was dissolved in 10 mL water to yield a 1 mg/mL stock solution of each compound (free base). The stock solutions were further diluted with water to give standard solutions containing 0.1, 1, 10, 20 and 40 μg/mL. Aliquots of 0.1 mL plasma were spiked with the standard solutions to yield the calibration solutions containing 0.05, 0.5, 5, 10 and 20 μg/mL of each compound. The standard curves were constructed by plotting GlcN:IS or GVG:IS peak height ratio versus the added concentration of GlcN or GVG. A 30 μg/mL mannosamine HCl (IS) solution was used as the internal standard (IS). Fmoc-chloride was used as the derivatizing reagent at a concentration of 8 mM in acetonitrile. Borate buffer (0.2 M) was prepared by dissolving 6.18 g boric acid in 425 mL water followed by pH adjustment to 8.5 using NaOH (10 M). A 300 mM solution of amantadine was prepared in acetonitrile/water (1:1, v/v) and was used as a scavenger for the unreacted Fmoc-chloride.

To 0.1 mL of rat plasma was added 50 μL of IS and proteins were precipitated with 200 μL of cold acetonitrile followed by 1 min vortex-mixing and centrifugation for 3 min at 10,000 g. 100 μL of the supernatants were transferred into clean test tubes and 50 μL of each borate buffer and a freshly prepared Fmoc-chloride solution were added. After 1 min vortex-mixing the samples were incubated in a water bath at 30º C for 30 min. Subsequently, 50 μL of amantadine

solution was added and samples were diluted with 1 mL acetonitrile/water (1:1) followed by injection of aliquots (5 μL) into the HPLC.

Three calibration curves (0.05, 0.5, 5, 10 and 20 μg/mL of each compound) were prepared on the same day to determine intra-day variability. The experiment was repeated on three different days to determine the inter-day variability. The accuracy was determined from % error = (mean observed concentration – added concentration) x100/added concentration. The coefficient of variation (CV%) was used to estimate the assay precision.

The percent recovery of GlcN, GVG, and IS from plasma was estimated from % recovery = (compound peak height in plasma sample/compound peak height in water sample) x 100.

In order to determine the short term stability, samples (0.05, 0.5, 5 and 20 μg/mL) were derivatized and analyzed at 0, 4, 8 and 24 h post-derivatization. In order to measure the freeze and thaw stability, samples were prepared and kept in the freezer at -20º C for 24 h. The samples were removed from the freezer and allowed to thaw at room temperature followed by refreezing for another 24 h. Aliquots of the samples were derivatized and analyzed after each freeze-thaw cycle. At the end of the stability experiments, the % accuracy and CV% were calculated.

4.2.4. Bioavailability of ester derivatives

Under oxygen/methoxyflurane anaesthesia, animals were cannulated in their right jugular vein as previously described (Gilzad-Kohan and Jamali, 2012). Briefly, a

polyethylene cannula (Dow Corning Corp., Midland, MI, USA) tipped with 2 cm of silastic tubing (Becton Dickinson, Sparks, MD, USA) was inserted into their right jugular vein. Rats were allowed to recover overnight. Food was withdrawn from the animals 12 h before dosing. They were randomly assigned into four different groups (n=8-11/group) and received either GlcN, or one of its ester prodrugs (GVG, FFG, or VGG) at the equivalent to 100 mg/kg of GlcN and serial blood samples were collected just before the compound administration and at 0.25, 0.5, 0.75, 1, 1.5, 2, 3, and 4 h post dose. Samples were collected into heparinized tubes and plasma separated immediately by centrifugation. Total urine output was also collected over a period of 24 h. All samples were stored at -20º C until analyzed.

4.2.5. Fecal stability of GVG

Fecal pellets were collected from healthy rats (one pellet from each rat; n = 6) and placed in 50 mL centrifuge tube, softened by addition of 25 mL deionised water, vortex-mixed for 10 min and centrifuged at 2500 rpm for 8 min. The fecal suspension (1 mL) was transferred into a 1.5 mL microcentrifuge tube and centrifuged at 8,300 g for 5 min. The supernatant was immediately spiked with GVG (1.25 mg per each sample). The first set of samples was incubated at 37º C for 2 h to assess the effect of aerobic intestinal micro-flora. Air was removed from the tubes of the second set of samples (n=6) by blowing nitrogen (N_2) into the test tubes. Thereafter samples were incubated at 37º C for 2 h to assess the effect of anaerobic intestinal micro-flora. The third set of samples was placed in a 4º C

refrigerator for 2 h in order to study the effect of the feces compositions on GVG stability. A GVG solution (1.25 mg) with addition of no feces was set separately as a negative control. Control sample was incubated for 2 h at 37º C. At the end of the experiment the amount of GVG was determined using HPLC.

4.2.6. Effect of inflammation on GVG bioavailability

Animals were randomly divided into two groups: Inflamed or healthy control (n = 6). Adjuvant arthritis was developed in the inflamed animals by injecting 0.2 ml of *Mycobacterium butyricum* in squalene (50 mg/mL) into the tail base. Healthy rats were injected with the same volume of saline. On day 14 when the signs and symptoms of arthritis appear (Gilzad-Kohan and Jamali, 2012), all animals were cannulated in their right jugular vein. After an overnight recovery period, a dose of GVG (equivalent to 100 mg/kg of GlcN base) was administered and serial blood samples were collected before dosing and at 0.25, 0.5, 0.75, 1, 1.5, 2, 3, and 4 h post dosing. The total urine output was also collected for the determination of GVG and GlcN.

4.2.7. GVG efficacy in prevention of adjuvant arthritis

Rats were randomly assigned to six different groups (n=6 /group): Control-Healthy, Control-Inflamed, GlcN-20, GVG-20, GVG-30, and GlcN-90. On day one, all inflamed rats were injected 0.2 ml of *Mycobacterium butyricum* in squalene (50 mg/mL). Control-Healthy animals were injected 0.2 mL of normal saline. GlcN or GVG administration was commenced on the first day of

experiment as a preventive measure. In this study we chose 2 daily GlcN equivalent dosage regimens: First, 30 mg/kg GVG vs 90 mg/kg GlcN to confirm efficacy at doses higher than the minimum effective GlcN dose (40 mg/kg, (Agahzadeh-Habashi et al., 2013), and second, 20 mg/kg GVG versus 20 mg GlcN, a regimen below the minimum effective GlcN dose.

During the experiment body weight gain and paw thickness (using a caliper with a sensitivity of 25 µm, Mitutoyo Canada Inc., Toronto, ON) was measured daily. On the last day of experiment, serum nitrite was measured using a previously described method (Grisham et al., 1995). Nitrite standard curves were linear over the concentration range of 3 - 200 µM ($r^2 \geq 0.99$, coefficient of variation $\leq$10%).

On day 18 after injection of the adjuvant, the AI was calculated according to a previously described method (Piquette-Miller and Jamali, 1995). A maximum score of 14 could be assigned to each animal as follows: On a 0–4 basis hind paws were scored where zero was no virtual sign of arthritis, one was involvement of a single joint, two was involvement of more than one joint and/or ankle, three was involvement of several joints and ankle with moderate swelling, and four was involvement of several joints and ankle with severe swelling. Each forepaw was scored on a 0–3 basis scoring system where 0 was involvement of none of the joints, one was involvement of single joint, two was involvement of more than one joint and/or wrist, and three was involvement of wrist and joints with moderate-to-severe swelling.

4.2.8. Data treatment and statistical analysis

The non-compartmental approach was used to calculate bioavailability parameters. The area under plasma concentration-time curve (AUC) 0-4 h post-dose was calculated using the trapezoidal method. The peak concentration (C_{max}) and the time of its attainment (t_{max}) were the experimentally observed values. Data are presented as mean ± SD. Differences between two means were assessed using the Student's t-test. To compare three or more means, one-way ANOVA followed by the Bonferroni post-test was used. Statistical analyses were carried out using Prism software (GraphPad Software Inc., San Diego, CA, USA) at $p < 0.05$.

4.3. Results

4.3.1. Simultaneous HPLC assay for GlcN and GVG

The HPLC peaks for Fmoc derivatized GlcN and mannosamine (IS) appeared as pairs of equal sized and resolved anomers at 11.6 and 14.1 min for the IS and 12.7 and 15.5 min for GlcN (Figure 4.1). The second peak of each compound was used in order to quantify the samples. GVG resolved as a single peak at a retention time of 17.7 min. There was no interfering peak in the spiked rat plasma samples.

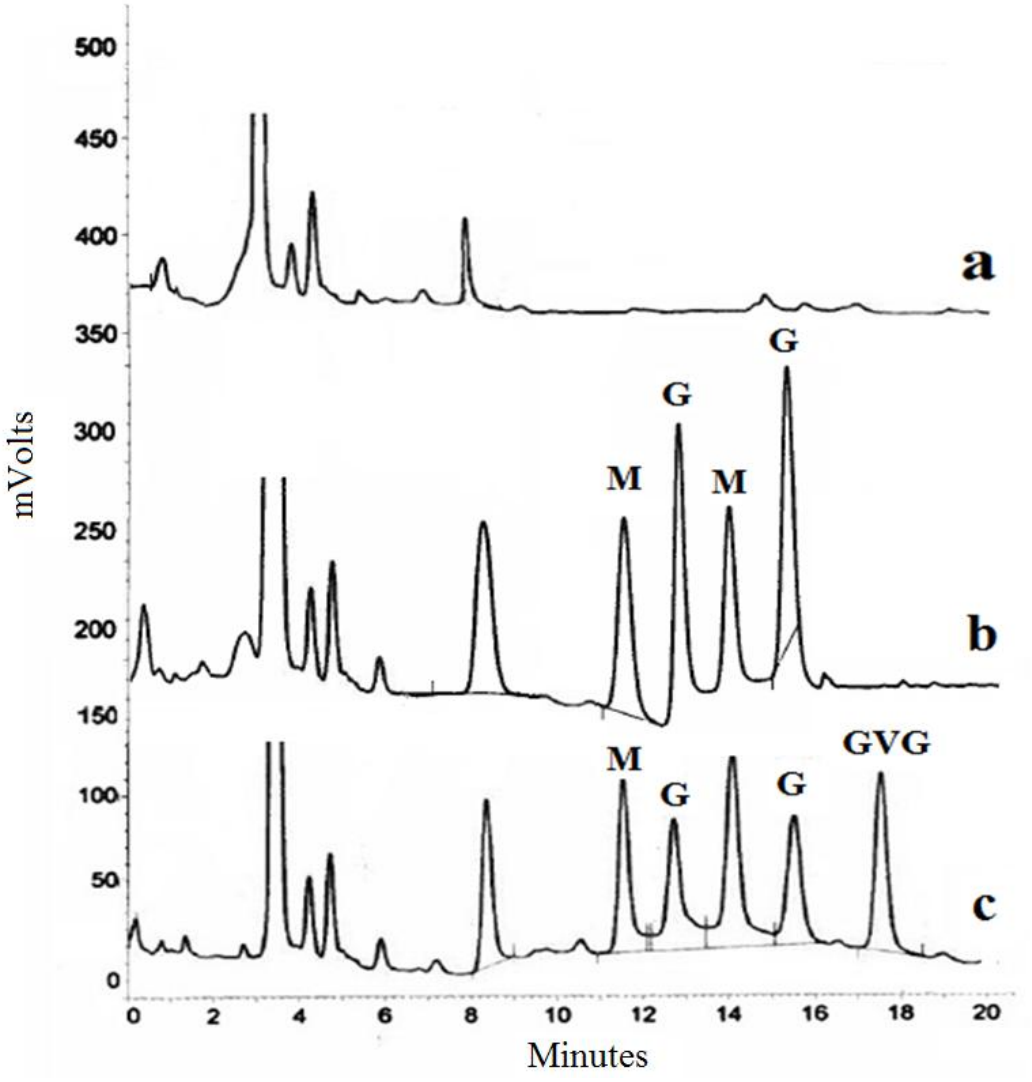


Figure 4.1. HPLC chromatograms of (a) blank rat plasma, (b) rat plasma at t=4 h post-dosing (GVG 100 mg/kg); only GlcN peaks appears in plasma after GVG administration, and (c) blank rat plasma spiked with 1 µg/mL GlcN and GVG. Keys: M, mannosamine (IS); G, GlcN; GVG, Gly-Val-COO-GlcN

The assay was linear over the range of 0.05-20 µg/mL for both GVG and GlcN in rat plasma ($r^2 \geq 0.99$). The lowest limit of quantification was set at 50 ng/mL for both compounds. The peak height measurement approach provided lower variability in response than that of the peak area, hence, the former was used

throughout. The inter- and intra-day variations were less than 10% for all concentrations and the accuracy ranged from -1.4 to 2.4% for GlcN and -6 to 8% for GVG (Table S3).

The percent recovery of the IS from rat plasma was 96.1±3. The % recovery of GVG was 99±5.3, 98.2±5.1, 97±3.7, 95.9±9.2, and 94.2±7.3 for the 0.05, 0.5, 5, 10, and 20 μg/mL samples in rat plasma. Percent recovery of GlcN from rat plasma appeared to be 105±6.1, 97.8±7, 103.2± 4.3, 103.3±2.2, and 99.8±6.2% from the 0.05, 0.5, 5, 10, and 20 μg/mL samples.

Derivatized GVG and GlcN were stable in all plasma samples during the analysis process and 24 h after derivatization; stability was > 91 % for all samples. GVG and GlcN also appeared to be stable in rat plasma after three freeze and thaw cycles (Table S4).

4.3.2. Bioavailability of GlcN versus its ester derivatives

Figure 4.2 depicts the mean plasma concentration-time curve of GlcN and figure S7 shows the spaghetti graph of plasma concentration-time curve of GlcN in individual Sprague Dawley rats after administration of equivalent doses (100 mg/kg of GlcN base) of GlcN or its ester derivatives. All compounds were rapidly absorbed ($t_{max} \leq 0.5$ h). However, among all tested compounds, only GVG showed a significantly higher oral bioavailability as compared to GlcN (Table 4.1). FFG and VGG only showed an insignificant trend toward increased bioavailability as compared to GlcN. GVG also exhibited a significant increase in percent of dose excreted in the urine as compared to the other tested compounds.

No significant differences were noticed among GlcN, FFG, and VGG in any of the bioavailability parameters.

No intact GVG was detected in plasma or urine after its oral administration.

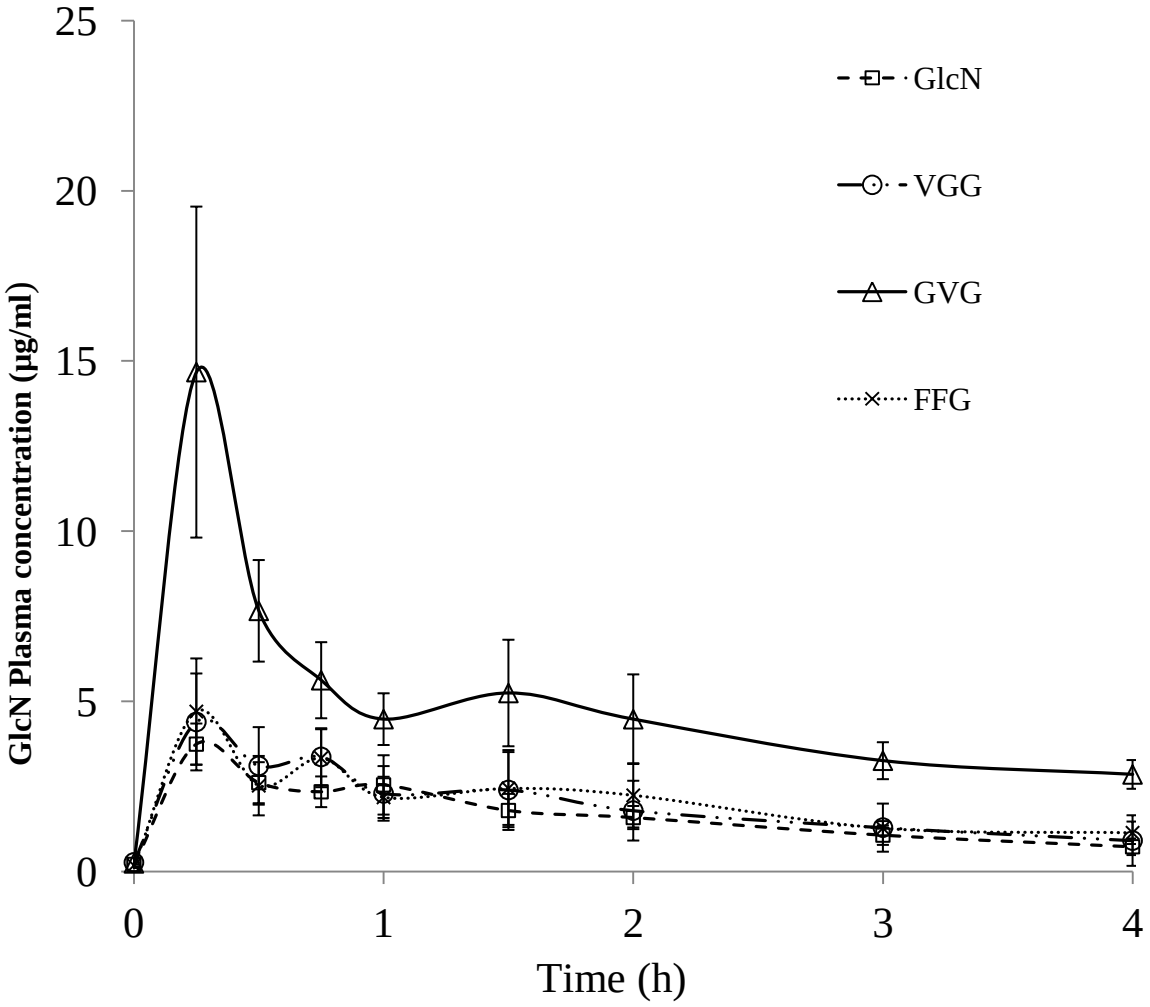


Figure 4.2. GlcN mean plasma concentration (±SD) versus time profile following oral administration of GlcN or the ester derivatives of GlcN to rats (n=8-11/group). All doses were equivalent to100 mg/kg of GlcN base.

Table 4.1. Bioavailability parameters of GlcN and its ester derivatives

Compound	GlcN	VGG	FFG	GVG
C_{max} (µg/ml)	3.7 ± 0.6^{a}	4.4 ± 1.4^{a}	4.7 ± 1.6^{a}	14.6 ± 4.9^{b}
$AUC_{0\text{-}4}$ (µg.h/ml)	6.6 ± 1.2^{c}	7.8 ± 1.6^{c}	8.1 ± 1.5^{c}	19.2 ± 3.7^{d}
% of dose excreted in the $urine_{0\text{-}24}$	2.2 ± 0.3^{e}	2.6 ± 0.5^{e}	2.8 ± 0.7^{e}	6.8 ± 0.6^{f}

For all compounds, a single oral dose equivalent to 100mg/kg of GlcN base was orally administered to the animals (n=8-10/group). Data are shown as mean± SD. Different superscript characters indicate significant differences between means in a row ($p < 0.05$).

4.3.3. Fecal stability of GVG

Figure 4.3 depicts the percent remaining of GVG after 2 h incubation under control (no feces), aerobic, and anaerobic conditions at 37 and 4º C. Incubation of GVG with rat faces resulted in almost 33% loss in all samples. There were no significant differences between the amounts of GVG that remained at the end of the experiment under aerobic, anaerobic, or 4º C conditions. At the end of the experiment, approximately 97% of GVG remained intact in the control samples.

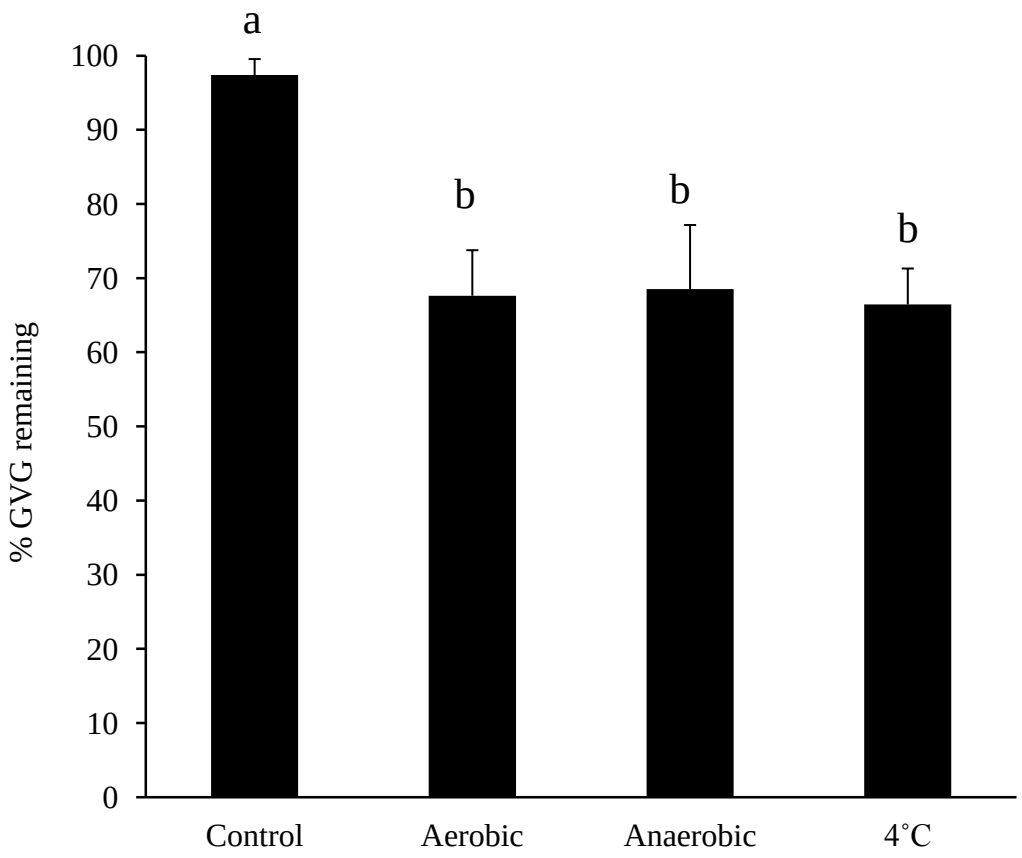


Figure 4.3. The average percent GVG remaining in rat feces after 2 h incubation at 37º C (control, aerobic and anaerobic) or at 4º C. Error bars represent standard deviation. The same character indicates no significant difference between means at $p < 0.05$; n=6/group.

4.3.4. Effect of inflammation on GVG bioavailability

Inflammation did not significantly alter the oral bioavailability of a single oral dose of GVG (100 mg/kg of GlcN base) as no significant differences were noticed between the groups in C_{max}, AUC_{0-4} and the percent of dose excreted in the urine in 24 h (Figure 4.4 and Table 4.2).

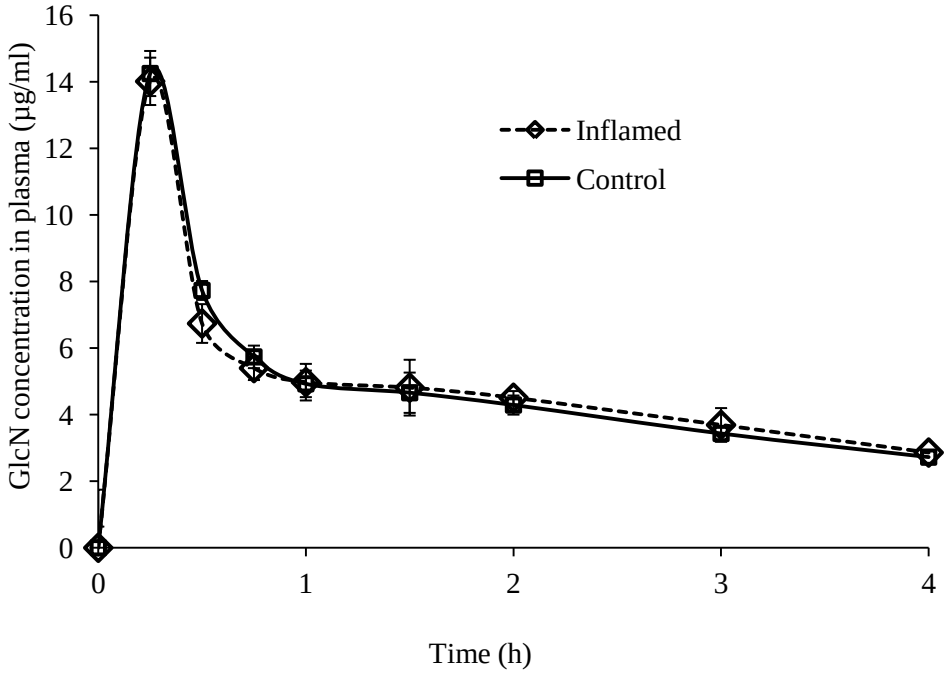


Figure 4.4. Mean (n=6/group) plasma concentration (±SD) versus time profiles of GlcN in control and inflamed rats following oral administration of GVG (single dose equivalent to 100 mg/kg GlcN base).

Table 4.2. Bioavailability parameters of GVG in control and inflamed rats (n=6 /group).

Group	Inflamed	Control
C_{max} (µg/ml)	14.01 ± 1.7	14.25 ± 0.6
AUC_{0-4} (µg.h/ml)	19.1 ± 1.1	18.97 ± 0.5
% of dose excreted in the urine$_{0-24}$	7.1 ± 0.6	6.9 ± 0.5

4.3.5. GVG efficacy in prevention of adjuvant arthritis

Adjuvant arthritis emerged in the rats (Control-Inflamed) that received *Mycobacterium butyricum* but were not treated with either GlcN or GVG. They

exhibited significant increases in paw thickness (Figure 4.5) as compared to the Control-Healthy animals and, at the end of the experiment (day 18) had an AI of 9.1 ± 0.8. No sign of arthritis was observed in the Control-Healthy group or in the rats that were treated with 90 mg/kg/day GlcN (GlcN-90) or 20 and 30 mg/kg/day GVG. Administration of 20 mg/kg/day GlcN (GlcN-20) did not prevent AA but the severity of the disease was significantly less than those observed for Control-Inflamed (AI, 4.1 ± 1.1) (Table S5).

Similar observations were made with the weight gain (Figure 4.6) and serum nitrite levels (Figure 4.7). Serum nitrite was elevated in inflamed but not treated animals. GVG or GlcN administration in GlcN-90, GVG-30 and GVG-20 animals was able to keep the serum nitrite concentration at normal levels; however administration of 20mg/kg/day of GlcN (GlcN-20) could not normalize the elevated serum nitrite. Inflammation significantly reduced weight gain in the inflamed animals, which was normalized by GVG or GlcN treatment in GlcN-90, GVG-30 and GVG-20 groups. Animals in the GlcN-20 group did not gain weight as much as control animals; however, their average weight gain was significantly higher than inflamed but not treated animals.

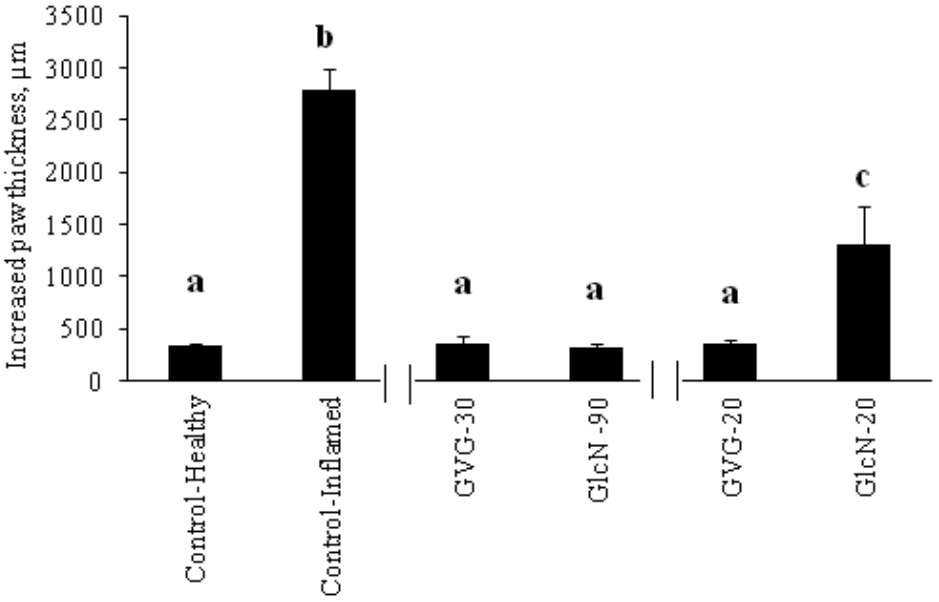


Figure 4.5. Average increase in paw thickness at the end of the experiment. Error bars represent standard deviation. The same character indicates no significant difference between means at $p < 0.05$; n = 6/group.

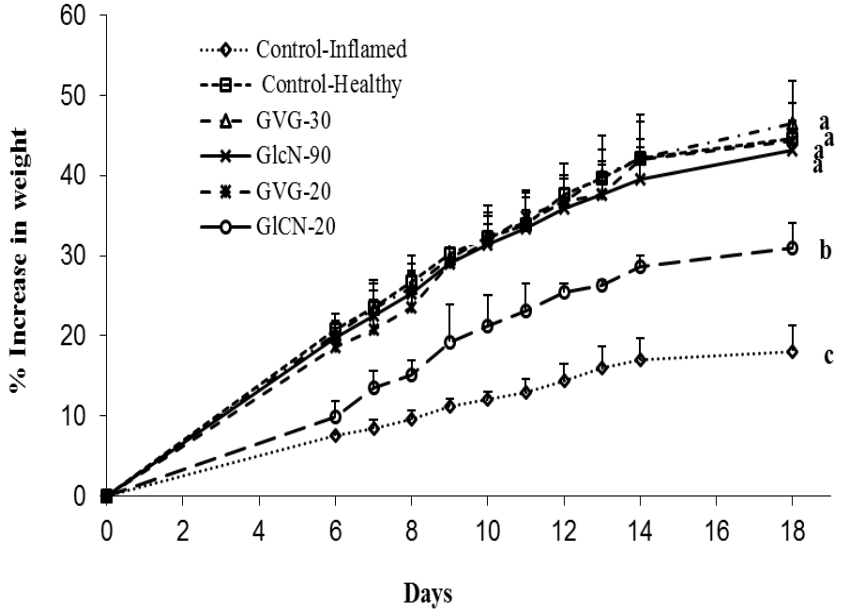


Figure 4.6. The average percent weight gain (+SD) from baseline. Error bars represent standard deviation. The same character indicates no significant difference between means at $p < 0.05$.

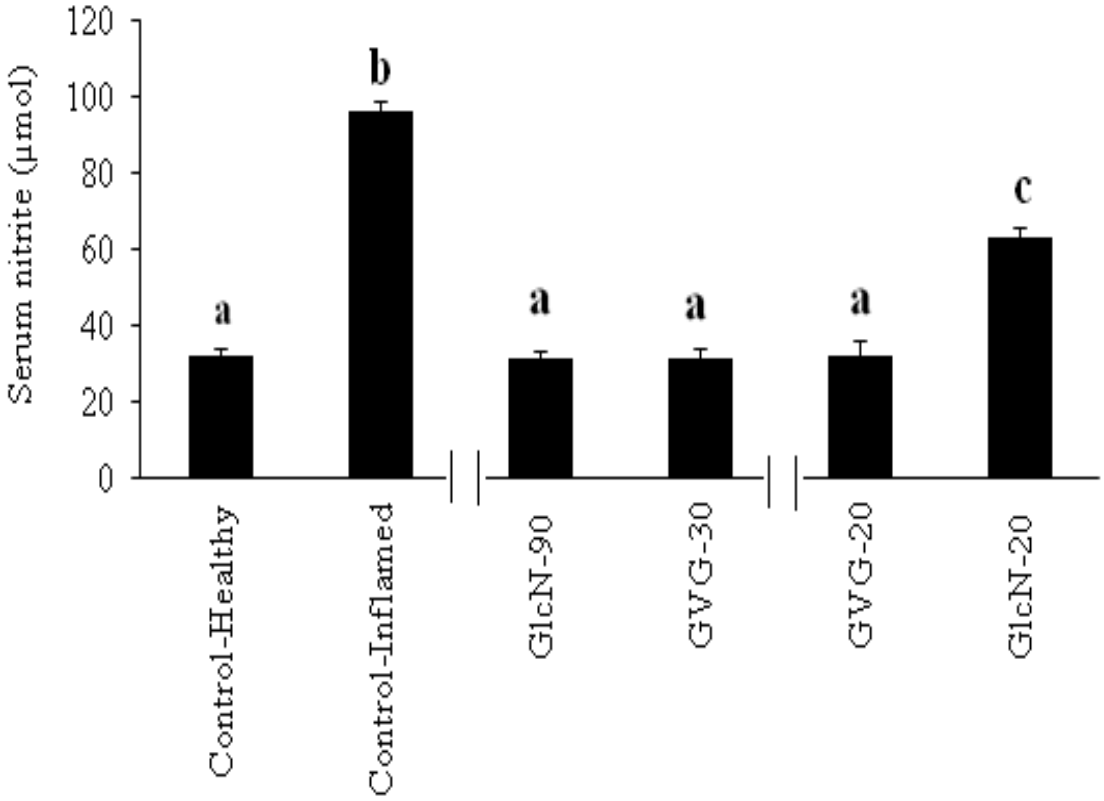


Figure 4.7. The effect of inflammation and prevention with GlcN or GVG on serum nitrite concentration in inflamed rats (n = 6/group); error bars represent standard deviation. The same character indicates no significant difference between means at $p < 0.05$.

4.4. Discussion

4.4.1. Assay of GVG and GlcN

We developed a sensitive HPLC assay for simultaneous measurement of GlcN and its ester derivative, GVG, based on fluorescence detection. As neither GlcN nor GVG contain fluorophore groups, we derivatized them to Fmoc-GlcN and -GVG. Fmoc-Cl has been used since 1980s for HPLC analysis of amino acids

(Einarsson et al., 1983). It rapidly reacts with primary and secondary amines under alkaline conditions. The recovery of GlcN and GVG from plasma samples ranged between 97.8-105 and 94.2-99 %, respectively. The solvent gradient program and the addition of 0.1% acetic acid to the mobile phase resulted in sharper and more resolved peaks (Figure 4.1). Most of the plasma interfering peaks eluted before the appearance of the peaks of interest. The derivatization method yields two equal sized but completely resolved anomers (stereoisomers of a cyclic sugars that differ in their configuration at the anomeric carbon) peaks (Jamali and Ibrahim, 2010). The excessive amount of the derivatizing agent was removed by adding amantadine to form a hydrophobic complex that elutes at the end of the run time and before the next injection. To prevent pump pressure build-up, the injection volume was set at 5 μL.

The method was linear over the examined range, accurate and reproducible with the minimum quantifiable concentration of 50 ng/ml for both compounds (inter- and intra-day variations<10%; accuracy, -1.4 to 2.4% for GlcN and -6 to 8% for GVG).

4.4.2. Bioavailability

All of the three examined di-peptide GlcN esters demonstrated trends toward a greater oral bioavailability than the parent compound but the change reached statistical significance only for GVG. This is in agreement with the previously reported enhanced permeability across the gut membrane likely facilitated by *PepT1* (Gilzad-Kohan et al., 2013). Our observation may be proven useful in

predicting oral absorption of these compounds following administration to humans, since rats appear to be suitable predictors of drug absorption by humans (Cao et al., 2006). This, however, may not hold true for the expression levels and patterns for metabolizing enzymes in the gut and liver. The superiority of GVG over other examined derivatives is in agreement with our previous observation that it has higher permeability through the everted rat gut (Gilzad-Kohan et al., 2013). The high permeability of GVG is due, likely, to the recognition of the conjugate by the *PepT1*, as a specific *PepT1* substrate; Gly-Sar completely inhibited transport of GVG (Gilzad-Kohan et al., 2013). In general, the 3-dimensional conformation properties of a substrate govern whether a substrate has affinity for *PepT1* (Bailey et al., 2000; Bailey et al., 2006; Rubio-Aliaga and Daniel, 2008). GVG seems to have the appropriate stereochemical and conformational features. There are several other examples in which researchers have attempted to improve oral absorption of various poorly absorbed drugs via attachment to amino acids or dipeptides in order to resemble *PepT1* substrates, mostly through ester bonds (Anand et al., 2004; Purifoy et al., 1993; Szczech, 1996; Weller et al., 1993). Similar to GVG, the glycine-valine ester prodrug of acyclovir appeared to be more efficiently absorbed across the intestinal mucosa as compared to other ester derivatives (Anand et al., 2004; Purifoy et al., 1993; Szczech, 1996; Weller et al., 1993).

GVG has been shown to be relatively stable in the gut, readily crosses the gut wall and rapidly yields the parent drug upon incubation with liver homogenates (Gilzad-Kohan et al., 2013). Although it has been shown that GVG

remains intact during movement from mucosal toward the serosal side in the rat small intestine (Gilzad-Kohan et al., 2013), we were not able to detect any intact GVG in the systemic circulation. In other words GVG seems to be transported into the portal vein and then cleaved into GlcN and the di-peptide moiety either in the liver and/or in plasma.

In general, the activation of the prodrug such as GVG after its in vivo hydrolysis is not expected to raise toxicity concerns since the only activation byproducts are a dipeptide and/or amino acids and the parent drug (Anand et al., 2004; Krylov et al., 2013).

The low bioavailability of GlcN is attributed to at least two mechanisms, the inefficiency of the involved transporters (i.e., GLUT-2) and the loss due to the uptake by the gut microflora (Ibrahim et al., 2012). It has been shown that incubation of 1.25 mg GlcN with rat faces results in 95% loss. For GVG only 33% loss was detected over a 2 h incubation period (Figure 4.3). The mechanism of this loss is remains unclear as it occurred under various conditions (aerobic, anaerobic and or 4º C) that may put the mere involvement of microflora into question. Nevertheless, the observed lower fecal instability of GVG as compared with GlcN may contribute to the greater bioavailability of the former.

4.4.3. Efficacy

The rat model of AA mimics human RA and has been used extremely for decades to assess the efficacy of antirheumatic agents (Rosenthale and Capetola, 1982). We used this model to compare the anti-inflammatory effects of GVG with GlcN.

Assuming a three-fold greater bioavailability of GVG as compared with GlcN, we first compared 30 mg/kg/day of the former with 90 mg/kg/day (GlcN equivalent) of the latter and noticed that the treatments were equally effective in preventing AA (Figure 4.5), reduced body weight gain (Figure 4.6) and increased serum nitrite levels (Figure 4.7). Considering the fact that oral administration of GVG yields only GlcN in the systemic circulation, one can suggest that the greater potency of GVG is due to the corresponding higher GlcN bioavailability after administration of the prodrug as compared to the parent compound. To further confirm the observation, we compared the two compounds following daily treatment with 20 mg/kg/day (GlcN equivalent), which was known to be below the GlcN effective dose range (Agahzadeh-Habashi et al., 2013). The GVG and not GlcN regimen was effective in completely preventing the experimental arthritis. This further confirms that at a dosage level that GlcN is not fully effective, GVG prevents the emergence of AA.

4.4.4. Effect of inflammation on GVG pharmacokinetics

Inflammation may cause alteration in expression of some transporters (Cressman et al., 2012) and potentially alter bioavailability of drugs. This appears to not be the case for GVG, assuming a major involvement of *PepT1* in its absorption (Gilzad-Kohan et al., 2013) since the plasma GlcN concentration-time curves following GVG and GlcN in equal doses (GlcN equivalent) were almost superimposable (Figure 4.4). Similarly, according to Radeva et al. (2007), experimentally-induced rat colitis, another inflammatory condition, does not alter

the bioavailability of cephalexin or valacyclovir, two known *PepT1* substrates despite certain modifications in the transporter mRNA expression (Radeva et al., 2007).

4.5. Conclusion

A novel dipeptide ester of GlcN, GVG, is found to have an increased bioavailability assessed using a novel HPLC method. GVG exhibited increased anti-inflammatory potency through a greater GlcN delivery into the systemic circulation. The pharmacokinetics of GlcN delivered through GVG is not influenced by AA.

Supporting Information

Additional tables (S3-S5) related to CV and Accuracy of the simultaneous HPLC assay for GlcN and GVG, short term and freeze and thaw stability of GVG and GlcN in rat plasma samples and AI scores, and figure (S7) related to the spaghetti graph of the plasma concentration-time curve of GlcN in individual Sprague Dawley rats after administration of equivalent doses (100 mg/kg of GlcN base) of GlcN or its ester derivatives are available in the supporting information in the appendix.

Chapter 5

5.1. General discussion and conclusion

In this project, we are reporting for the first time that GlcN is effective in reducing signs of AA after its emergence (Fig.2.1) in addition to its preventative effect reported previously (Hua et al., 2005). These beneficial effects of GlcN were associated with decreased levels of the pro-inflammatory biomarker, serum nitrite (Fig.2.3) and enhanced body weight gain (Fig. 2.2). The mechanism for the anti-inflammatory effect of GlcN is not clear but it has been attributed to its ability to suppress neutrophil functions (Hua et al., 2005), to inhibit NF-kB activation (Hwang et al., 2010), to suppress the maturation of naïve CD4+ T cells to Th2 cells (Kim et al., 2011), and to induce tissue TGF β1 and connective tissue growth factor (Ali et al., 2011).

We examined the beneficial effects of GlcN following a dosage regimen of 300 mg/kg/day that yields a plasma peak concentration of approximately 16 µg/mL (Fig.2.7) that is substantially higher than those recorded for humans following the commonly used dosage of 1500 mg/day (<3.4 µg/mL) (Aghazadeh-Habashi and Jamali, 2011). This, however, does not imply that lower doses are ineffective in the treatment of rat AA. Indeed, doses as low as 40 mg/kg/day that corresponding to GlcN plasma mean peak concentration of 1.32 ± 0.24 µg/mL, demonstrates preventative effects on emergence of AA in the rat (Agahzadeh-Habashi et al., 2013).

It is known that inflammatory conditions result in serious cardiovascular complications (Gerli and Goodson, 2005; Kapetanovic et al., 2011) and down regulation of calcium channel and β-adrenergic target proteins, hence reduced

response to some pharmacotherapeutic approaches (Kulmatycki and Jamali, 2005). Our present data confirms the adverse effect of inflammation on these target proteins and reduced response to verapamil, a calcium channel blocker. Moreover, we have demonstrated for the first time that GlcN restores these conditions by both preventing and controlling the disease. One of the most common approaches in curtailing inflammation is the use of NSAIDs. These drugs, however, have their own cardiovascular side effects that limit their use (Davies and Jamali, 2004; Harirforoosh and Jamali, 2009). Our present data on the beneficial effects of GlcN may suggest a safe (Anderson et al., 2005; Sawitzke et al., 2010) alternative to NSAIDs in the treatment of inflammatory conditions. In addition, we have demonstrated that, unlike NSAIDs, treating healthy rats with GlcN does not result in any alteration of the examined cardiovascular factors.

Glucosamine either as a preventive measure or as a treatment tool completely restored the diminished response to verapamil as well as the down regulation of the 190 kDa Cav1.2 subunit protein.

It is also known that inflammation reduces the efficiency of the hepatic drug-metabolizing enzymes (Morgan et al., 2008) resulting in increased plasma concentration of drugs such as verapamil that are efficiently metabolized before reaching the systemic circulation. The present data suggest that both prevention and control of AA by GlcN results in rebuilding of this metabolic pathway, hence normalizing, at least in part, the pharmacokinetics of verapamil (Fig.2.6).

GlcN is a drug with substantial presystemic clearance and hence has a low oral bioavailability (>20%) in both humans (Setnikar et al., 1984) and rats

(Aghazadeh-Habashi et al., 2002). However, unlike verapamil that demonstrates substantial reduced clearance under inflammatory conditions, GlcN pharmacokinetics were not influenced by AA (Fig.2.7). This is likely due to the fact that for GlcN, the first-pass loss upon oral administration is attributed to its clearance in the gut rather than the liver (Aghazadeh-Habashi et al., 2002). It appears that the mechanism involved in the gut clearance of GlcN is not significantly influenced by inflammation.

However, GlcN has low bioavailability, which limits its beneficial therapeutic effects (Aghazadeh-Habashi and Jamali, 2011). Designing prodrugs to target a specific receptor in the gastrointestinal (GI) tract in order to enhance bioavailability is a widely utilized method in drug development (Han and Amidon, 2000). The prodrugs are usually inactive entities that quickly yield the parent drug as soon as they enter the systemic circulation. Among several intestinal transporters, *peptide transporter 1 (PepT1)* has attracted a great deal of attention in recent years (Han and Amidon, 2000; Yu et al., 1996). *PepT1* shows broad substrate specificity and transports di- and tri-peptides. Introducing an amino acid or di-peptide moiety into the molecular structure of parent drugs via an ester or amide bond is a common strategy in this matter (Anand et al., 2004; Weller et al., 1993). New drug conjugates should have a desired physicochemical and intestinal stability. On the other hand they should convert to their parent drug as soon as they cross the GI membrane and enter the systemic circulation. The site of prodrug cleavage might be the plasma or the liver.

We synthesized and characterized several GlcN mono-amino acid and di-peptide ester and amide conjugates in order to find a GlcN prodrug with enhanced gut permeability. We used an in-solution synthesis method to synthesize the amide derivatives (Figure 3.2B) and a solid-phase approach to synthesize the ester derivatives (Figure 3.2A). Since the amine group of GlcN is several fold more reactive than the primary and secondary hydroxyl groups (QI-WEI et al., 2002), several amide prodrugs were synthesized easily in-solution without protecting the hydroxyl groups. As mentioned above, the ester derivatives were synthesized using a solid-phase approach. There are several reasons to use the solid-phase approach including convenience, acceleration of the overall process and the ability to achieve good yields of purified products (Amblard et al., 2006). This allowed the development of a large number of candidate prodrugs. In addition, an in-solution method for synthesis of ester prodrugs requires the protection of the amine groups which, in turn, could add several steps to the process.

One of our goals of this study was to synthesize GlcN derivatives with desirable physical stability. GlcN is available on the market as sulfate and HCl salts with the latter being the salt in the most of the commercially available products for which clinical data are available (Herrero-Beaumont et al., 2007; Noack et al., 1994; Rovati et al., 2012). The bioequivalence and possible identical efficacy of the two salts has been discussed elsewhere (Aghazadeh-Habashi and Jamali, 2011); however, since the sulfate salt is known to lack physical stability (unless formulated as crystalline GlcN sulfate with additional stabilizing salts) (Russell et al., 2002), we aimed to synthesize new GlcN derivatives with

enhanced chemical stability. The stable derivatives do not require the addition of other stabilizing salts such as KCl. This approach, in turn, provided the opportunity to reduce the size of the administrable bulk. A reduction in the size of the finished product is of primary importance, particularly for elderly patients. The currently available data regarding the stability of the HCl salt is not conclusive; however the stability of the finished products can be an issue since GlcN HCl crystals are hygroscopic. Further investigations are needed in order to assess the stability of the HCl salt. Newly synthesized compounds were tested for their physicochemical stability. All of the synthesized GlcN prodrugs that have been studied here appear to have a desirable chemical stability. Next, pH stability of the newly synthesized compounds was also studied at two different pH's, 2 and 7.4, which correspond to the normal pH of different segments of the gastro-intestinal tract. All the studied compounds exhibited a high stability in the aforementioned pH conditions.

In addition, all the prodrugs were investigated for their GI stability, their ability to be biotransformed into the parent drug in the liver, and their transportability through the everted rat gut. GI stability is another prerequisite for suitable orally administered prodrug candidates, for if they cleave before reaching their site of absorption, they will lose their property to enhance the bioavailability of the parent drug (Anand et al., 2004). Amide and ester derivatives demonstrated varied susceptibility to the hydrolyzing intestinal enzymes. Gly-Val-COO-GlcN (GVG), Phe-Phe-COO-GlcN (FFG), and Val -Gly-COO-GlcN (VGG) demonstrated cleavage half-lives of around 16-17 min in the intestinal

homogenate, which was the highest stability among all other ester prodrugs. In another study (Anand et al., 2004), a series of acyclovir di-peptide prodrugs were synthesized and tested *in vitro* and *in vivo*. According to the authors, intestinal homogenate hydrolysis studies revealed that a minimum cleavage half-life of 15 min was desirable in order to have a potential candidate with increased bioavailability. Among all the synthesized ester derivatives only GVG, FFG, and VGG met this requirement. However, none of the amide conjugates showed GI instability. In fact, all of the amide prodrugs were too stable to release GlcN even when incubated with liver homogenates. In contrast, all of the ester prodrugs were rapidly cleaved to yield GlcN after incubation with liver homogenates in less than 15 min. In this step we conclude that the successful candidate(s) should be an ester conjugate rather than an amide conjugate.

In order to improve bioavailability, in addition to an acceptable stability profile, GlcN prodrugs needed to demonstrate an acceptable transportability via *PepT1*. Previously, the affinity of several di-peptides toward *PepT1* has been studied (Bailey et al., 2006). Many of the studied di-peptide sequences exhibited high affinity toward the transporter; however high affinity may not always be translated into high transporter-mediated transportation after oral administration because the substrates might only bind to the transporter without being translocated (Anand et al., 2004). We utilized the permeability test through the everted rat gut in order to determine the transportability of the GlcN derivatives. GVG fulfilled this criterion better than other derivatives, showing a significant increase in the cumulative amount of GlcN inside the jejunum sacks. We

assumed that the increase in the permeability of the GVG prodrug was possibly due to the recognition of the conjugate by the *PepT1*. This is most likely due to the fact that this conjugate met the general requirements concerning the structures of the *PepT1* substrates, including possession of a di- or tri-peptide skeleton, high affinity toward the transporter, and an appropriate small size (Bailey et al., 2006). FFG and VGG also showed increases in the cumulative amount crossing through the gut wall; these, however, did not reach statistical significance.

As mentioned above, the increase in the permeability of the Gly-Val-COO-GlcN (GVG) prodrug was possibly due to the recognition of the conjugate by the *PepT1*. To prove this assumption, we performed a permeability inhibition test with Gly-Sar, a specific *PepT1* substrate. As depicted in Figure 3.3B, Gly-Sar inhibited transport of Gly-Val -COO-GlcN, indicating penetration by way of the *PepT1*.

At this step, we concluded that the GlcN di-peptide conjugate, GVG, demonstrated the most desirable chemical and physical stability and the greatest gut permeability of GlcN as compared to GlcN and the other synthesized derivatives. The uptake of this conjugate was efficiently mediated by *PepT1*, as it was significantly inhibited in the presence of Gly-Sar. We, therefore, suggest GVG to be a desired GlcN prodrug in order to increase GlcN bioavailability.

Next we examined the bioavailability of the three di-peptide GlcN esters, which demonstrated trends toward greater gut permeability than the parent compound. The change reached statistical significance only for GVG. This is in agreement with the previously reported enhanced permeability across the gut

membrane likely facilitated by *PepT1* (Gilzad-Kohan et al., 2013). Our observation may prove useful in predicting oral absorption of these compounds following administration to humans, since rats appear to be suitable predictors of drug absorption by humans (Cao et al., 2006). This, however, may not hold true for the expression levels and patterns for metabolizing enzymes in the gut and liver. The superiority of GVG over other examined derivatives is in agreement with our previous observation that it has higher permeability through the everted rat gut (Gilzad-Kohan et al., 2013). As mentioned above, the high permeability of GVG is due, likely, to the recognition of the conjugate by the *PepT1*, as a specific *PepT1* substrate; Gly-Sar completely inhibited transport of GVG (Gilzad-Kohan et al., 2013). In general, the 3-dimensional conformation properties of a substrate govern whether a substrate has affinity for *PepT1* (Bailey et al., 2000; Bailey et al., 2006; Rubio-Aliaga and Daniel, 2008). GVG seemed to have the appropriate stereochemical and conformational features. There are several other examples in which researchers have attempted to improve oral absorption of various poorly absorbed drugs via attachment to amino acids or dipeptides in order to resemble *PepT1* substrates, mostly through ester bonds (Anand et al., 2004; Purifoy et al., 1993; Szczech, 1996; Weller et al., 1993). Similar to GVG, the glycine-valine ester prodrug of acyclovir appeared to be more efficiently absorbed across the intestinal mucosa as compared to other ester derivatives (Anand et al., 2004; Purifoy et al., 1993; Szczech, 1996; Weller et al., 1993).

GVG has been shown to be relatively stable in the gut, to readily cross the gut wall and to rapidly yield the parent drug upon incubation with liver

homogenates (Gilzad-Kohan et al., 2013)**.** Although it has been shown that GVG remains intact during movement from mucosal toward serosal side in the rat small intestine (Gilzad-Kohan et al., 2013), we were not able to detect any intact GVG in the systemic circulation. In other words GVG seems to be transported into the portal vein and then cleaved into GlcN and the di-peptide moiety either in the liver and/or in plasma.

In general, the activation of the prodrug such as GVG after its in vivo hydrolysis is not expected to raise toxicity concerns since the only activation byproduct are a dipeptide and/or amino acids and the parent drug (Anand et al., 2004; Krylov et al., 2013).

The low bioavailability of GlcN is attributed to, at least, two mechanisms, the inefficiency of the involved transporters (i.e., GLUT-2) and the loss due to the uptake by the gut microflora (Ibrahim et al., 2012). It has been shown that incubation of 1.25 mg GlcN with the rat faces results in 95% loss. For GVG only 33% loss was detected over a 2 h incubation period (Figure 4.3). The mechanism of this loss is remains unclear as it occurred under various conditions (aerobic, anaerobic and or 4º C) that may put the mere involvement of microflora into question. Nevertheless, the observed lower fecal instability of GVG as compared with GlcN may contribute to the greater bioavailability of the latter.

Next we examined the efficacy of GVG in prevention of AA. The rat model of AA mimics human RA which has been used extensively for decades to assess the efficacy of antirheumatic agents (Rosenthale and Capetola, 1982). We used this model to compare the anti-inflammatory effects of GVG with GlcN.

Assuming a three-fold greater bioavailability of GVG as compared with GlcN, we first, compare 30 mg/kg/day of the former with 90 mg/kg/day (GlcN equivalent) of the latter and noticed that the treatments were equally effective in preventing AA (Figure 4.5), reduced body weight gain (Figure 4.6) and increased serum nitrite levels (Figure 4.7). Considering the fact that oral administration of GVG yields only GlcN in the systemic circulation, one can suggest that the greater potency of GVG is due to the corresponding higher GlcN bioavailability after administration of the prodrug as compared to the parent compound. To further confirm the observation, we compared the two compounds following daily treatment with 20 mg/kg/day (GlcN equivalent), which was known to be below the GlcN effective dose range (Agahzadeh-Habashi et al., 2013). The GVG and not GlcN regimen was effective in completely preventing the experimental arthritis. This further confirms that at the dosage level that GlcN is not fully effective, GVG prevents the emergence of AA.

Inflammation may cause alterations in expression of some transporters (Cressman et al., 2012) and potentially alter bioavailability of drugs. This appears to not be the case for GVG, assuming a major involvement of *PepT1* in its absorption since the plasma GlcN concentration-time curves following GVG and GlcN in equal doses (GlcN equivalent) were almost superimposable (Figure 4.4). Similarly, according to Radeva et al. (2007), experimentally-induced rat colitis, another inflammatory condition, does not alter the bioavailability of cephalexin or valacyclovir, two known *PepT1* substrates, despite certain modifications in the transporter mRNA expression (Radeva et al., 2007).

As a conclusion, a novel dipeptide ester of GlcN, GVG, is found to have an increased bioavailability as assessed using a novel HPLC method. GVG exhibited increased anti-inflammatory properties through a greater GlcN delivery into the systemic circulation. The pharmacokinetics of GlcN delivered through GVG is not influenced by AA.

5.2. Future directions and studies

In our initial screening in order to find a glucosamine (GlcN) prodrug with enhanced bioavailability, GVG showed pharmaceutical superiority compared to GlcN in term of bioavailability and fecal stability in animal subjects. GVG successfully fulfilled the requirements for developing a pharmaceutical ingredient from a nutraceutical. However the following tests and studies are necessary before the newly synthesized compound can be used in humans.

- Toxicological examination to establish safety profile of GVG

- Bioavailability study on human subjects

- Clinical trial on human subjects to show efficacy of GVG

Chapter 6

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Appendix

Supporting information

Supporting figures

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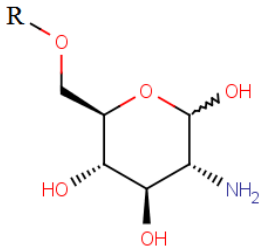

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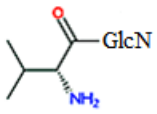

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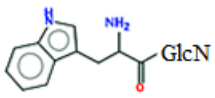

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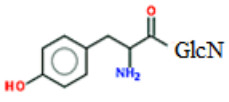

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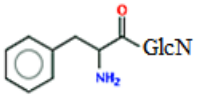

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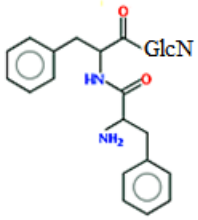

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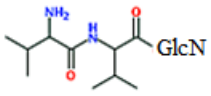

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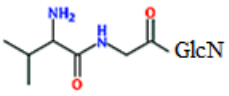

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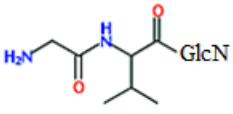

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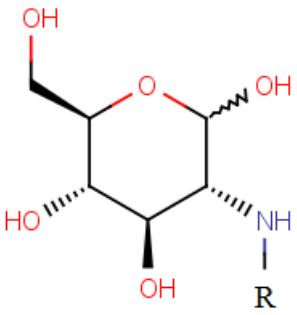

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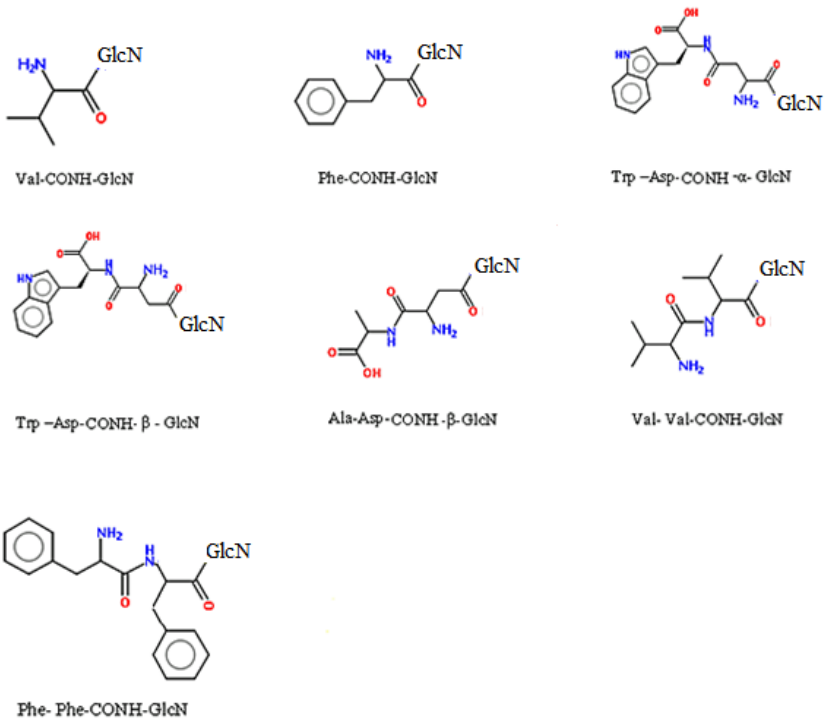
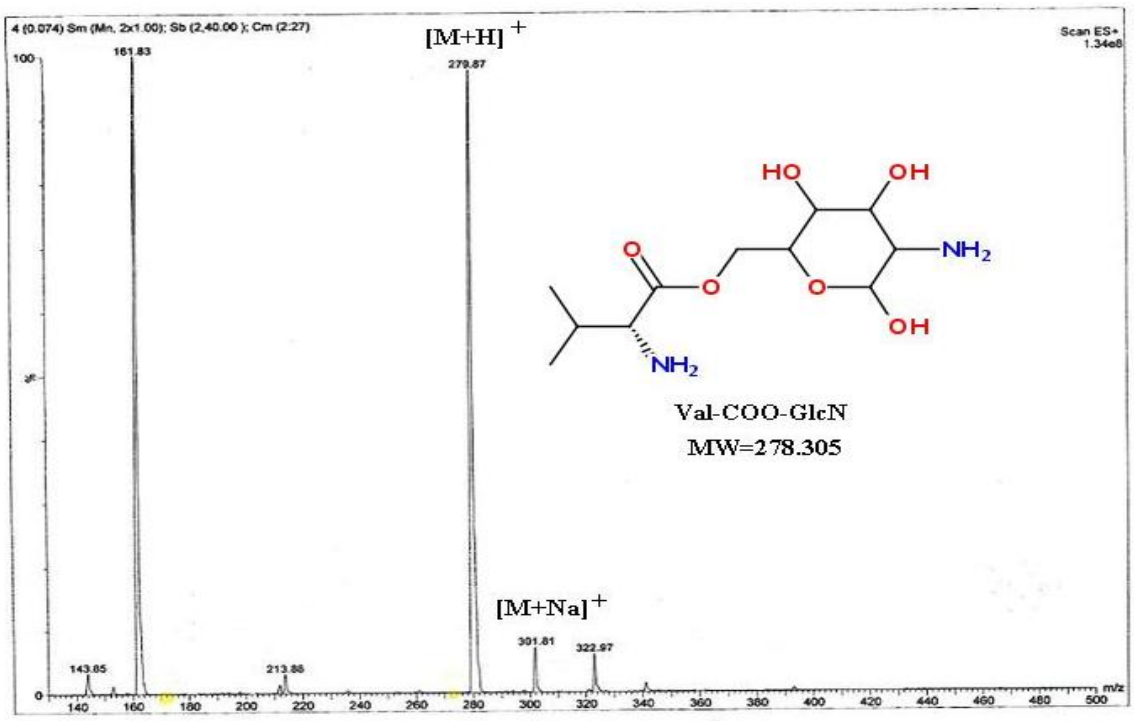


Figure S1. Chemical structure of ester and amide peptide-GlcN derivatives synthesized and investigated in this study.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161.83
279.87
[M+Na]+
143.85
213.88
301.81
322.97
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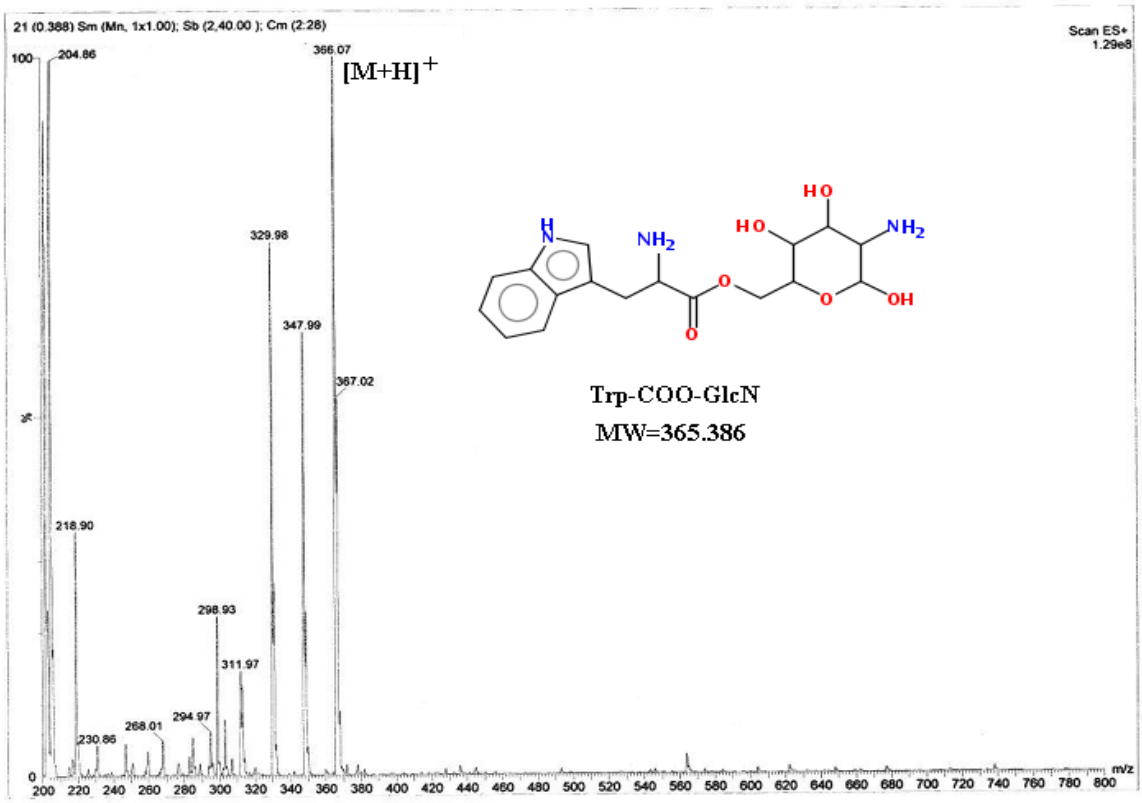
NH2
O
O
O
OH
NH2
Val-COO-GlcN
MW=278.305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366.07
329.98
347.99
367.02
218.90
298.93
311.97
294.97
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OH
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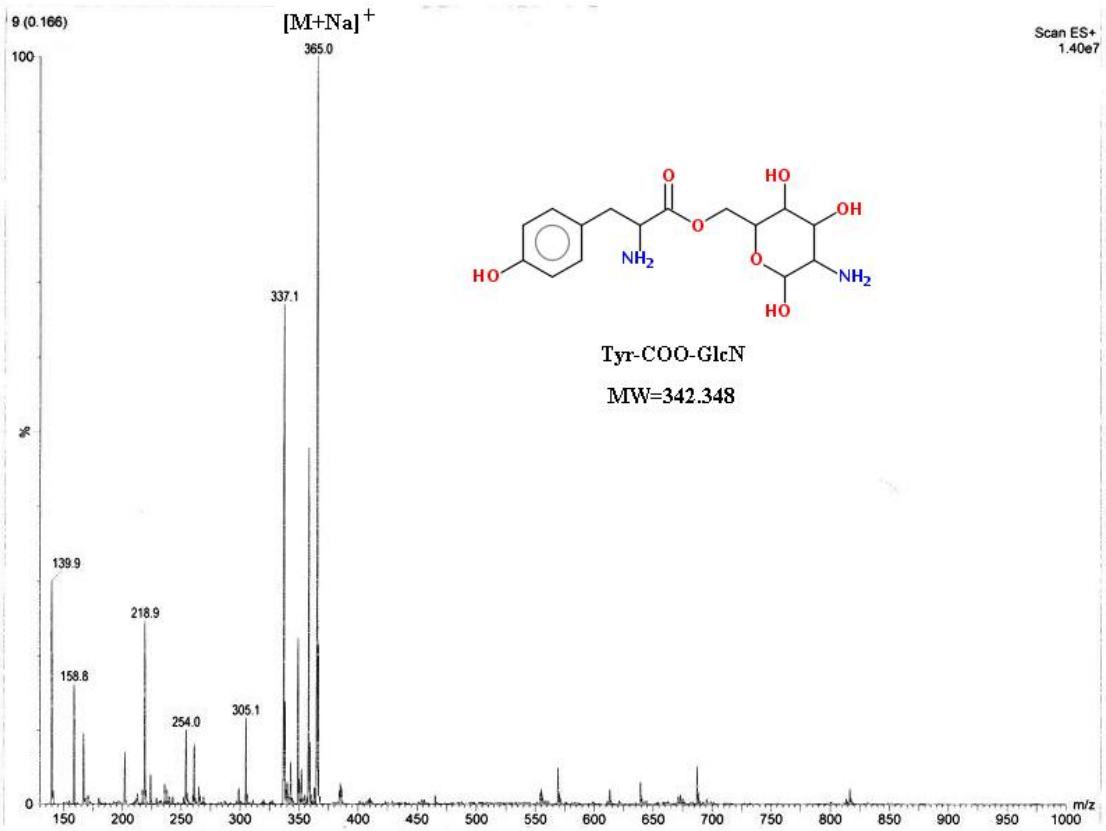

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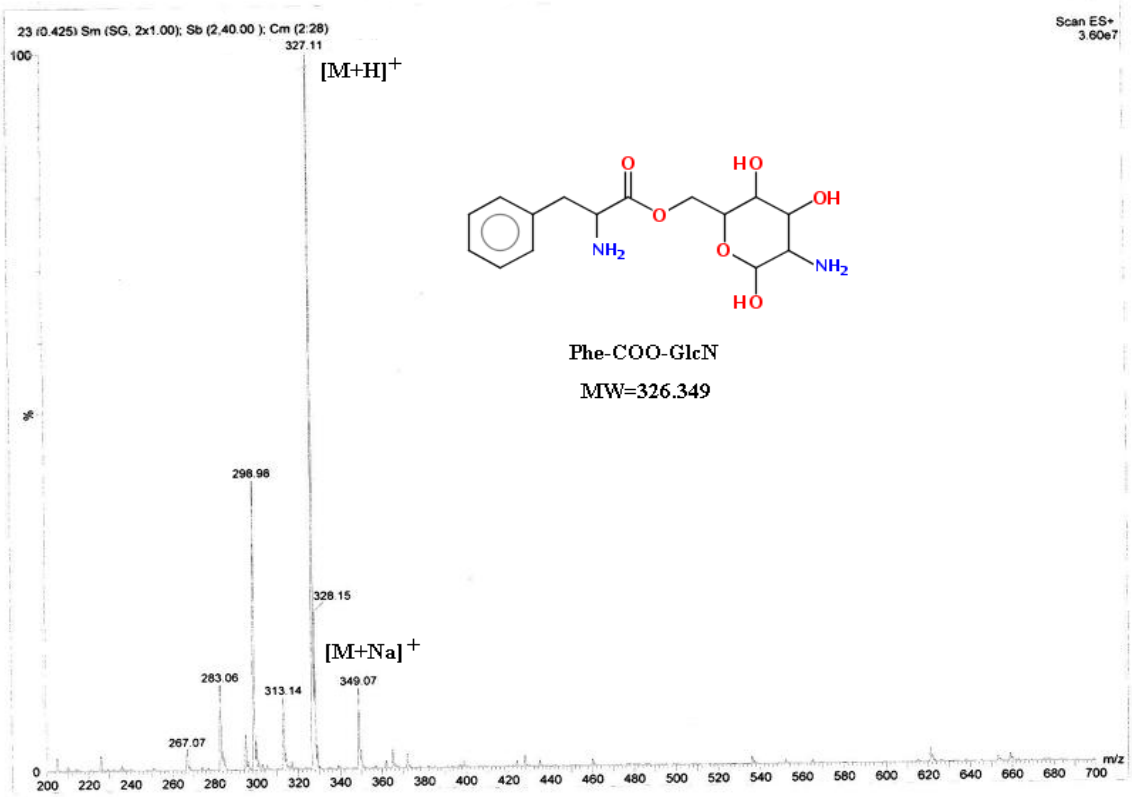

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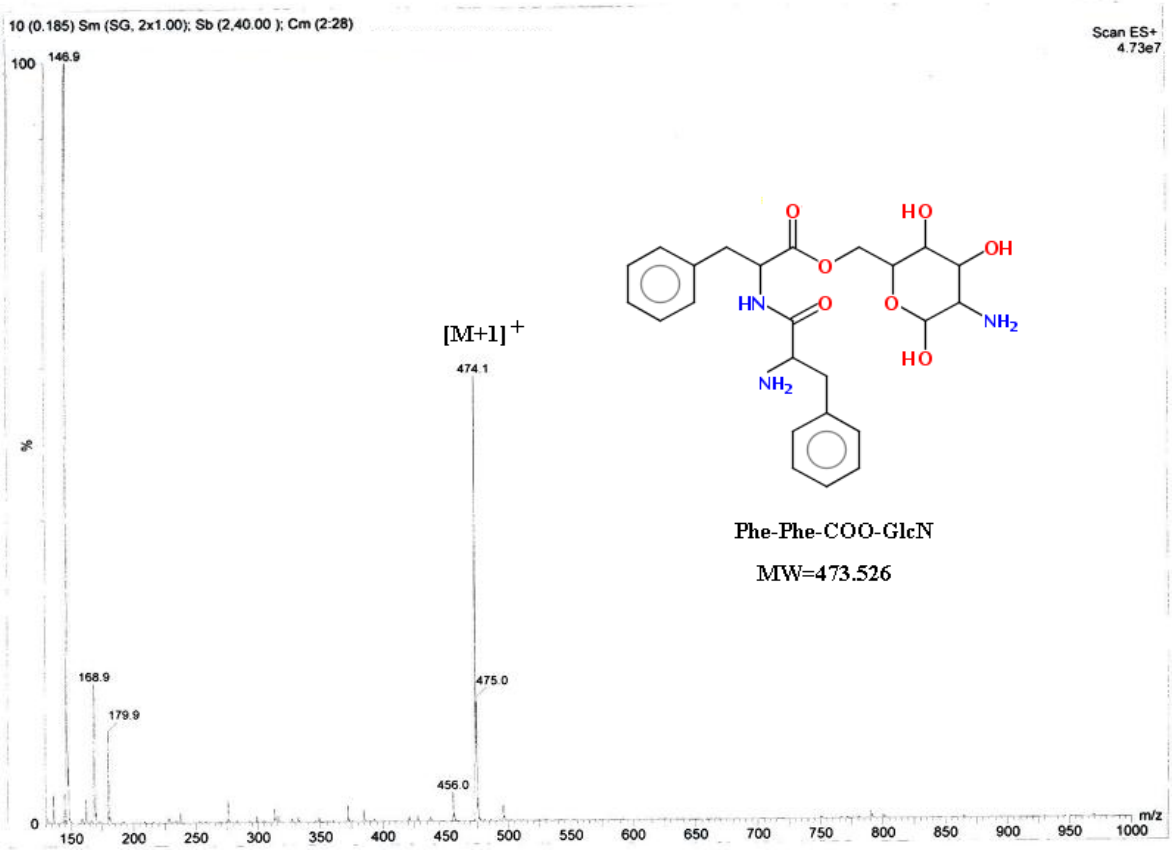
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100
146.9
%
[M+1]+
474.1
168.9
179.9
475.0
456.0
0
150 200 250 300 350 400 450 500 550 600 650 700 750 800 850 900 950 1000
m/z
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O
HN
O
O
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HO
NH2
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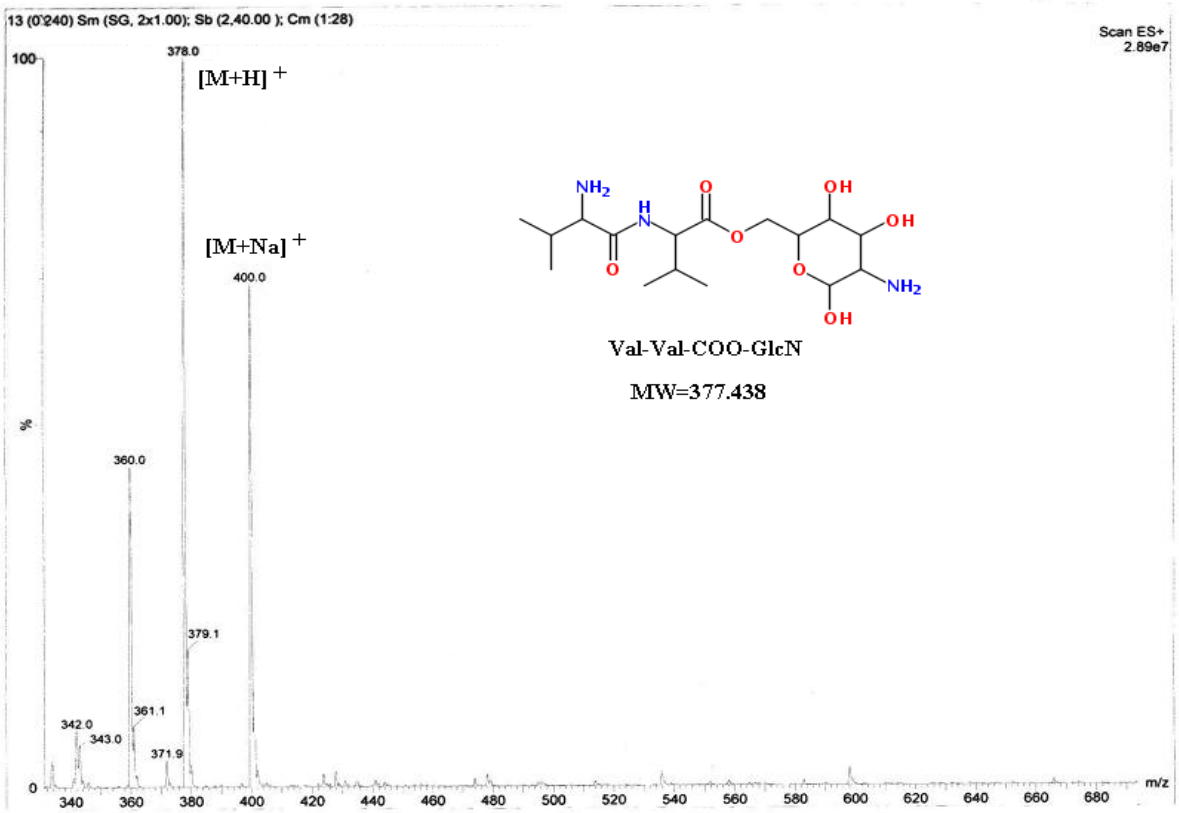
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100
378.0
[M+H]+
[M+Na]+
400.0
%
360.0
379.1
342.0
343.0
361.1
371.9
0
340 360 380 400 420 440 460 480 500 520 540 560 580 600 620 640 660 680
m/z
NH2
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NH2
OH
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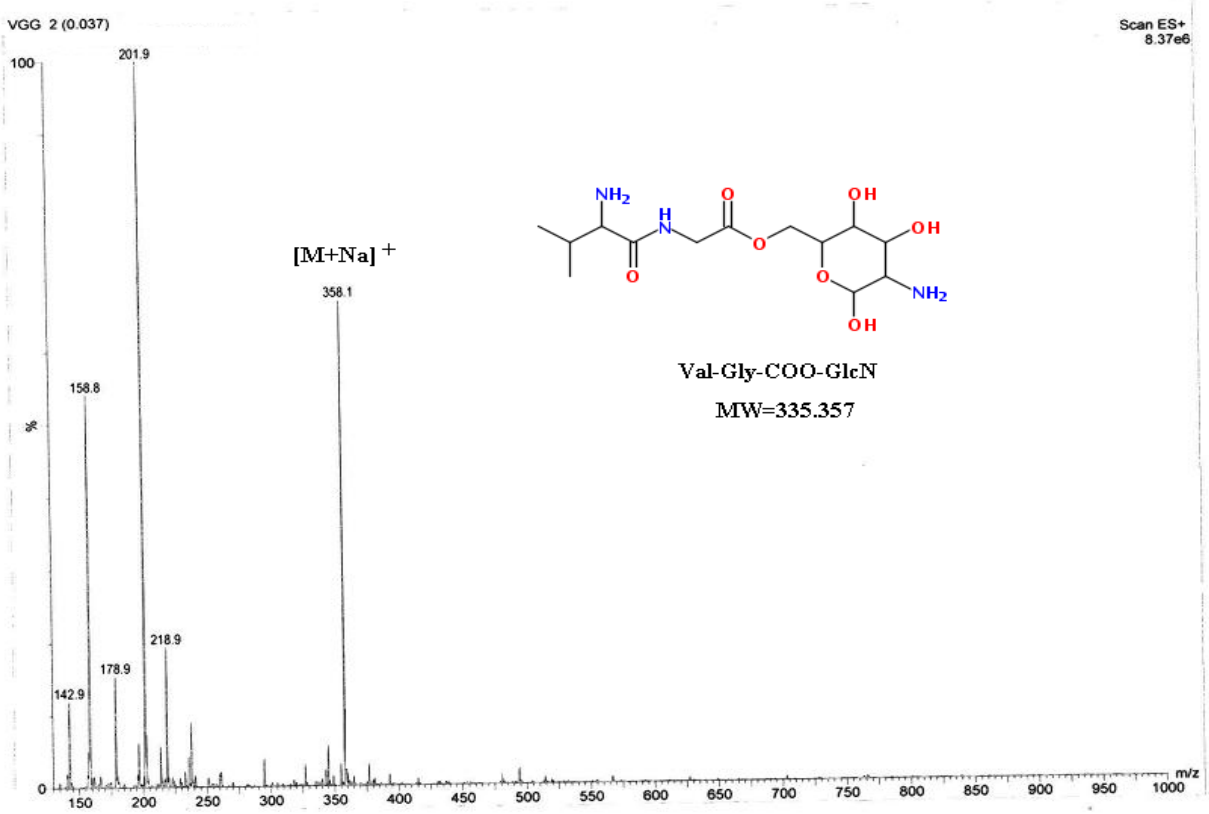
MW=377.438



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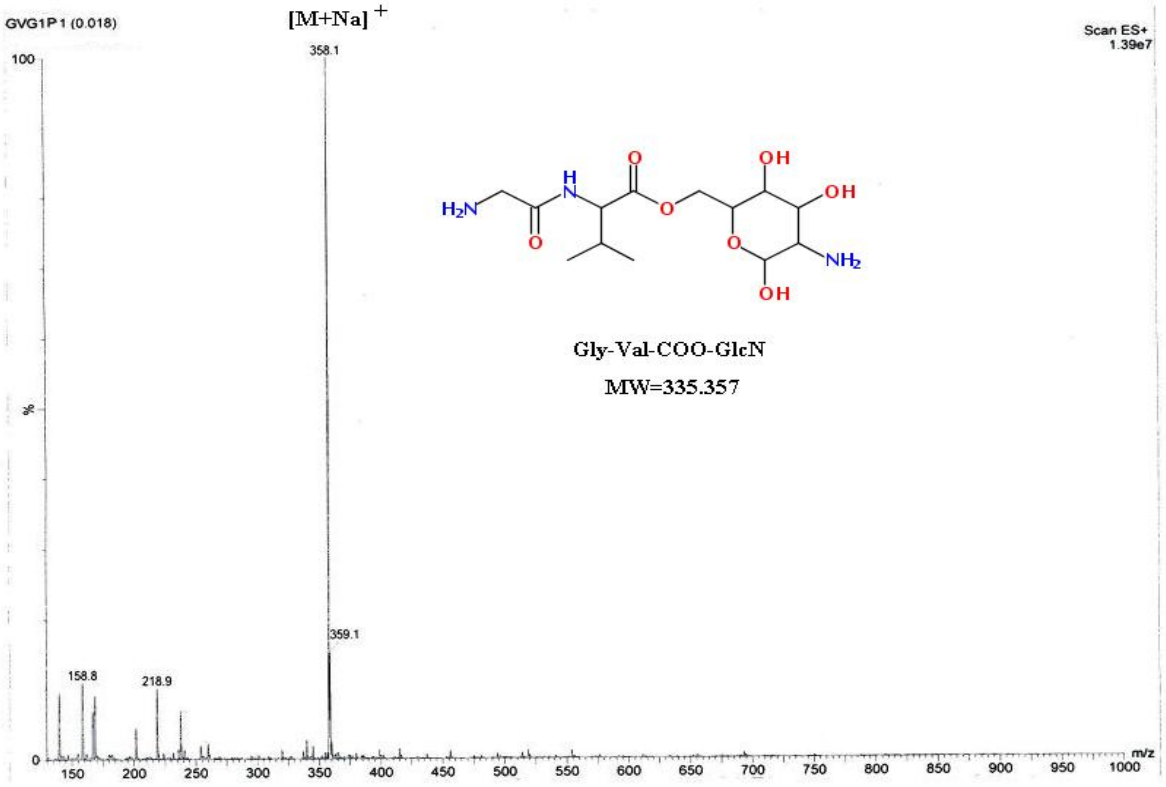

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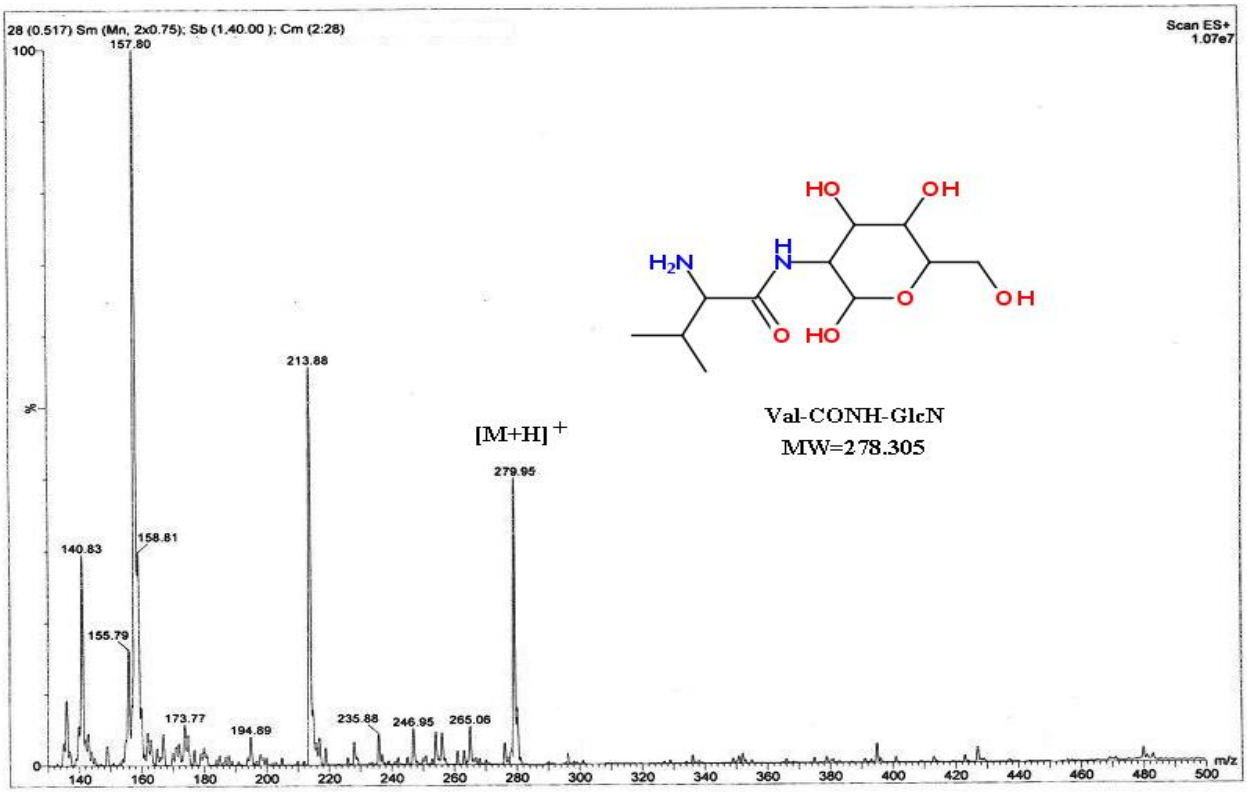
MW=335.357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100
%
0
157.80
213.88
279.95
140.83
158.81
155.79
173.77
194.89
235.88
246.95
265.06
[M+H]+
m/z
140 160 180 200 220 240 260 280 300 320 340 360 380 400 420 440 460 480 500
HO
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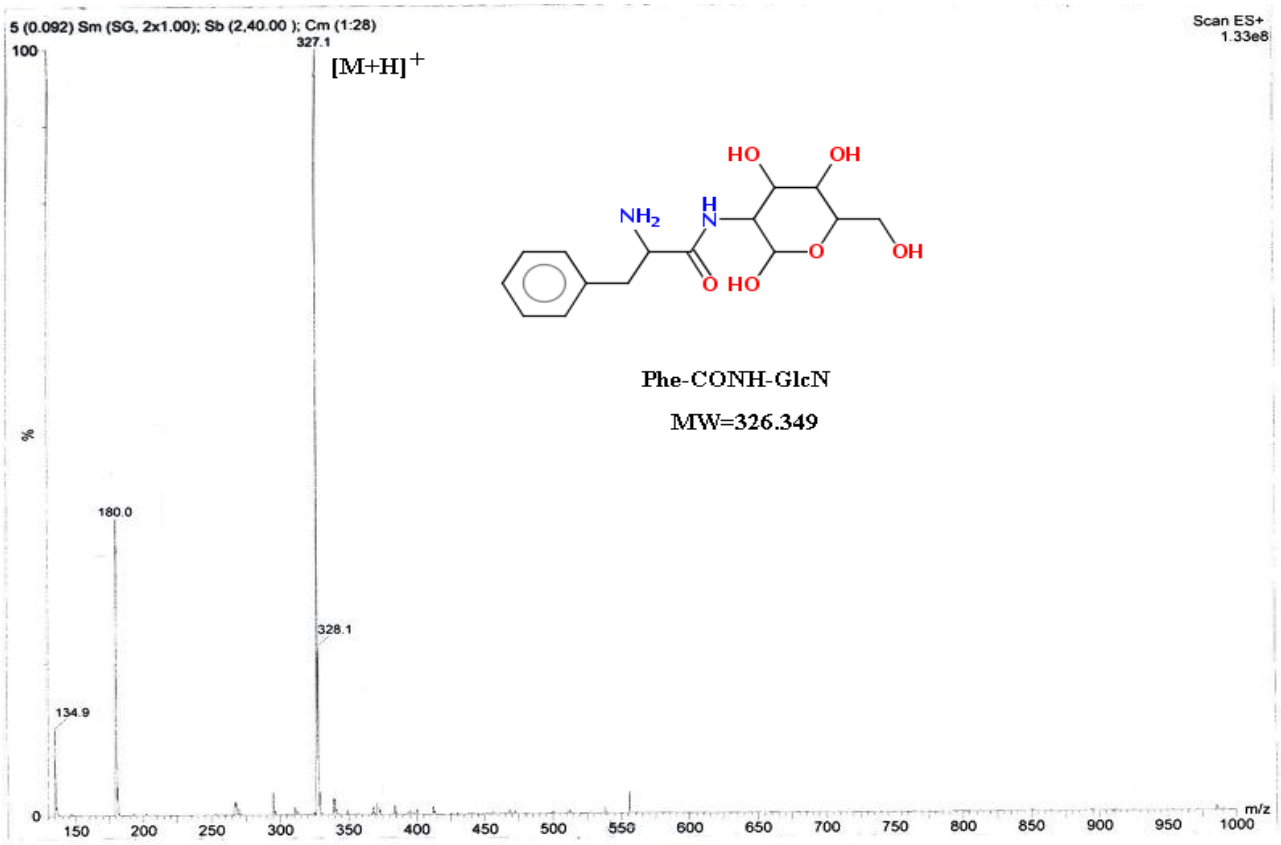
N
H
O
O
HO
OH
Val-CONH-GlcN
MW=278.305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100
%
0
327.1
[M+H]+
180.0
328.1
134.9
m/z
150 200 250 300 350 400 450 500 550 600 650 700 750 800 850 900 950 1000
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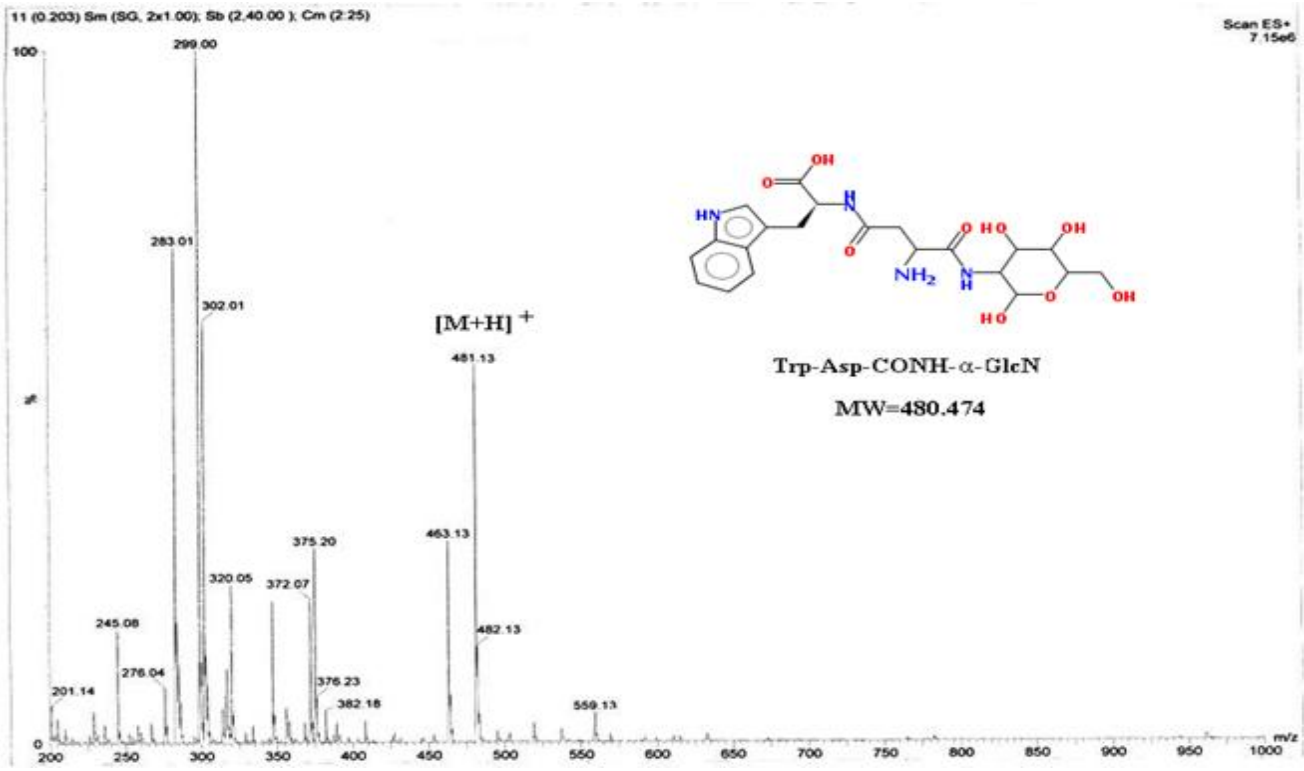
O
O
HO
OH
Phe-CONH-GlcN
MW=326.349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100
%
0
299.00
283.01
302.01
[M+H] +
481.13
463.13
375.20
320.05
372.07
245.08
482.13
276.04
376.23
201.14
382.18
559.13
200
250
300
350
400
450
500
550
600
650
700
750
800
850
900
950
1000
m/z
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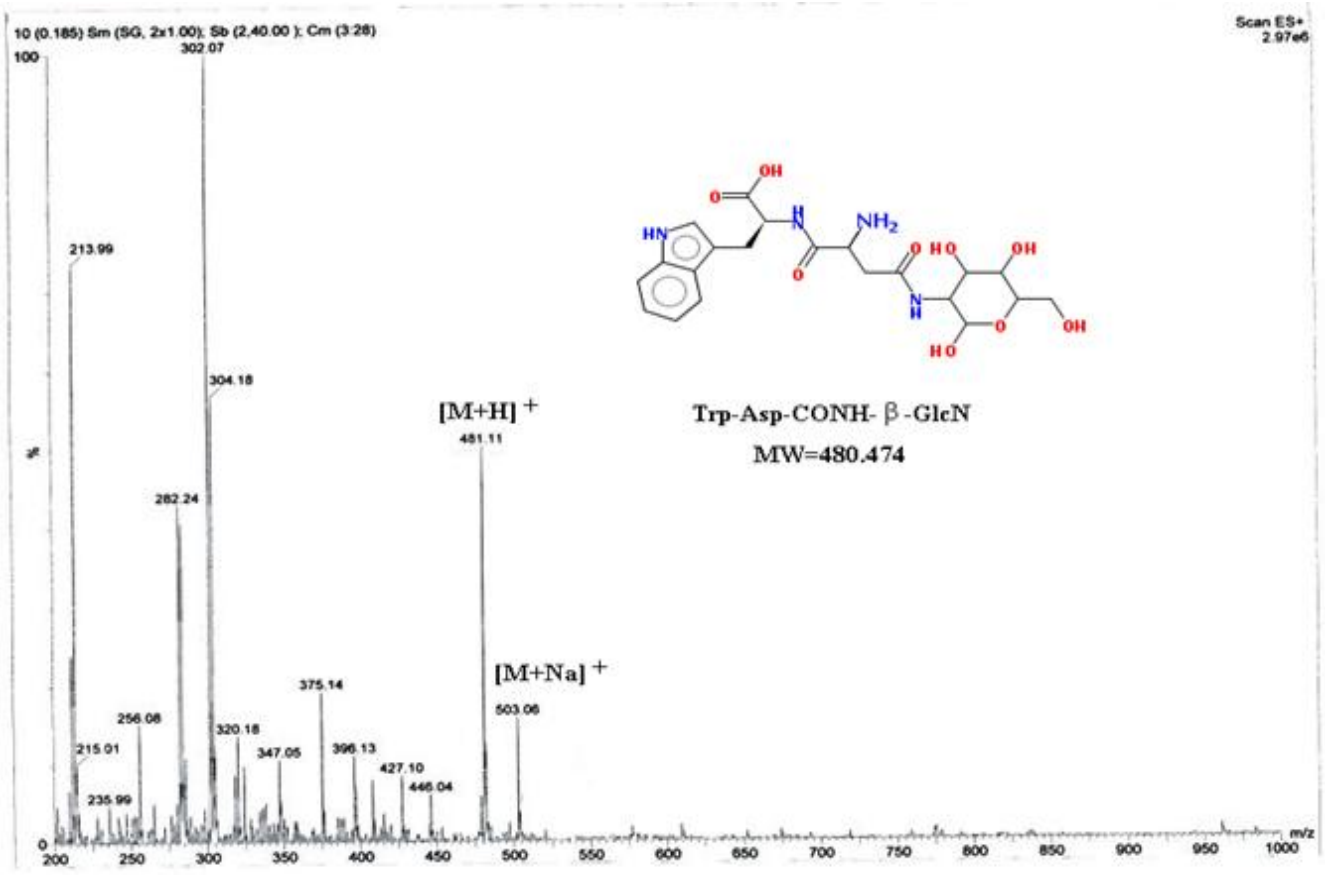
MW=480.474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2.97e6
100
%
0
302.07
213.99
304.18
[M+H] +
481.11
282.24
[M+Na] +
503.06
375.14
256.08
320.18
215.01
347.05
396.13
427.10
446.04
235.99
200
250
300
350
400
450
500
550
600
650
700
750
800
850
900
950
1000
m/z
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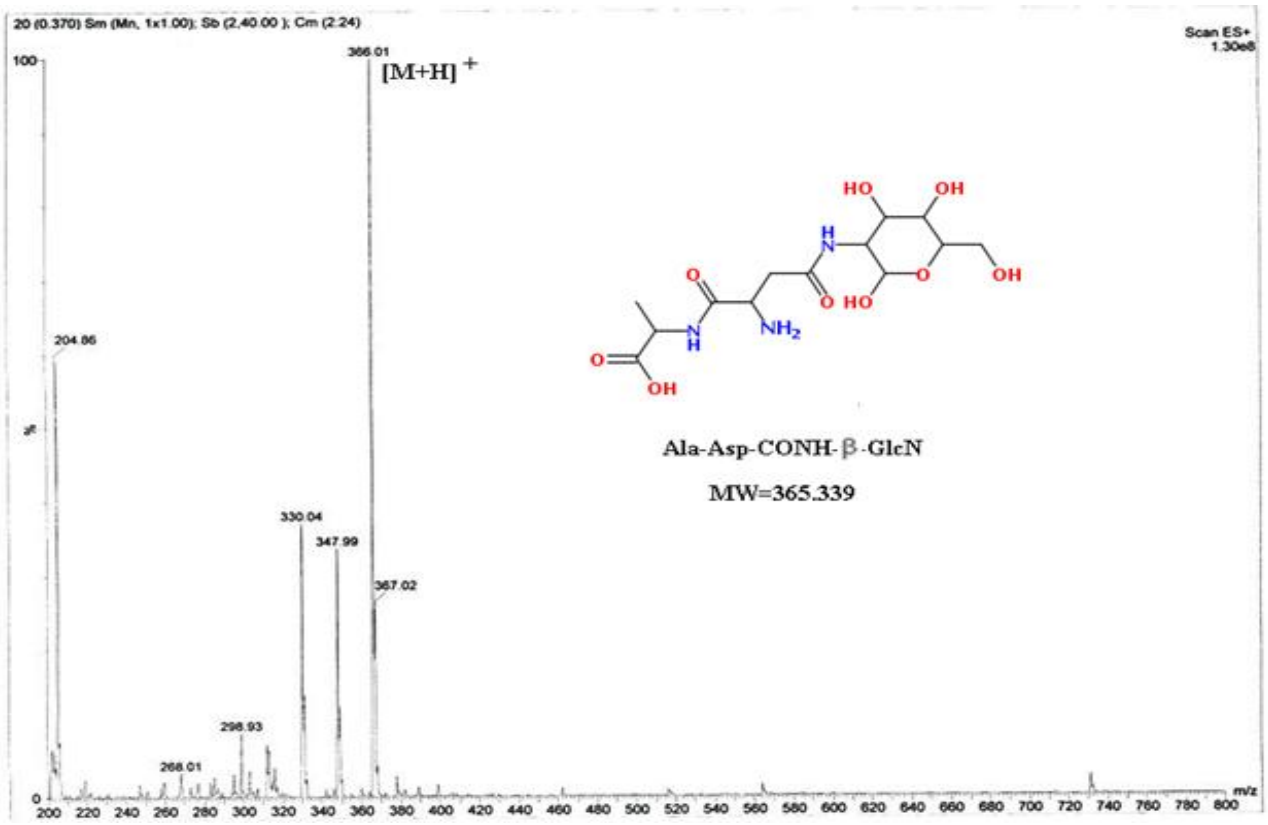
MW=480.474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[M+H] +
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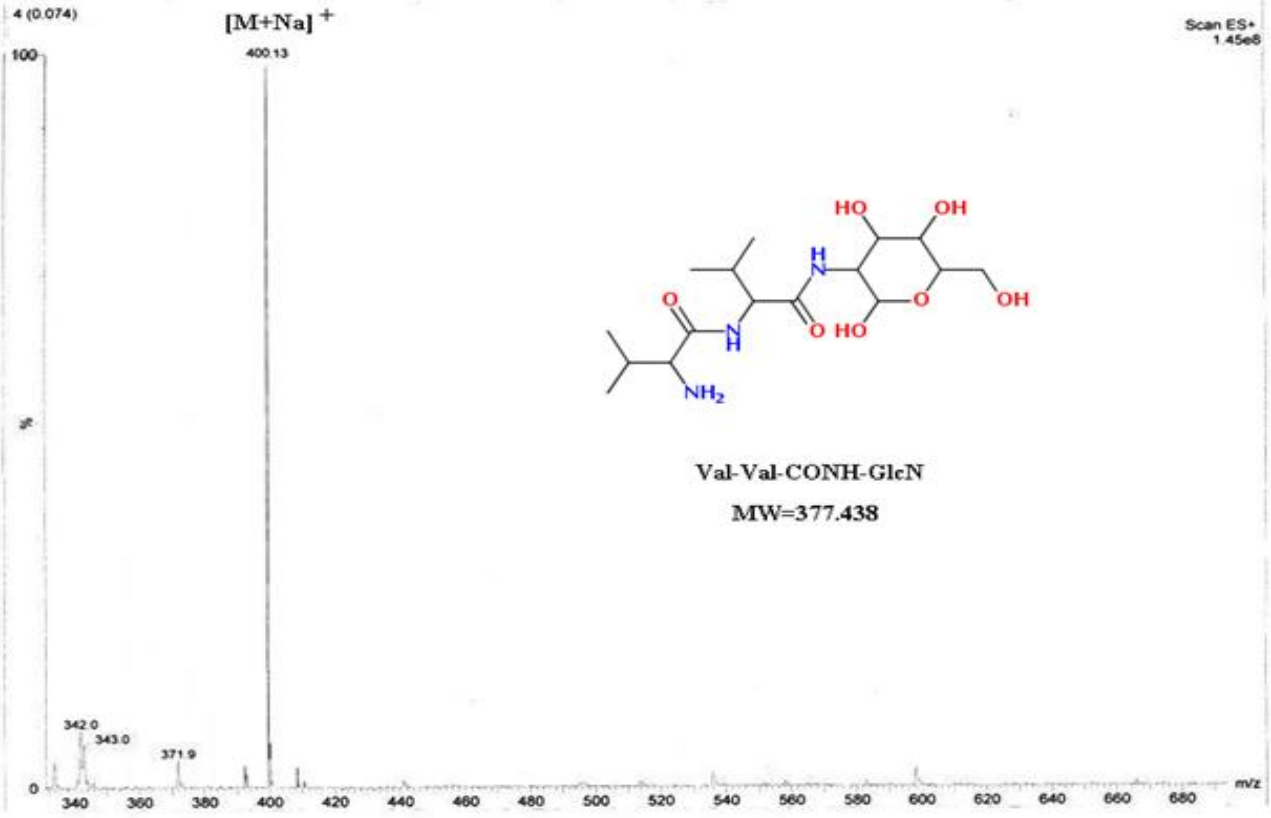
268.01
Ala-Asp-CONH-β-GlcN
MW=365.339


4 (0.074)
[M+Na] +
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1.45e8
400.13
342.0
343.0
371.9
Val-Val-CONH-GlcN
MW=377.438

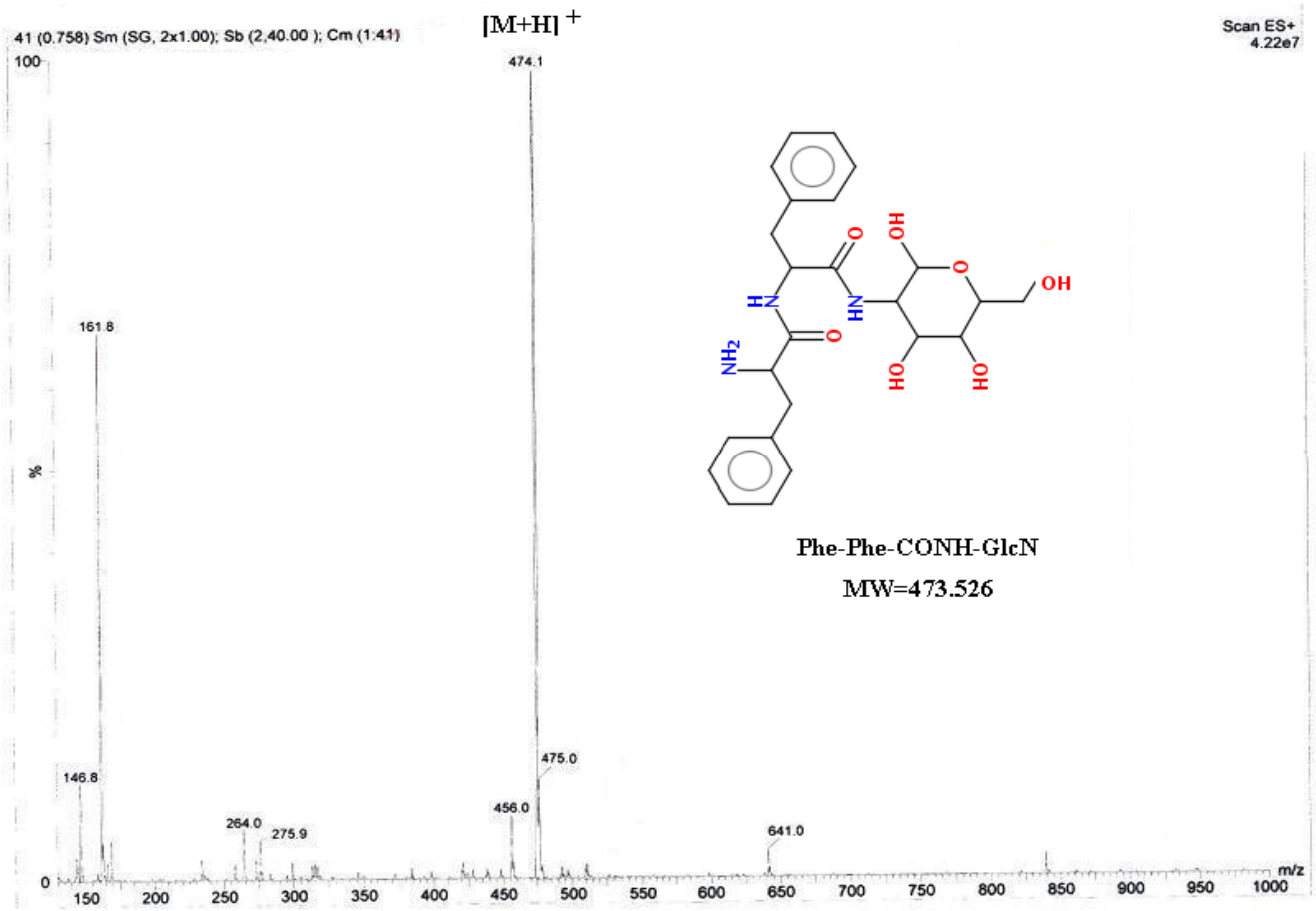


Figure S2. Mass spectra of the synthesized peptide-GlcN derivatives.

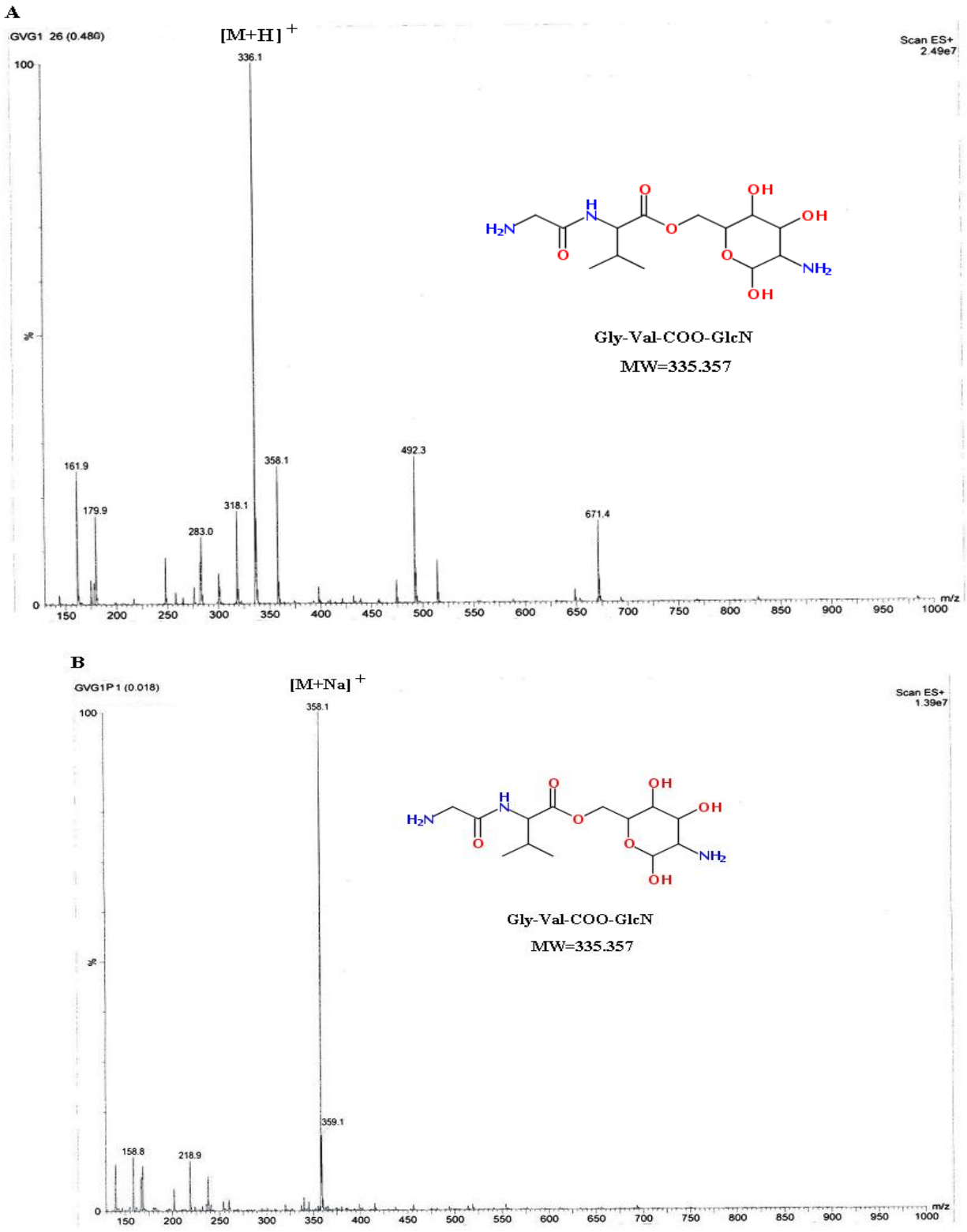


Figure S3. Mass spectra of the Gly-Val-COO-GlcN (GVG) conjugate before (A) and after (B) purification using *Diaion HP-20* resin.

^{1}H NMR [CD_3OD, 400 MHz]: [D_2O, 400 MHz]: δ 5.40 (d, 1H, J = 2.9 Hz, H1α), 4.95 (d, 1H, J = 7.5 Hz, H1β), 4.30-4.65 (m, 3H, H5, H6a and H7), 4.06-4.14 (m, 1H, H6b), 3.90 (s, 2H, H10), 3.66-3.85 (m, 1H, H4), 3.45-3.65(m, 1H, H3). 3.00 and 3.30 (m, 1H, H2), 2.20-2.40 (m, 1H, H8), 0.99 (d, 6H, J = 8.6 Hz, H9).

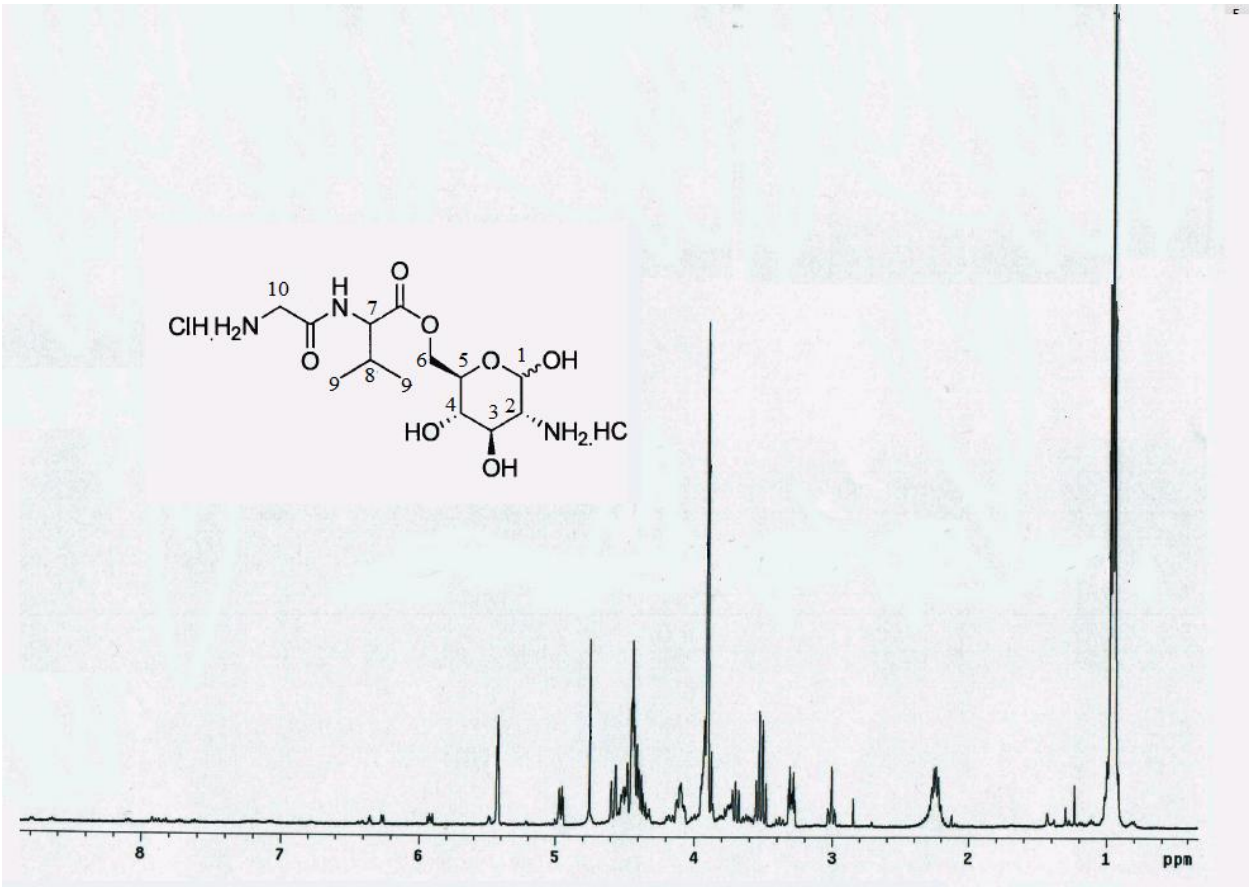


Figure S4. ^{1}H NMR spectra result for the purified Gly-Val-COO-GlcN**.**

^{13}C NMR [D_2O, 150 MHz]:δ 172.8 (C12), 167.2 (C7), 92.7 and 89.1 (C1α and C1β), 73.5, 71.7 and 69.5 (C3, C4 and C5), 63.8 (C6), 58.5 (C8), 56.5 (C2), 40.2 (C13), 30.0 (C9), 18.1 and 17.1 (C10 and C11).

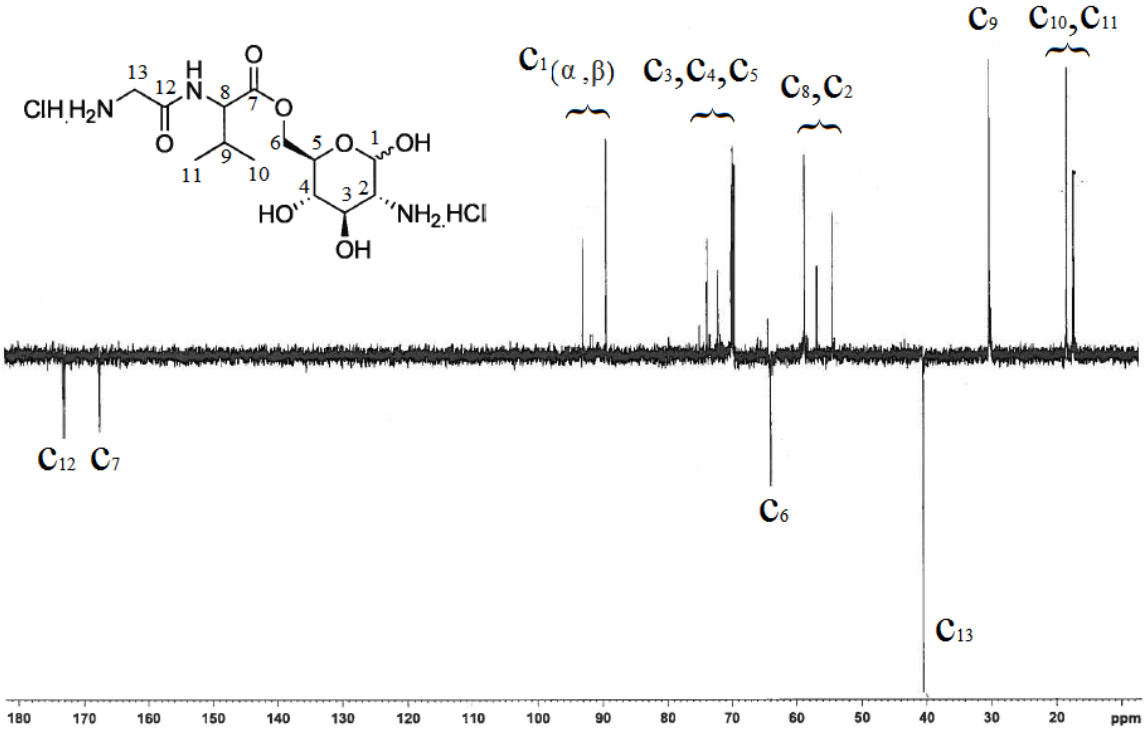
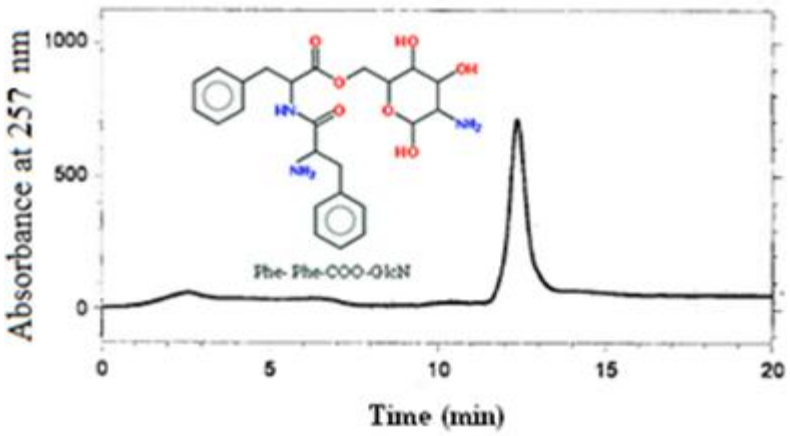
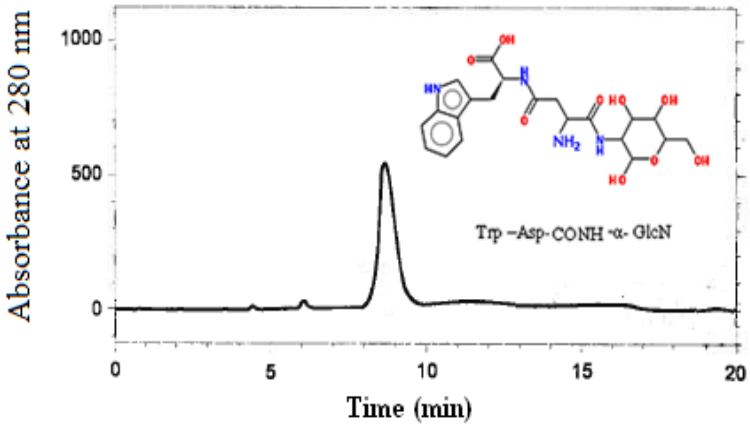
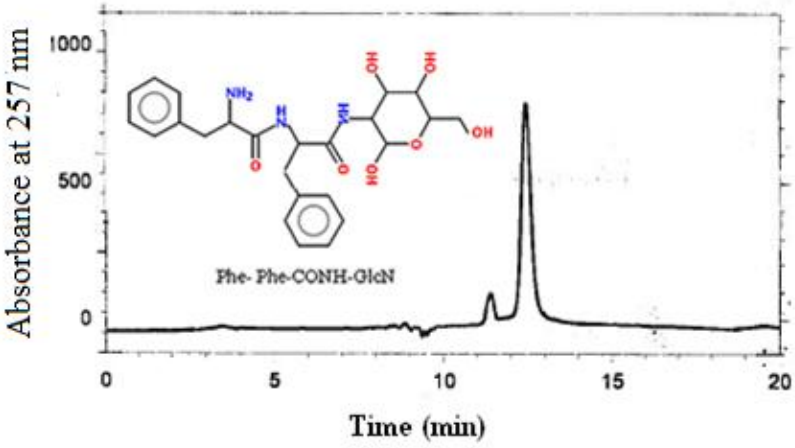
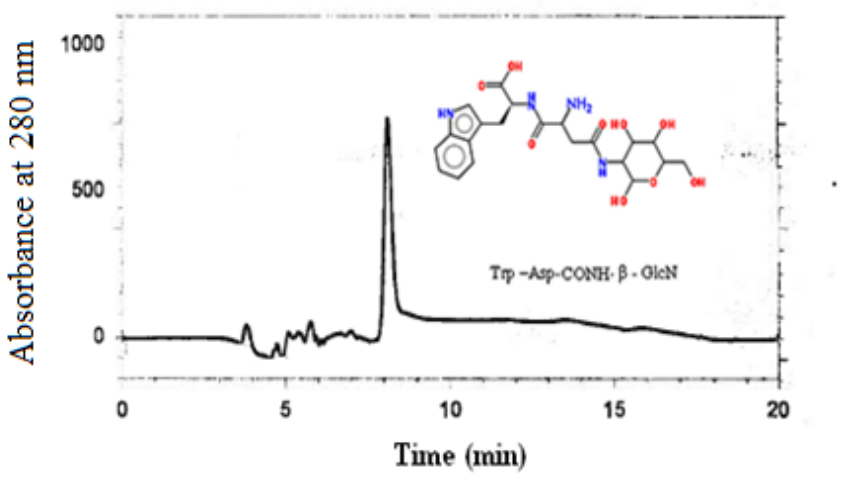


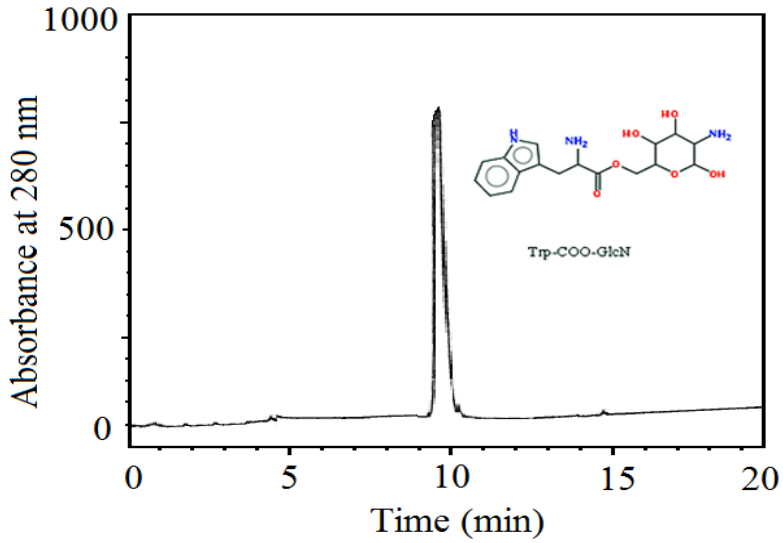
Figure S5. ^{13}C NMR spectra result for the purified Gly-Val-COO-GlcN**.**

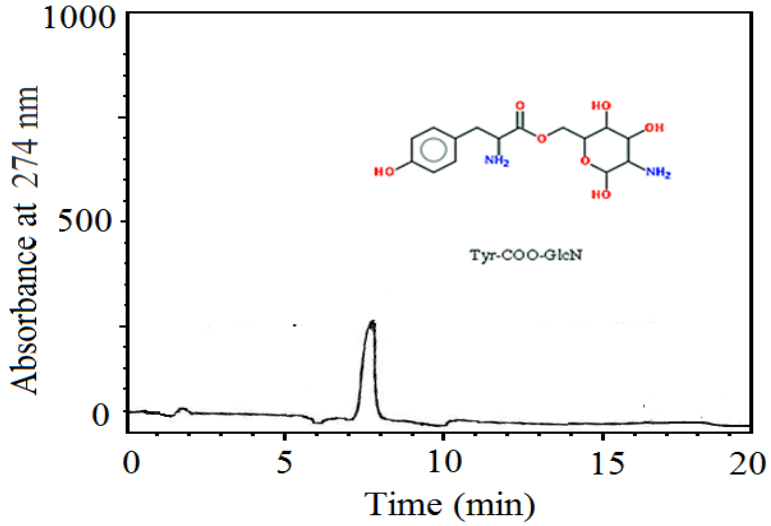

Absorbance at 257 nm
1000
500
0
0
5
10
15
20
Time (min)
Phe- Phe-COO-GlcN


Absorbance at 280 nm
1000
500
0
0
5
10
15
20
Time (min)
Trp –Asp-CONH -α- GlcN


Absorbance at 257 nm
1000
500
0
0
5
10
15
20
Time (min)
Phe- Phe-CONH-GlcN


Absorbance at 280 nm
1000
500
0
0
5
10
15
20
Time (min)
Trp –Asp-CONH- β - GlcN


Absorbance at 280 nm
1000
500
0
0
5
10
15
20
Time (min)
Trp-COO-GlcN


Absorbance at 274 nm
1000
500
0
0
5
10
15
20
Time (min)
Tyr-COO-GlcN

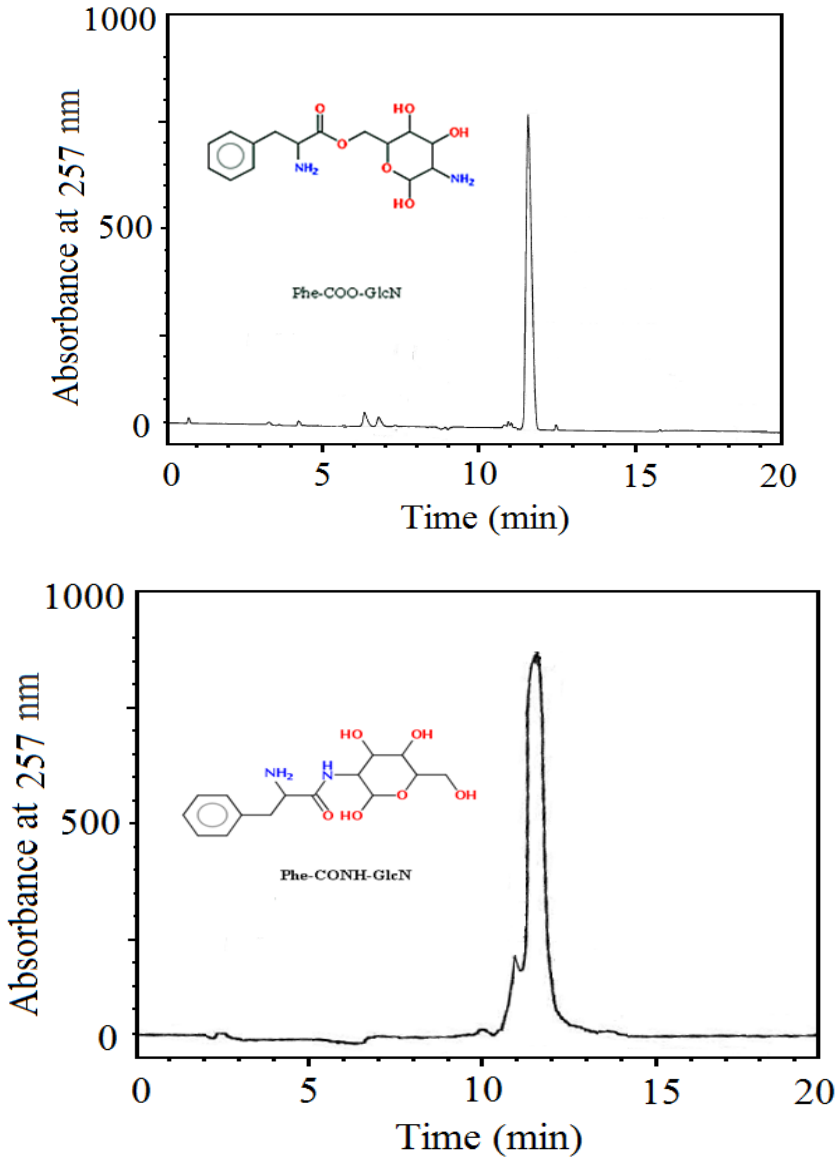
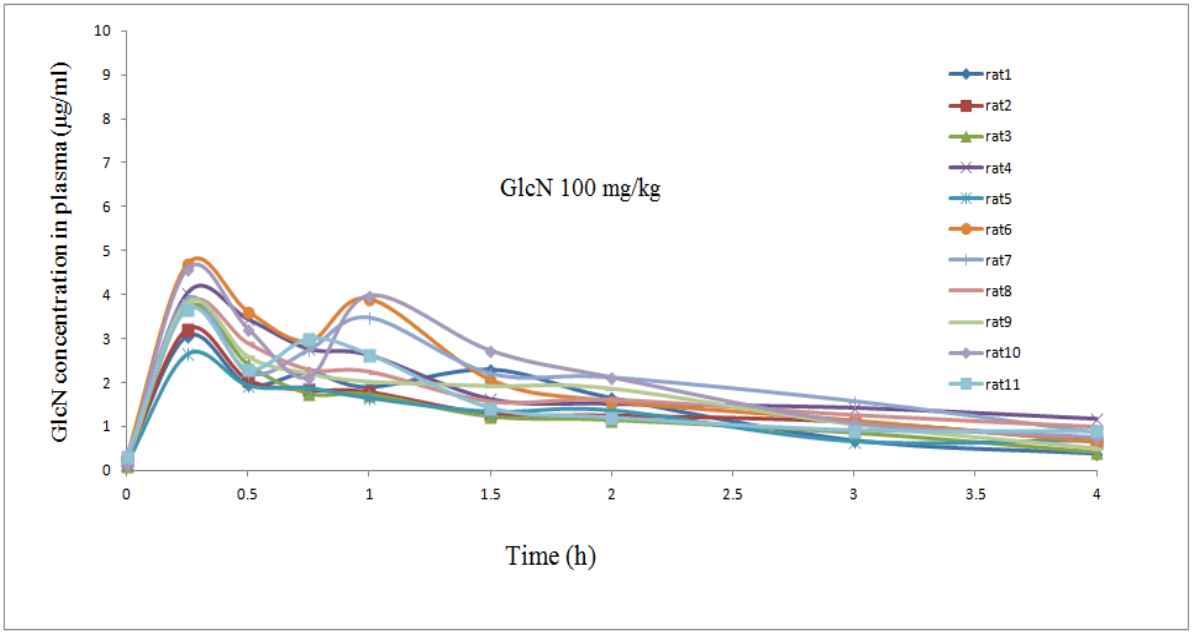
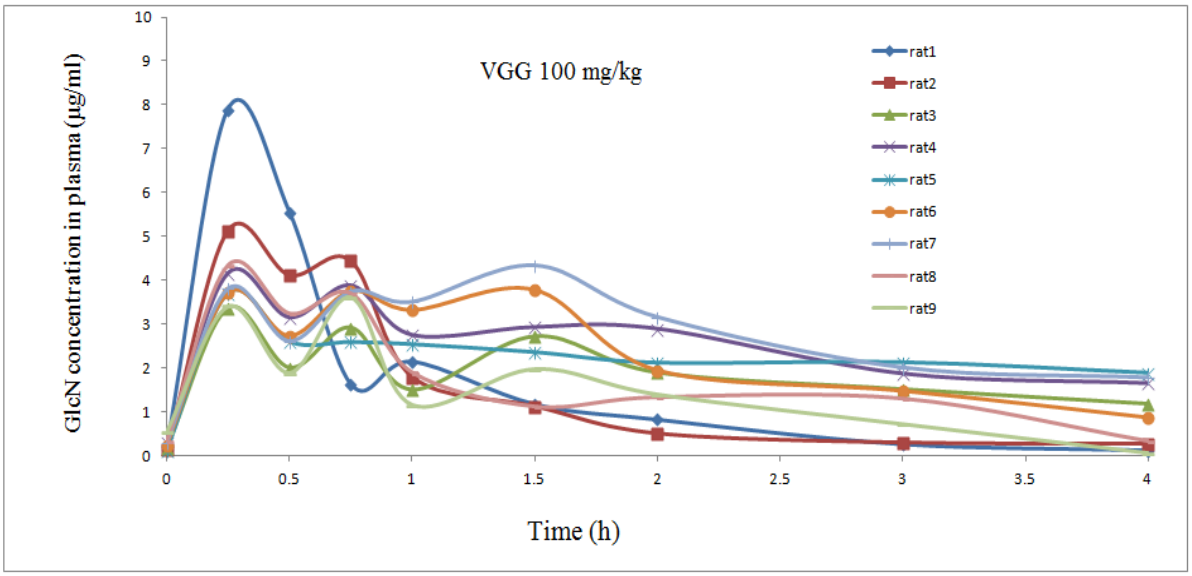
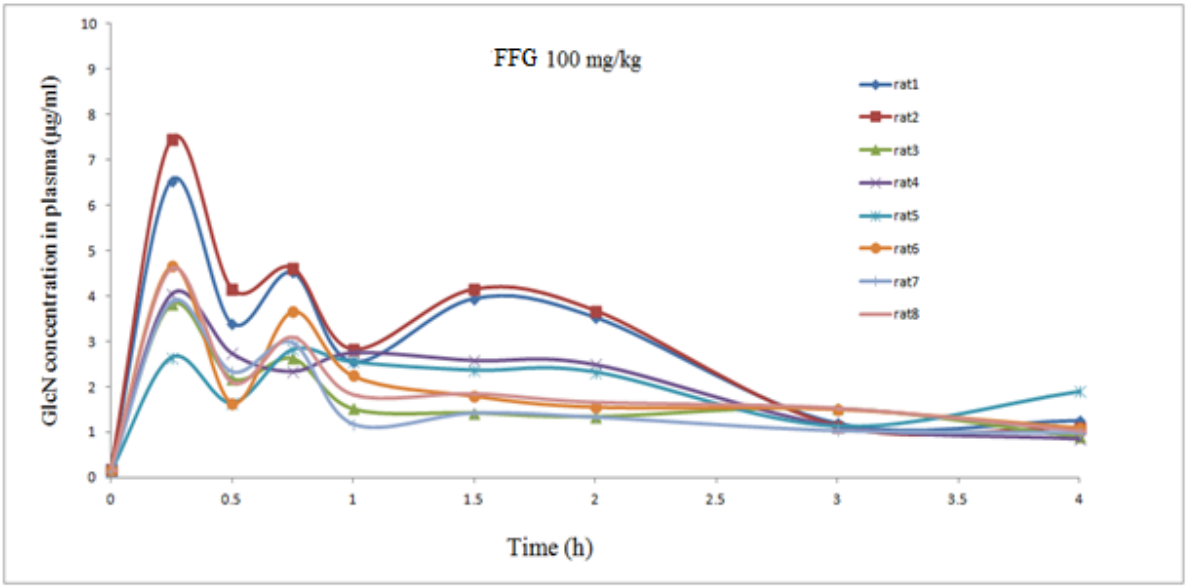


Figure S6. HPLC chromatogram of the purified peptide-GlcN derivatives, which were identified by the direct HPLC methods; these compounds possess a fluorophore or chromophore group in their structure. . The Mobile phase compositions and assay conditions for the direct HPLC methods have been detailed in Table 3.1.


GlcN 100 mg/kg
GlcN concentration in plasma (µg/ml)
Time (h)
rat1
rat2
rat3
rat4
rat5
rat6
rat7
rat8
rat9
rat10
rat11


VGG 100 mg/kg
GlcN concentration in plasma (µg/ml)
Time (h)
rat1
rat2
rat3
rat4
rat5
rat6
rat7
rat8
rat9



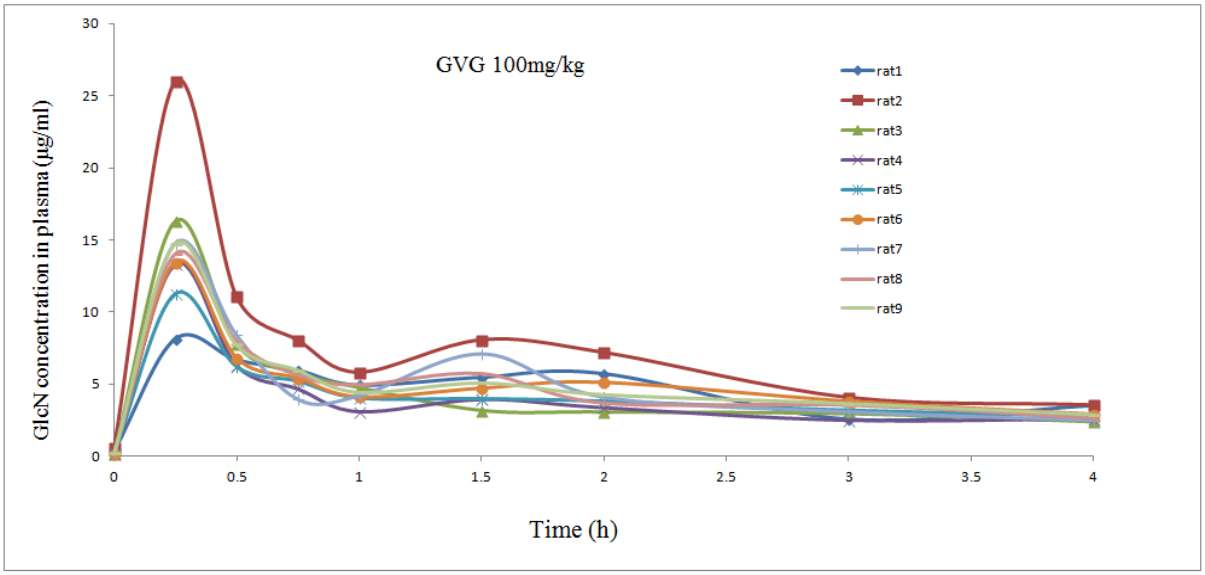


Figure S7. The spaghetti graph of plasma concentration-time curve of GlcN in individual Sprague Dawley rats after administration of equivalent doses (100 mg/kg of GlcN base) of GlcN or its ester derivatives. Notice that Y axis scale for GVG is different from other derivatives.

Supporting tables

Table S1. Mobile phase compositions and assay conditions for the direct HPLC methods. These methods were developed for the GlcN derivatives with a chromophore or fluorophore present in their structures.

Peptide-GlcN derivatives	Composition of the mobile phase in RP-HPLC (v/v)	Composition of the mobile phase in NP-HPLC (v/v)	Retention time (min)	λ_{max}	Fluorescence		Purity[1]
					EX	EM	
Trp-COO-GlcN	N/A	hexane/ethanol/IPA/TEA (92:4:4:0.1)	9.8	280	284	348	99.1
Tyr-COO-GlcN	N/A	hexane/ethanol/IPA/TEA (90:5:5:0.1)	7.8	274	274	303	95.3
Phe-COO-GlcN	N/A	hexane/ethanol/IPA/TEA (93:3:4:0.1)	11.7	257	257	282	98.3
Phe-Phe-COO-GlcN	ACN/water/AA (50/50/0.00)	N/A	12.3	257	263	285	97.2
Phe-CONH-GlcN	N/A	hexane/ethanol/IPA/TEA (93:3:4:0.1)	11.5	257	257	282	91.1
Trp -Asp-CONH-α-GlcN	ACN/water/AA (60/40/0.001)	N/A	8.8	280	278	340	96.3
Trp -Asp-CONH-β-GlcN	ACN/water/AA (60/40/0.001)	N/A	8.1	280	278	340	92.4
Phe-Phe-CONH-GlcN	ACN/water/AA (50/50/0.001)	N/A	12.5	257	263	285	90.1

RP= Reversed phase; NP= Normal phase; ACN= acetonitrile; AA= acetic acid; IPA= Isopropanol; TEA= Tri-ethylamine

[1] The purity of the peptides was determined by the direct HPLC methods.

Table S2. Thermal and pH stability of the peptide-GlcN derivatives based on the remaining amounts of the pro-drugs at the end of the experiments

Peptide-GlcN derivatives	**% remaining**		
	60 ºC	**pH=2**	**pH=7.4**
Val-COO-GlcN	96.6	96.9	97.3
Trp-COO-GlcN	97.3	97.8	98.9
Tyr-COO-GlcN	97.2	97.5	98.7
Phe-COO-GlcN	96.9	96.3	97.9
Phe-Phe-COO-GlcN	97.1	97.2	98.1
Val-Val-COO-GlcN	96.7	98.3	98.6
Val -Gly-COO-GlcN	96.6	97.1	97.8
Gly-Val-COO-GlcN	97.2	97.3	98.3
Val-CONH-GlcN	>98	>98	>98
Phe-CONH-GlcN	>98	>98	>98
Trp -Asp-CONH-α-GlcN	>98	>98	>98
Trp -Asp-CONH-β-GlcN	>98	>98	>98
Ala-Asp-CONH-β-GlcN	>98	>98	>98
Val-Val-CONH-GlcN	>98	>98	>98
Phe-Phe-CONH-GlcN	>98	>98	>98

Table S3. CV and Accuracy of simultaneous HPLC assay for GlcN and GVG

Concentration, µg/mL			CV(GVG)	CV(GlcN)	Accuracy(GVG)	Accuracy(GlcN)
Added	Observed (GVG)	Observed (GlcN)	Percent			
Intra-day						
0.05	0.054±0.003	0.053±0.001	5.56	1.88	8	6
0.5	0.51±0.04	0.48±0.03	7.84	6.25	2	-4
5	4.95±0.3	5.05±0.4	6.06	7.92	-1	1
10	9.63±0.35	9.93±0.19	3.63	1.91	-3.7	-0.7
20	20.4±0.53	19.74±0.32	2.6	1.62	2	-1.3
Inter-day						
0.05	0.053±0.004	0.051±0.002	7.55	3.92	6	2
0.5	0.47±0.04	0.49±0.03	8.51	6.1	-6	-2
5	4.93±0.27	5.12±0.25	5.48	4.88	-1.4	2.4
10	10.12±0.15	9.86±0.25	1.48	2.53	1.2	-1.4
20	20.2±0.36	20.39±0.46	1.78	2.26	1	1.95

Table S4. Short term and freeze and thaw stability of GVG and GlcN in rat plasma samples

Concentration, µg/mL			CV(GVG)	CV(GlcN)	Accuracy(GVG)	Accuracy(GlcN)
Added	Observed (GVG)	Observed (GlcN)	Percent			
Short term stability (24h)						
0.05	0.049±0.004	0.054±0.003	8.16	5.60	-2	8
0.5	0.54±0.05	0.51±0.02	9.30	3.92	8	2
5	5.03±0.4	5.1±0.3	7.95	5.88	0.6	2
10	9.79±0.25	10.2±0.28	2.55	2.75	-2.1	2
20	19.73±0.73	19.97±0.19	3.70	0.95	-1.35	-0.15
Freeze/thaw stability						
0.05	0.055±0.003	0.051±0.004	5.45	7.84	10	2
0.5	0.53±0.04	0.49±0.01	7.55	2.04	6	-2
5	5.1±0.36	5.18±0.38	7.06	7.34	2	3.6
10	10.21±0.21	10.8±0.85	2.06	7.87	2.1	8
20	19.87±0.35	19.59±0.96	1.76	4.90	-0.65	-2.05

Table S5. Arthritic index (AI) on day18 post-adjuvant injection (n=6/group). GVG or GlcN administration was commenced at day 1.

	Arthritic index (AI) ± SD
Control-Inflamed	9.1 ± 0.8
Control-Healthy	0
GVG-30	0
GlcN -90	0
GVG-20	0
GlcN-20	4.1 ± 1.1