

Chapter 2

Application of Prodrug Chemistry to GLP-1

Abstract It is impressed upon the reader how a prodrug at the biologically active N-terminal end of GLP could be utilized to extend and improve the pharmacodynamics of this peptide hormone. The quality of an ideal prodrug and the different strategies that could be potentially considered for constructing peptide-based prodrugs are described. One could use enzymes for the conversion of a prodrug to a drug but here we relied on pH and temperature for generating the drug from the prodrug. This is because these two attributes are virtually invariant physiologically and thus the strategy could be widely used. Thiol prodrugs are described, but they are relatively unstable in physiology and thus would likely cleave too fast, thereby resulting in rapid clearance of the prodrug in its active form. Finally, the intramolecular cyclization reaction of dipeptide esters and amides to form diketopiperazine or diketomorpholine is considered as an example of a chemoreversible prodrug. The chemistry is reasonably straightforward and allows at least four points (stereochemistry of substituent groups, nature of the nucleophile and leaving group as shown) where structure can be stereochemically controlled to refine the rate of formation with release of the active peptide.

Keywords Ideal prodrug • Diketopiperazine • DKP • Diketomorpholine • DMP • Active imidazole • Pharmacodynamics • Therapeutic index

2.1 Introduction

It is hypothesized that a prodrug at the N-terminal end of GLP will extend and improve the pharmacodynamics of this peptide hormone (Fig. 1.5a and b).

The ideal prodrug should be soluble in water at a pH of 7.2 and 37 °C, and it should be stable in the powder form for long-term storage. It should be immunologically

silent and biologically inactive when injected in the body, and be quantitatively converted to the active drug within a defined period of time. Our greatest interest lies in prodrugs with a $t_{1/2}$ of between 10 and 100 h (weekly, or even monthly duration) under physiological conditions (pH of 7.2 and 37 °C).

During the course of this research, we identified four GLP analogs that are physically and chemically stable, and whose conversion from prodrug to the active drug form under normal physiological conditions ranges within the optimal range. One of these peptides converts to GLP with a half-life of 64 h. The in vivo extension in duration of action of this magnitude would constitute the longest acting peptide prodrug ever designed. These analogs possess a minimal alteration to the native amino acid sequence, and this should minimize potential adverse immunogenic affects. The bioactivities of these synthetic peptides have been determined using in vitro cellular assays.

At the beginning of this research, many strategies were considered for constructing our prodrugs. It was contemplated that the protecting groups of the prodrug could be cleaved by enzymes as reported for nucleotide prodrugs [1]. However, we decided to design a prodrug that would slowly convert to the parent drug at physiological conditions of 37 °C and pH 7.2 driven by inherent chemical instability. The pH and temperature were relied upon for this conversion, as they are virtually and physiologically invariant. We are seeking a prodrug that converts quantitatively to the drug under physiological conditions without the aid of any enzyme. The establishment of prodrug chemistry at the N-terminal end of GLP should be translatable for use with other peptides where this specific site is vital to bioactivity.

We decided to synthesize a chemical derivative of GLP that would convert to GLP spontaneously as stated above. A few possible choices were contemplated before deciding that our primary target in prodrug chemistry would be diketopiperazine formation. The N-terminal histidine is important for the potency of our drug, and a reversibly modified histidine side chain was considered, as has been reported for thyrotropin-releasing hormone (TRH) [2]. However, we dispensed with this approach since this prodrug chemistry is specific to the imidazole ring. This means that the prodrug chemistry requires the presence of a histidine, and possibly also at an N-terminal site.

Thiol esters could also be synthesized by thioesterification [3]. However, a thiol ester is relatively unstable in physiology and thus would likely cleave too fast, thereby resulting in rapid clearance of the prodrug in its active form. Additionally, the use of thiol-based chemistry is fraught with other difficulties, such as instability of disulfide bonds. We decided to forgo this course of action.

Our work therefore focused on making amide and ester prodrugs at the N-terminus that upon cleavage of the suitable amide or ester bond generates the desired drug. Esters are normally more labile than amides; however they are easily hydrolyzed by the ubiquitous serum esterases [4]. Hence, amide bond based prodrugs were a much more attractive design, although the risk of peptidase degradation may potentially complicate in vivo application.

In considering amide prodrugs, it is reported that histidyl-proline amide cyclizes to a cyclo (His-Pro) [5] at a pH of 7 and 37 °C with a $t_{1/2}$ of 140 min, with the release of NH_3 . The imidazole ring is purported to be playing a catalytic role at this pH. Hence, other dipeptides that did not have a histidine cleaved more slowly, and tripeptides did not cleave at all [5]. Thus, it seemed from this work that there might be a basic difference in the rate of diketopiperazine formation from the cleavage of a secondary amide (as in a tripeptide) as compared to a primary one (as in a dipeptide amide).

In another paper [6], the intramolecular aminolysis of Phe-Pro-p-nitroanilide (Phe-Pro-pNA) to Phe-Pro-diketopiperazine (Phe-Pro-DKP) was studied as a function of pH. The pH-rate plot showed that the rate of the formation of the DKP was dependent on the degree of ionization of the N-terminal amino group, with the unprotonated free amine being more reactive than the protonated form. In their experiment, the authors used an activated, strongly electron withdrawing p-nitroanilide dipeptide instead of a natural tripeptide. This was because these p-nitroanilide dipeptides dissociate by DKP formation more rapidly than amino acid amides (i.e., natural dipeptides), thereby greatly facilitating their use in kinetic studies. The calculated $t_{1/2}$ of conversion of a Gly-Pro-p-nitroanilide (Gly-Pro-pNA) dipeptide to Gly-Pro-diketopiperazine (Gly-Pro-DKP) under physiological conditions was about 120 h. This is consistent with the previous assertion that there is a significant difference in the dissociation rate between a primary (around 140 min as in previous example [5]) and an activated secondary amide. It is also important to note that in a natural tripeptide, the half-life of the DKP formation would be further extended since there is no electronic assistance from the pNA.

In both these papers [5, 6], it seems that the presence of proline in the C terminus of the dipeptide extension accentuates the formation rate of the DKP. This is likely due to contribution of the cis-proline conformer in the facilitation of the dipeptide's adoption of an optimal steric conformation for formation of DKP. The observations with modifications of an N-terminal residue upon the rate of DKP formation at pH 7.0 have been more varied. Reports suggest that it might depend on the pKa of the residue [5], on its bulk [7], or on the conformational stability of the resulting DKPs [6].

We also envisioned modification of a hydroxyl group at what otherwise would be the N-terminus to prepare a depsi-peptide and thus make an ester prodrug. An ester can cleave hydrolytically [8, 9] or via the formation of five- [17, 18] or six-membered rings (like DKP). The ester prodrugs of floxuridine (FUdR) [9] convert by general ester hydrolysis, i.e., a nucleophilic attack by water on the ester carbonyl. For the most part, the bulk of the pro-moiety influenced the hydrolysis of the FUdR prodrug (the Val ester prodrugs dissociated the slowest). In another paper, the authors studied the cyclization of the dipeptide esters in paracetamol to form a diketopiperazine [10]. They observed that they could obtain differential time action depending on the dipeptide structure. They also considered the possibility that in paracetamol, the drug release might have been via the general mechanism of ester hydrolysis and not the formation of a DKP ring. However, they eliminated it as in that case the nature of the dipeptide would have a lesser effect

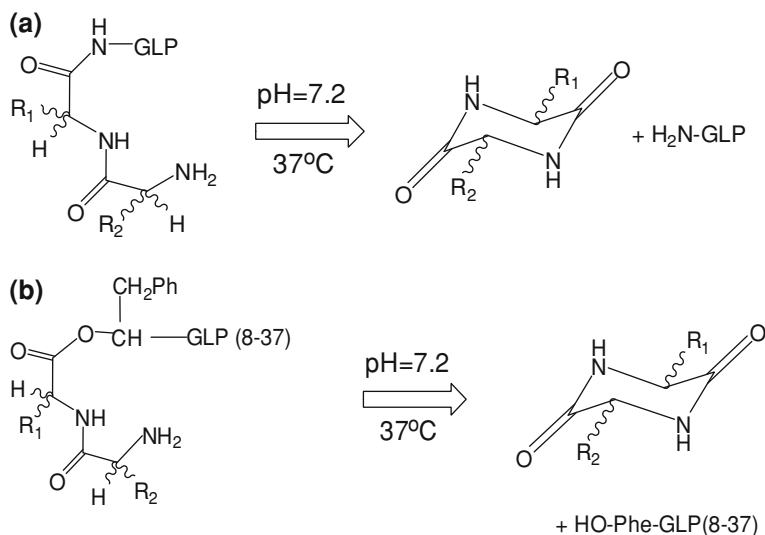


Fig. 2.1 Cleavage of amide (a) and ester prodrugs (b)

on reactivity. The proof of labile esters cleaving very fast under physiological conditions is exemplified by the fast cleavage of the dipeptide esters in paracetamol where all the prodrugs had a $t_{1/2}$ of less than 20 min [10].

In another investigational report, this time with cyclosporine-A prodrugs [11, 12] it has also been seen that through modulating the chemical nature of dipeptide esters it was possible to get conversion rates at physiological conditions ranging from minutes to several hours, but not longer. In these papers [10, 11], it seems that the presence of a minimally bulky glycine residue in the C terminus of the dipeptide extension accentuates the rate of formation of the DKP. This might be because of less bulk and the preferred conformational effect of glycine. However in the cyclosporine prodrug [11], it seems unexpected that the presence of a proline in the C terminus of the DKP actually attenuates the rate of the conversion.

In another investigation [13], the C terminal amides of glycine were rapidly hydrolyzed at 25 °C and a pH of 7 when the N-terminus was N-hydroxyethylated. The $t_{1/2}$ of bis-N-2-hydroxyethylglycinamide is three hours. In this case, the C terminal amide bond is activated by H bonding with the N-hydroxyethyl group. However, there was no practical way that one could modify the structure of the N-hydroxyethyl group as the precise Vander Waal radii were required to activate the amide group.

Consequently, we explored the intramolecular cyclization reaction of dipeptide esters and amides to form diketopiperazine as an example of a chemoreversible prodrug (Fig. 2.1). This chemistry is reasonably straightforward and allows at least four points (stereochemistry of R_1 and R_2 , nature of the nucleophile and leaving group as shown in Fig. 2.1a and b) where structure can be stereochemically controlled to refine the rate of formation with release of the active peptide.

Finally, they can also be prepared from readily available alpha-amino acids using established chemistry [14].

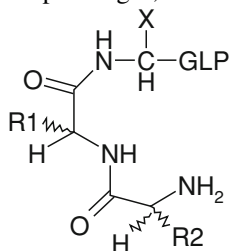
As shown below (Fig. 2.1a and b), prodrugs of varying half-lives were designed by modifying *R1* and *R2*. In the reaction below, there is an N-terminal histidine residue (native peptide), and an amide bond is being broken (Fig. 2.1a). In the second equation, the ester bond of phenyllactic acid (hydroxyl phenylalanine) is being broken (Fig. 2.1b). Though it might be beneficial to use the histidine in the 7th position of GLP, alternatives to the native N-terminal histidine were utilized for synthetic and analytical ease. Once the chemistry of a longer acting prodrug is established, it is plausible to return to the histidine or for that matter any other suitably potent amino acid at the N-terminus.

Thus, we propose to make a prodrug that will slowly convert ($t_{1/2}$ between 10 and 100 h) to the parent drug at physiological conditions of 37 °C and pH 7.2 so that they could be administered at a weekly or monthly frequency. As far as possible, a native sequence shall be used in the prodrug so as to minimize the chances of an immunological response. We rely on the pH and the temperature for this intramolecular conversion, as they are virtually invariant. It is of essence to note that this reaction should be concentration independent.

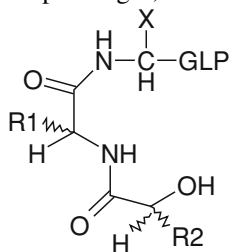
Four such peptides were identified with protracted half-lives, with minimal potency as compared to the drug in the luciferase-based bioassay. These prodrugs regained their potencies after incubation in PBS buffer at a pH of 7.2 and temperature of 37 °C.

Prodrugs in this book can be broadly classified into four different types:

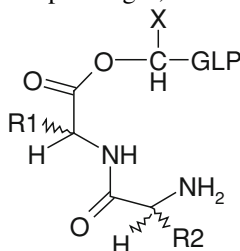
1. An amine nucleophile cleaving an amide bond (Class 1): This will dissociate with the formation of the corresponding 2,5-diketopiperazine.



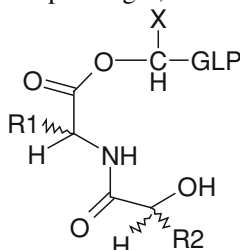
2. A hydroxyl nucleophile cleaving an amide bond (Class 2): This will dissociate with the formation of the corresponding 2,5-diketomorpholine.



3. An amine nucleophile cleaving an ester bond (Class 3): This will dissociate with the formation of the corresponding 2,5-diketopiperazine.



4. A hydroxyl nucleophile cleaving an ester bond (Class 4): This will dissociate with the formation of the corresponding 2,5-diketomorpholine.



The initial analysis was performed with the crude peptides. The rate of this reaction was not different from the pure peptides as this is an intramolecular cyclization. Additionally, it is also possible to observe our molecules of interest (both the prodrug and the drug) with a HPLC and MALDI analysis even in the midst of contaminating material. In most cases, an excellent mass balance for the disappearance of the prodrug and the appearance of the drug was observed. After we were satisfied that the crude prodrug had the required $t_{1/2}$, the prodrug was purified and a standard luciferase-based bioassay was conducted to obtain relative potencies.

The experimental design of our bioassay was based on the general principles of “reporter gene technology” [15] (Fig. 2.2). In our case, the luciferase-based reporter gene assay for cAMP detection was used. The changes in the intracellular cAMP concentrations [16] caused by the GLP receptor-mediated interactions are detected by the changes in the expression level of the luciferase gene. The transcription of this gene is regulated by the cAMP response-element binding protein (CREB) binding to cAMP response element (CRE).

This is an artificially created test system where the luciferase gene is downstream to the CRE which resembles nature’s response system all the way up to the point of gene expression where the luciferase gene is expressed. This modification is necessary as the concentration of activated luciferase is easier to measure than that of cAMP.

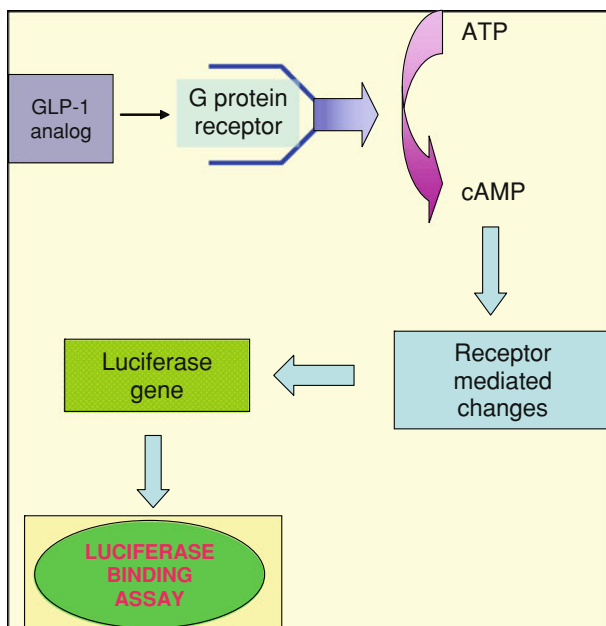


Fig. 2.2 Pictorial explanation of luciferase-based reporter gene assay

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