Toxicodynamics of Methemoglobin (MtHb) Inducers

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ABSTRACT: There is a need in New Zealand for a new, more advanced generation of toxins to minimize the impact of invasive animals. A new pest control agent, *para*-aminopropiophenone (PAPP), represents a lead candidate presently undergoing registration for the humane control of stoats and feral cats. It exhibits low toxicity to most bird species, no secondary poisoning risk, and has a simple and highly effective antidote. PAPP induces methemoglobinemia (MtHb), which acts to prevent oxygen from binding to red blood cells. This reduces oxygen supply to the brain, causing animals to become lethargic, sleepy, and unconscious prior to eventual death in 1 to 2 hours. Despite such promise, to date no previous study has comprehensively examined the effect of modifying the structure of PAPP on MtHb induction. Using PAPP as a platform, this paper describes the design, synthesis, and bioevaluation of PAPP-like red blood cell toxins. The impacts of introducing groups of varying electronic nature at different positions on the PAPP molecule are presently being evaluated. Analogues are synthesized and their MtHb-inducing properties determined using an *in vitro* assay to establish a formal structure-activity profile. *In vivo* evaluation in rats is used to assess both their acute toxicity and humaneness of potential candidates. Structure-activity profiles are discussed with the objective of optimizing the bioavailability and potency of PAPP-like compounds to target, high priority pests, particularly rodents.

KEY WORDS: chemical synthesis, *in vitro* assay, *in vivo* assay, lipophilicity, MtHb induction, PAPP, *para*-aminopropiophenone, pro-drug, red blood cell toxins, selectivity, structure-activity relationships

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INTRODUCTION

Pest Control in New Zealand

In New Zealand, pest problems are unique. Unlike Australia, where many native plants contain natural toxins (e.g., sodium fluoroacetate) as a defence against animals, New Zealand flora developed in the absence of mammalian herbivores without the need for such chemical defences. Likewise, our native ground-dwelling birds are extremely susceptible to introduced predators such as rats (*Rattus* spp.), possums (*Trichosurus vulpecula*), and stoats (*Mustela erminea*) (Eason et al. 2008).

Since European settlement in New Zealand in 1840, native forest coverage has been reduced from 53% to around only 23% of the original land area occupied (Atkinson and Cameron 1993). New Zealand has now lost over 40% of its pre-human land bird fauna (Clout 1997). If we are to maintain the natural heritage of our forest landscapes, there needs to be an effective means of controlling these animal pests.

Traditional toxic baits such as the anticoagulant brodifacoum, along with non-anticoagulants such as sodium fluoroacetate (1080), have proven effectiveness in the field; however, the use of 1080 has come up against some serious opposition in recent years (Hansford 2009). The use of these agents carries some serious drawbacks, such as high secondary poisoning risks and animal welfare concerns (Eason and Wickstrom 1997).

PAPP (*para*-aminopropiophenone), a compound currently under research and development for pest control in New Zealand by Connovation Ltd., represents a promising alternative to 1080 (Eason et al. 2008). PAPP is one of the only non-anticoagulant vertebrate pesticides developed in the last 30 years. Studies have shown that PAPP can be used in the humane control of stoats and feral cats (*Felis catus*), with low toxicity to non-target species such as birds (Fisher et al. 2008). In addition, PAPP poses low secondary poisoning risks, and its effects may be reversed through treatment with a very simple and readily available antidote, methylene blue (Savarie et al. 1983, Umbreit 2007). With these factors in mind, PAPP was envisaged as a suitable platform for extended chemical synthetic structure activity relationship studies.

This paper describes the design, synthesis, and bioevaluation of PAPP-like red blood cell (RBC) toxins. The effect of introducing groups of varying electronic nature at different positions on the PAPP molecule is examined. Analogues synthesized are measured for their MtHb-inducing properties using an *in vitro* assay to establish a formal structure-activity profile that provides insights into the pharmacodynamic properties of PAPP-like molecules.

We present an introduction to the biochemical mechanism of toxicity of PAPP and PAPP-like molecules, a discussion about the factors to be considered in the design of PAPP-like molecules, results from *in vitro* and *in vivo* experiments on PAPP analogues, and subsequent conclusions.

The Biochemical Mechanism of Toxicity of PAPP and PAPP-like Molecules

Methemoglobinemia (MtHb) is the phenomenon by which PAPP causes its toxic effect. MtHb occurs when the central iron atom of a heme group situated on the hemoglobin protein is oxidized from the ferrous (Fe^{2+}) to

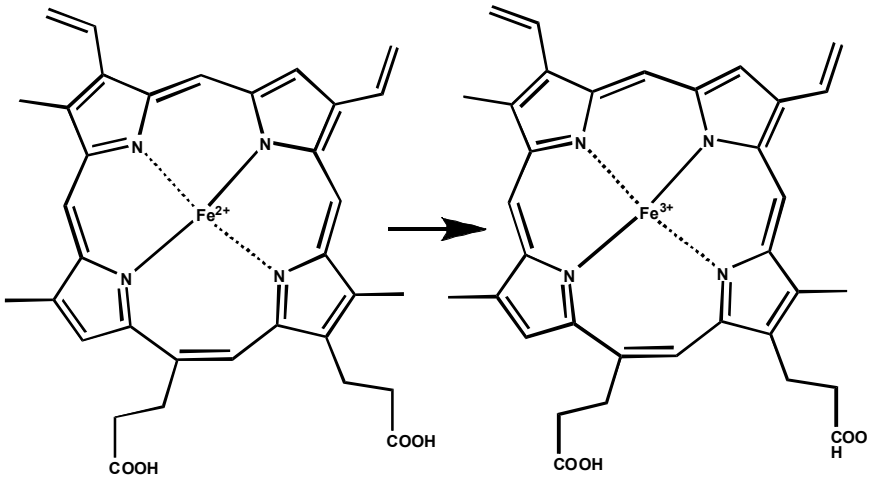


Figure 1. Oxidation of the heme group.

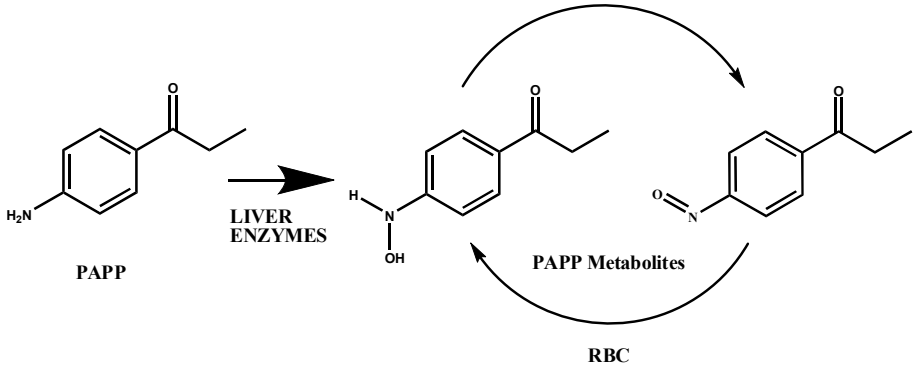


Figure 2. Biochemical pathways of PAPP.

is the ferric (Fe^{3+}) form (Figure 1) (Ash-Bernal et al. 2004, Hall et al. 1986, Norton and Smith 1976). Hemoglobin a globular protein within red blood cells that acts to deliver oxygen to vital organs such as the brain (Norton and Smith 1976).

This oxidation of the iron atom situated on the heme causes a 'left shift' in the oxygen dissociation curve, which essentially results in a higher affinity for O_2 by the remaining heme iron, which in turn causes the hemoglobin to loses its ability to combine reversibly with molecular oxygen (Martin et al. 1995). This reaction causes an irreversible binding of a heme iron to oxygen, which subsequently leads to the other hemoglobin subunits holding on to oxygen more tightly. This impairs the ability of hemoglobin to deliver oxygen to vital organs such as the brain, leading to tissue hypoxia, cyanosis, impaired aerobic respiration, metabolic acidosis, and in severe cases, death (Goldstein and Doull 1973, Hall et al. 1986, Wright et al. 1999).

Factors to Consider in the Design of PAPP-like Molecules

Conversion of PAPP to its Active N-Hydroxy Metabolite

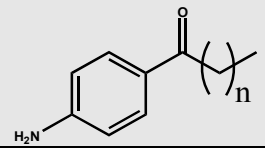
One of the first factors to consider in the design of PAPP-like molecules is extent of conversion of the parent amine to its active *N*-hydroxy metabolite (Figure 2). This conversion of PAPP to the corresponding *N*-hydroxy derivative is performed predominantly in the liver and is widely accepted as the toxic metabolite for induction of MtHb (Graffe et al. 1964). If this process could be further understood at a biochemical level, it could be manipulated to further enhance MtHb induction.

Red Blood Cell Permeability

Another factor to consider is red blood cell permeability. The red cell membrane is generally lipophilic in nature, so for passive diffusion of molecules to occur through the membrane, the lipophilicity of the molecule must be similar to that of the membrane. Lipophilicity of a molecule can be measured empirically by Log P, the partition coefficient. The goal of our experiment was to elucidate the particular window of lipophilicity where molecules will passively diffuse into the red cell and thus elicit toxicity.

This parameter of lipophilicity for PAPP-like molecules can be manipulated by varying the alkyl chain length of the ketone at the *para* position. Log P is changed without compromising the active component of the molecule, the aromatic amine. The greater the number of carbons situated on the alkyl chain of the ketone, the higher the Log P of the molecule (Table 1). These results indicate that the lipophilicity of these molecules is significant at the level of the red blood cell, and that this significance holds even when at the level of the whole organism. This experiment also served as a satisfactory validation experiment of our *in vitro* MtHb assay.

Table 1. Structure activity relationships for PAPP analogues comparing the induction of MtHb *in-vitro* in red blood cells with previously published in-vivo data.

Structure	Formal Abbrev.	Log P	*In Vitro* MtHb (%)	*In Vivo* LD_{50} (mg/kg) (Pan, Savarie et al. 1983)
				
n = 0	PAAP	0.54	4.5 ±0.9	381 (360 - 404)
n = 1	PAPP	1.20	12.8 ±1.9	221 (197 - 248)
n = 2	PABP	1.61	32.5 ±2.7	84 (56 - 126)
n = 3	PAVP	2.03	40.9 ±2.8	84 (56 - 126)
n = 4	PACP	2.45	4.5 ±1.0	216 (177 - 263)

PAPP's Biochemical Mode of Action – Redox Cycling

It is widely known that PAPP is a potent MtHb inducer (Beutler and Mikus 1961, Bright and Marks 1983, Vandenbelt et al. 1944), but few synthetic studies have explored this in depth in terms of its biochemical mode of action – redox cycling (Kiese 1966). It is known that aniline possesses low levels of MtHb activity, but not comparable to that of PAPP (Smith et al. 1967). Benzocaine, however, conveys similar MtHb induction compared to PAPP *in vitro* (Coleman and Kuhns 1999). The ketone at the *para* position of PAPP and the ester at the *para* position of benzocaine exhibit a similar electronic effect on the aromatic ring of the aniline moiety (Hansch et al. 1991). This could possibly be involved in driving the redox cycling that takes place within the red blood cell, and consequently the level of MtHb induction.

The electronic effect of a substituent attached to an aromatic ring is measured by what is known as a Hammett Constant. The Hammett Constant is a very helpful tool for determining the influence of a substituent on the electronics of a molecule. Since these ester and ketone groups suggest there may be a link between electronic influence and MtHb induction, it was hypothesized that manipulation of this side chain could help elucidate the electronic contribution of this substituent and the role it plays in redox cycling and MtHb induction.

Pharmacokinetics, Pro-drug Strategies, and Selectivity

Finally, other factors to consider in the design of PAPP-like toxicant are pharmacokinetics, pro-drug strategies, and selectivity. The active site of PAPP is situated at the nitrogen atom of the amine functionality, where PAPP is converted to the active *N*-hydroxy metabolite (Graffe et al. 1964). When PAPP is administered to the animal, it is converted to the *N*-hydroxy derivative in the liver through the action of CYP enzymes, which upon subsequent systemic circulation proceeds to cause its effect on the RBC (Graffe et al. 1964). This process, however, can be significantly retarded through the attachment of various groups to the nitrogen atom to inhibit conversion to the active *N*-hydroxy metabolite (Figure 3).

The rationale behind this is that the target species displays high levels of hydrolytic enzymatic expression, i.e., rats can convert this 'protected' pro-drug form of PAPP to the free amine much more readily than other domestic mammals such as cats and dogs. A recent study conducted by Yamauchi et al. (2002) demonstrated that the *N*-deacetylation activity of rats was significantly more than that of dogs and monkeys (Table 2). Although PAPP conveys low toxicity to most bird species (Fisher et al. 2008), this pro-drug strategy could confer even greater selectivity towards the target species.

Table 2. A comparison of *N*-deacetylase activity between species.

Species	*N*-deacetylase activity (nmol/min/mg) in the liver (Yamauchi, Ueda et al. 2002)
Rat	52.1 ±7.6
Dog	0.2 ±0.3
Monkey	7.3 ±0.4

METHODS

Chemistry

General

Compounds were synthesized at the Department of Chemistry at the University of Auckland, New Zealand. Purification procedures were performed by flash chromatography and thin layer chromatography (TLC). Structures of the compounds synthesized were confirmed by ^{1}H nuclear magnetic resonance (NMR) and ^{13}C NMR analysis on a Bruker DRX400 spectrometer.

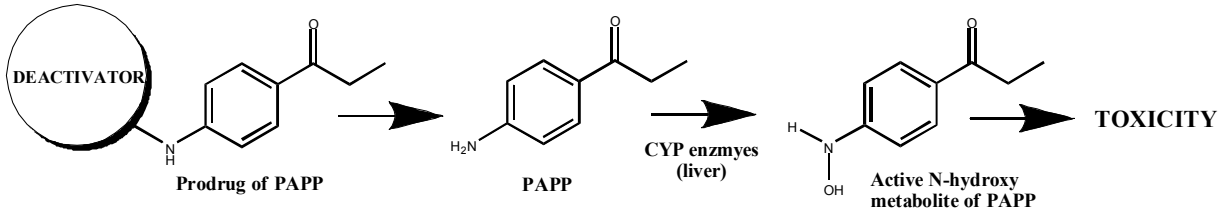


Figure 3. The pro-drug strategy for PAPP.

Red Blood Cell Permeability

In order to evaluate the significance of RBC permeability, a series of various alkyl chain length derivatives of PAPP were synthesized. These analogues were synthesized by the acylation of a phenol using the appropriate acid chloride subsequent Fries rearrangement (Commarieu et al. 2002), followed by a Smiles rearrangement (Mizuno and Yamano 2005) to furnish the desired alkyl chain derivatives.

PAPP's Biochemical Mode of Action – Redox Cycling

In order to evaluate the significance of the electronics of PAPP-like molecules in the redox cycling of the red blood cell, aniline, 4-nitroaniline, and 1,4-diaminobenzene were obtained from Sigma-Aldrich Ltd.

Pharmacokinetics, Pro-drug Strategies and Selectivity

In order to assess whether the pro-drug strategy would be effective for PAPP-like molecules, a series of pro-drugs of PAPP were synthesized. These pro-drugs were synthesized via the acylation (Schreivogel et al. 2006, Goslinski et al. 2002) of PAPP with an appropriate acid chloride or anhydride.

In Vitro Work

In vitro work was carried out using a MtHb assay adapted from Coleman et al. (1991) and Evelyn and Malloy (1938). Microsomes were prepared from pooled male and female Sprague-Dawley rats. Washed erythrocytes were obtained from male Sprague-Dawley rats. To microsomes (1 mg/ml) was added washed rat blood cells (resuspended to approximately 50% haematosis), NADPH co-factor (1 mM), and the PAPP-like molecule (100 μM) dissolved in dimethyl sulfoxide (1%) to give a final volume of 200 μL. The samples were mixed and maintained at 37°C for 1 hour. Following this, the samples were immediately put on ice and assayed for MtHb% using the CO-oximetry unit of an ABL700 blood-gas analyzer.

In Vivo Work

Males and female rats weighing 250 - 500 g were dosed by oral gavage (240 mg/kg) with PAPP and PAPP analogues as aqueous suspensions (120 mg/ml, dose volume 2ml/kg). All agents were dosed in as aqueous suspensions comprising of 1% Kelsol (seaweed extract) and 99% water. Following dosing, rate of onset of symptoms, duration of symptoms, and time to death were determined.

RESULTS

Red Blood Cell Permeability

Pan et al.'s study (1983) revealed that there was a structure-toxicity relationship between the alkyl chain length of PAPP-like molecules and oral toxicity in rats. An *in vitro* MtHb assay was conducted in order to reveal if there was any relationship between the already established *in vivo* data and toxicity at the level of the blood cell. The replication of this *in vivo* experiment *in vitro* was also envisaged to serve as a validation for the MtHb assay.

This experiment was replicated *in vitro*, with the results in general holding with Pan's *in vivo* data (Table 1, Figure 4). Analogues PABP and PAVP showed the greatest toxicity with PAPP and analogue PACP showing considerably less toxicity.

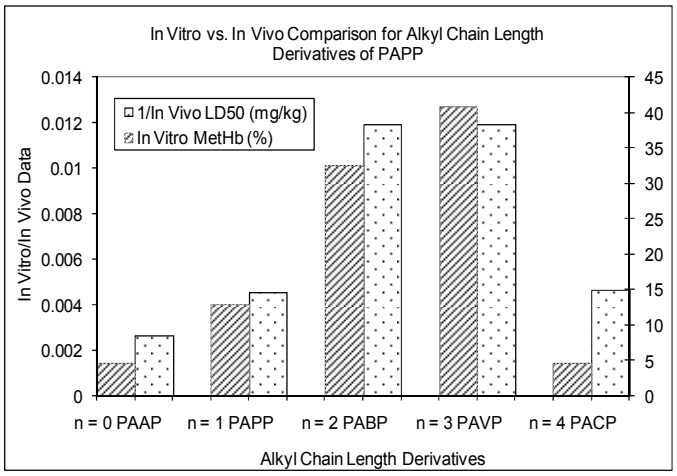


Figure 4. Structure activity relationships for PAPP analogues comparing the induction of MtHb *in-vitro* in red blood cells with previously published in-vivo data.

PAPP's Biochemical Mode of Action – Redox Cycling

To evaluate the effect of changing the electronics of the substituent situated at the *para* position of the aromatic amine, a decision was made to test aniline, consisting of no electronic influence on the active site, and two extremely different groups of electronic effect (i.e., a nitro group, an example of a strongly electron withdrawing group, and an amino group, a strongly electron donating group).

The results from the *in vitro* assay show that aniline and 1,4-diaminobenzene display a complete loss of MtHb activity, whereas 4-nitroaniline displayed ca. 50% the MtHb activity of PAPP (Table 3).

Pharmacokinetics, Pro-drug Strategies, and Selectivity

In vitro and *in vivo* compounds DC2009-2 and DC2009-42 exhibited low levels of MtHb activity, suggesting there was some cleavage by the rat enzymes to form PAPP and consequently the active *N*-hydroxy metabolite; however, the level of MtHb activity displayed by these compounds was not comparable to PAPP (Table 4).

DISCUSSION

Red Blood Cell Permeability

These results indicate that the lipophilicity of these molecules is significant at the level of the RBC, and that this significance holds at the level of the whole organism. This experiment also served as a satisfactory validation experiment of our *in vitro* MtHb assay.

PAPP's Biochemical Mode of Action – Redox Cycling

With the aromatic amine moiety unmodified in all the molecules tested (Table 3), this suggests the importance of the ketone moiety and the electronic influence that it

Table 3. MtHb toxicities from changing the electronic nature at the *para*-position of PAPP-like molecules.

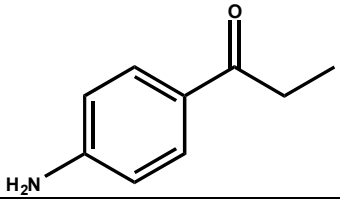
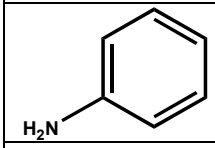
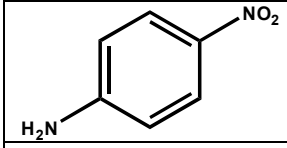
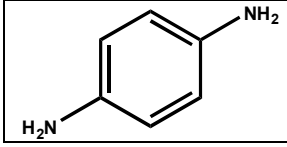
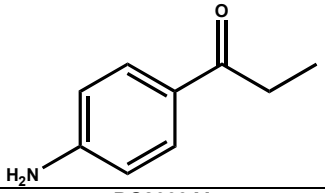
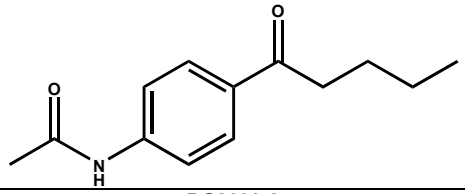
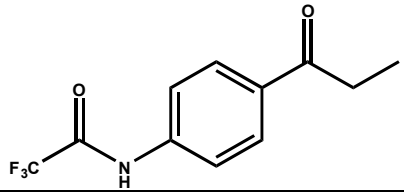
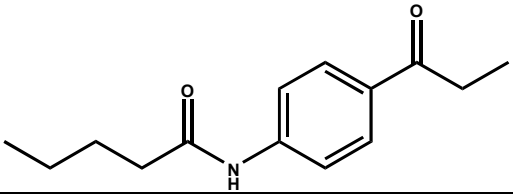
Structure	Name	Hammett Constant (σ)	*In vitro* MtHb activity compared with that of PAPP
	PAPP	+0.48	–
	Aniline	0	No activity
	4-Nitroaniline	+0.78	½-fold activity
	1,4-diaminobenzene	-0.66	No activity

Table 4. A comparison of *in vitro* MtHb activity vs. *in vivo* observations of symptoms upon oral administration of pro-drugs.

Compound	*In Vitro*	*In Vivo*			
	MtHb (%)	Dose Rate (mg/kg)	Time to Onset of Symptoms (mins)	Time to Death (mins)	Symptoms
PAPP 	9.6 ±1.6	240	15.0 ±1.0	89 ±47.8	Blue paws, quiet, ataxia, lethargy, vocalisation, peddling
DC2009-V 	0	240	41.3 ±4.0	–	Blue paws, quiet, ataxia, lethargy
DC2009-2 	7.4 ±1.2	240	12.5	–	Slight blue paw, lethargy, ataxia
DC2009-42 	1.7 ±0.7	240	11	–	Lethargy

introduces to the ring of the aromatic amine. The fact that any change in electronics at the *para* position substituent also brings about such a dramatic loss in MtHb activity suggests that electronic influence at this area is quite significant, and that there may possibly be an optimal electronic influence on the ring that promotes maximum redox cycling, hence high accumulation of the toxic agent. This variance in activity when different groups are attached to the ring meant that there is reduced scope for synthesis of PAPP or aniline-like analogues.

Upon consideration of this knowledge, it is proposed that perhaps a linker moiety can be introduced to increase the scope of analogues to be synthesized. The reasoning behind this is that by attaching groups of various electronic nature to the linker instead of directly to the ring, then perhaps this could dilute the electronic influence of the groups on the amine of the aromatic system. In this sense, the ketone moiety could be retained to conserve the desired electronic influence on the aromatic amine directly, but also to allow for a more subtle fine tuning of the electronics of the molecule in a remote fashion.

Pharmacokinetics, Pro-drug Strategies, and Selectivity

These results display proof of activation to PAPP and therefore validate the pro-drug concept for MtHb induction, and the potential of such a strategy to build selectivity into an already potent MtHb inducer, such as the analogue PAVP.

CONCLUSIONS

PAPP is a novel toxicant in that it exhibits low toxicity to most bird species, low secondary poisoning risk, and has a simple and highly effective antidote. Thus, PAPP constitutes a good platform to investigate and develop chemical synthetic structure-activity relationships. Factors to be considered in the design of PAPP-like molecules include extent of conversion of the PAPP to its active *N*-hydroxy metabolite, red blood cell permeability, PAPP's biochemical mode of action (redox cycling), pharmacokinetics, pro-drug strategies, and selectivity. Our results suggest that there is an optimum level of both lipophilicity and electronic balance required for increased MtHb induction, and that there is proof of concept for the pro-drug strategy for PAPP-like molecules. To date, we have confirmed in an *in vitro* test system with RBCs that analogue PAVP is more potent than PAPP.

ACKNOWLEDGEMENTS

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