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# Bi- or multifunctional peptide drugs

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## Abstract

Strategies for the design of bi- or multifunctional drugs are reviewed. A distinction is made between bifunctional drugs interacting in a monovalent fashion with two targets and ligands containing two distinct pharmacophores binding in a bivalent mode to the two binding sites in a receptor heterodimer. Arguments are presented to indicate that some of the so-called “bivalent” ligands reported in the literature are unlikely to simultaneously interact with two binding sites. Aspects related to the development of bi- or multifunctional drugs are illustrated with examples from the field of opioid analgesics. The drug-like properties of the tetrapeptide Dmt[1][DALDA] with triple action as a μ opioid agonist, norepinephrine uptake inhibitor and releaser of endogenous opioid peptides to produce potent spinal analgesia are reviewed. Rationales for the development of opioid peptides with mixed agonist/antagonist profiles as analgesics with reduced side effects are presented. Progress in the development of mixed μ opioid agonist/δ opioid antagonists with low propensity to produce tolerance and physical dependence is reviewed. Efforts to develop bifunctional peptides containing a μ opioid agonist and a cholecystokinin antagonist or an NK1 receptor antagonist as analgesics expected to produce less tolerance and dependence are also reviewed. A strategy to improve the drug-like properties of bifunctional opioid peptide analgesics is presented.

### Keywords

Bifunctional drugs; Mixed agonist/antagonists; Opioid analgesics; Opioid tolerance and dependence

## Introduction

It is now widely accepted that biological activity at a single receptor is often insufficient and recent research focused on ligands that have multiple activities (Morphy and Rankovic 2005; Cavalli et al. 2008). Bi- or multifunctional drugs may have much improved potency due to synergistic effects or may produce fewer side effects than compounds acting at a single target. Compared with drug combinations there are several advantages associated with bi- or multifunctional ligands, such as the more predictable pharmacokinetic (PK) and pharmacodynamic (PD) relationship that is a consequence of the administration of a single medicine. Differences in the relative rates of metabolism between patients can produce highly complex PK/PD relationships for multicomponent drugs (drug cocktails), leading to

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unpredictable variability between patients. Other advantages are improved patient compliance and a lower risk of drug-drug interactions compared to cocktails or multicomponent drugs (Edwards and Aronson 2000). A number of bifunctional drugs for the treatment of depression, schizophrenia, allergies, hypertension, inflammation or metabolic diseases are either already on the market or are in clinical trials (for a review, see Morphy and Rankovic 2005).

To design bifunctional drugs, three medicinal chemistry strategies may be used to join functionally distinct pharmacophores: i) pharmacophores connected directly or via a conjugate linker; ii) overlapping pharmacophores; and iii) highly integrated pharmacophores. Bifunctional compounds based on highly integrated pharmacophores have been discovered by chance or from screening of compound libraries, whereas bifunctional drugs containing two overlapping pharmacophores or two pharmacophores connected to each other either directly or via a linker can be designed. In the latter case, the design of the bifunctional ligands must be based on careful consideration of existing structure-activity relationships (SAR) of the two components, particularly with regard to the attachment sites of the pharmacophores to be connected. It is possible that the pharmacological characteristics (e.g. the receptor binding affinity and/or efficacy) of each component may be altered upon incorporation into the bifunctional construct. This needs to be verified in in vitro experiments before using the compound in in vivo studies. For example, it should not be assumed without verification that the “antagonist” component in the bifunctional ligand still has antagonist properties, as it could have partial or even full agonist activity.

A distinction has to be made between bifunctional compounds and bivalent ligands. Bifunctional drugs interact in a monovalent fashion with two different targets. In efforts to develop bivalent ligands, compounds containing two distinct pharmacophores linked via a rather long spacer are designed with the expectation that they might be able to interact simultaneously with two receptor binding sites such as, for example, in a receptor heterodimer. Theoretically, a bivalent ligand interacting with a receptor heterodimer in a bivalent manner would be expected to show greatly enhanced binding affinity on the basis of thermodynamic considerations (binding enthalpy increase and entropy advantage). In some reported studies on putative “bivalent” ligands an increase in binding affinity was indeed observed at a certain length of the linker in comparison with the binding affinities of the components by themselves. However, in most cases the binding affinity enhancements were quite modest and could perhaps be better explained by a monovalent interaction of one of the components in the “bivalent” ligand with one of the targeted receptor binding sites and an additional interaction with an accessory binding site. In this reviewer’s opinion, convincing studies in support of a bivalent binding mode of putative “bivalent” ligands interacting with heterodimeric G protein-coupled receptors (GPCRs) have not been reported to date. Furthermore, the very long spacer length (20–30 Å) required for the simultaneous binding of the two components in a bivalent ligand to a GPCR heterodimer would diminish the drug-like properties of such compounds.

In this article we review the development of bi- or multifunctional opioid peptides with high analgesic potency and/or reduced side effects as analgesic drugs or for the treatment of drug abuse.

## Development of opioid compounds as analgesics

The treatment of severe pain relies heavily upon opioid analgesics, most of which act via μ opioid receptors. Aside from the analgesic effect, morphine and other μ opioid analgesics produce a number of side effects, including inhibition of gastrointestinal motility, respiratory depression, tolerance and physical dependence, which often limit their use in pain treatment. Kappa opioid agonists have also been shown to be potent analgesics; however, their use is discouraged because of their sedative, dysphoric and psychotomimetic effects. Delta opioid

agonists are known to produce analgesic effects and were thought to hold some promise because they appeared to produce less physical dependence, less respiratory depression and less constipation than morphine. However, some of the early δ agonists produced convulsions in animals and δ opioid agonists efficacious enough as analgesics have yet to be developed. Thus, opioid agonists with selectivity for any of the three opioid receptor types (μ, δ,κ) all have limitations because of their side effects and/or weak analgesic activity. There is evidence to indicate that bi- or multifunctional opioid compounds with novel biological activity profiles may have therapeutic potential as potent analgesics with reduced side effects. Efforts to develop such compounds derived from opioid peptides are described in this review.

## [Dmt[1]]DALDA: a trifunctional opioid analgesic

The dermorphin-derived tetrapeptide [Dmt[1]]DALDA (H-Dmt-D-Arg-Phe-Lys-$NH_2$), containing 2′,6′-dimethyltyrosine (Dmt) in the 1-position of the peptide sequence, is a highly potent μ opioid agonist (Schiller et al. 2000). Its μ receptor binding affinity ($K_i^{\mu}$ = 0.143 nM) is about 7-fold higher than that of morphine and it has high μ receptor binding selectivity [$K_i$ ratio (μ/δ/κ) = 1/14700/156]. When incubated in sheep blood, [Dmt[1]]DALDA was found to be highly stable against enzymatic degradation, as was to be expected on the basis of its structural characteristics (Szeto et al. 2001). This compound showed extraordinary potency (3000 times more potent than morphine) in the rat tail-flick assay with intrathecal (i.th.) administration (Shimoyama et al. 2001). The observation that [Dmt[1]]DALDA has only 7-fold higherμ receptor binding affinity than morphine suggested that additional mechanisms may be implicated in producing the very potent analgesic effect of this compound at the spinal level. Indeed, [Dmt[1]]DALDA was shown to inhibit norepinephrine uptake in rat spinal cord synaptosomes with an $IC_{50}$ of 4.1μM (Shimoyama et al. 2001). Due to the latter effect, norepinephrine levels are increased and activation of both $\alpha_2$-adrenergic receptors and μ opioid receptors takes place which is known to produce an enhanced analgesic effect in a synergistic manner (Reimann et al. 1999). This interpretation is supported by the observation that the antinociceptive response to [Dmt[1]]DALDA is attenuated by the $\alpha_2$-adrenergic antagonist yohimbine (Shimoyama et al. 2001). Furthermore, it was shown that the i.th. antinociceptive effect of [Dmt[1]]DALDA was decreased after pretreatment (i.th.) with the κ opioid antagonist norBNI, or with antiserum against dynorphin A(1–17) or [Met[5]]enkephalin (Szeto et al. 2003). These results were interpreted to indicate that this peptide also acts as a releaser of dynorphin- and [Met[5]]enkephalin-like substances that act at κ and δ receptors to contribute to the extraordinary antinociceptive potency of [Dmt[1]]DALDA. Thus, [Dmt[1]]DALDA is a spinal analgesic with triple action: μ opioid receptor activation, inhibition of norepinephrine uptake and release of endogenous opioid peptides. In the rat tail-flick test, this peptide produced an antinociceptive effect that lasted four times longer than that of morphine when both compounds were administered i.th. at equipotent doses (3 × $ED_{50}$) (Shimoyama et al., 2001). Both its enzyme resistance and its slow clearance from the spinal cord due to its high positive charge may be the reason for the long duration of action of [Dmt[1]]DALDA. Another advantage of this compound is that it has a very low propensity to produce respiratory depression (Shimoyama et al. 2001). For all these reasons, [Dmt[1]]DALDA may be considered as a promising drug candidate for use in spinal analgesia. The only drawback of this compound is that it produced quite profound tolerance after chronic i.th. administration (Zhao et al. 2002; Ben et al. 2004). [Dmt[1]]DALDA was also 100–200-fold more potent than morphine in the mouse tail-flick test when given by the intracerebroventricular (i.c.v.) route (Neilan et al. 2001; Zhao et al. 2002).

Subcutaneous (s.c.) administration of [Dmt[1]]DALDA also produced a potent antinociceptive effect in the mouse tail-flick test. In comparison with s.c. morphine, s.c. [Dmt[1]]DALDA was 218 times more potent in one study (Neilan et al. 2001) and 36 times more potent in another (Zhao et al. 2002) and the duration of its analgesic effect (12h) was again much longer than

that of morphine (3h) at equipotent doses ($5 \times ED_{50}$). Furthermore, the results of a pharmacokinetic study using the sheep model (intravenous infusion) indicated that the elimination half-life of [$Dmt^1$]DALDA (2 hours) was four times longer than that of morphine (30 minutes) (Szeto et al. 2001). It appears that the prolonged analgesic effect of this peptide after systemic administration is due to both its metabolic stability and its long elimination half-life. Whereas [$Dmt^1$]DALDA did produce tolerance upon chronic s.c. administration to mice, only minimal cross-tolerance was seen with this compound given s.c. in morphine-tolerant mice (Neilan et al. 2001; Riba et al. 2002). This raises the possibility for the treatment of morphine-tolerant patients with [$Dmt^1$]DALDA for more effective pain treatment.

The potent analgesic effect produced by [$Dmt^1$]DALDA in the tail-flick assay with s.c. administration indicates that this peptide is capable of crossing the blood-brain barrier (BBB). This unexpected observation prompted a study on transcellular transport of [$Dmt^1$]DALDA and its cellular uptake (Zhao et al. 2003). [$^3$H][$Dmt^1$]DALDA was shown to readily translocate across Caco-2 cell monolayers. The peptide is taken up into Caco-2 cells in a concentration-dependent fashion until a steady-state is reached, suggesting that it is effluxed by a transporter. Uptake of [$^3$H][$Dmt^1$]DALDA into SH-SY5Y, HEK 293 and CRFK cells was also demonstrated. The mechanism of cellular uptake remains unclear, but it was established that it does not involve a transporter, receptor-mediated endocytosis or absorptive endocytosis. [$Dmt^1$]DALDA is a cationic-aromatic peptide (charge of 3+), consisting of alternating aromatic and basic amino acids. It is possible that the positively charged moieties of the peptide form a complex with negatively charged cell surface functionalities through electrostatic interactions and hydrogen bonding, followed by partitioning of the resultant ion pair complex into the lipid bilayer and migration across. Alternatively, [$Dmt^1$]DALDA may have membrane-destabilizing activity by altering the packing and organization of lipids, and the formation of a short-lived transient pore. The latter possibility has been suggested as a common mechanism for the rare group of membrane-crossing peptides with diverse structural characteristics (Rathinakumar and Wimley 2008).

## Compounds with an opioid agonist/opioid- or non-opioid-antagonist profile

There is evidence to indicate that compounds acting as agonist at one of the opioid receptors and as antagonist at another opioid or non-opioid receptor might have therapeutic potential as analgesics with reduced side effects or for the treatment of cocaine abuse. The development of such bifunctional compounds is reviewed in the following.

## Mixed opioid agonist/opioid- or non-opioid antagonists

### μ Agonist/δ antagonists

Selective δ opioid receptor blockade with a δ opioid antagonist has been shown to greatly reduce the development of morphine tolerance and dependence (Abdelhamid et al. 1991; Fundytus et al. 1995; Hepburn et al. 1997). A number of observations relevant to this finding have been made. Chronic administration of morphine resulted in an upregulation of δ opioid receptors in rats (Rothman et al. 1986). Using different strains of mice, it was demonstrated that the intensity of the withdrawal syndrome after chronic morphine treatment correlated with the level of δ binding sites (Yukhananov et al. 1994). An antisense oligodeoxynucleotide to the δ opioid receptor was shown to prevent the development of morphine tolerance and dependence after chronic morphine administration (Kest et al. 1996). Furthermore, in δ opioid receptor knockout mice morphine was shown to retain its μ receptor-mediated analgesic activity without producing tolerance with chronic administration (Zhu et al. 1999). All these observations suggest that a compound with a mixed μ opioid agonist/δ opioid antagonist profile could be expected to be an analgesic with low propensity to produce analgesic tolerance and physical dependence. Furthermore, it has been shown that the δ antagonist naltrindole enhanced

colonic propulsion (Foxx-Orenstein et al. 1998) and reversed alfentanil (a μ opioid agonist)-induced respiratory depression (Freye et al. 1992), suggesting that a mixed μ agonist/δ antagonist might also cause less inhibition of gastrointestinal transit and less respiratory depression than a μ agonist like morphine. Whereas numerous compounds with a mixed μ agonist/δ agonist profile have been reported, the development of mixed μ agonist/δ antagonists proved to be difficult. Earlier efforts to develop peptide and non-peptide compounds with this profile have been reviewed (Schiller et al. 1999b; Ananthan 2006).

The first reported compound with a mixed μ agonist/δ antagonist profile was the tetrapeptide amide H-Tyr-Tic-Phe-Phe-$NH_2$ (TIPP-$NH_2$; Tic = tetrahydroisoquinoline-3-carboxylic acid) which showed modest μ agonist potency and quite high δ antagonist activity in vitro (Schiller et al. 1992). Replacement of the $Tyr^1$ residue in this peptide with Dmt and reduction of the $Tic^2$-$Phe^3$ peptide bond produced the highly stable compound H-Dmt-TicΨ[$CH_2$NH]Phe-Phe-$NH_2$ (DIPP-$NH_2$[Ψ]), which turned out to be a potent, balanced μ agonist/δ antagonist with subnanomolar binding affinities for both μ and δ receptors (Schiller et al. 1995, 1999a). With DIPP-$NH_2$[Ψ] no clear distinction can be made between structural moieties that confer μ agonist properties to the molecule and moieties that are implicated in δ antagonist behavior. It thus represents a bifunctional compound of the integrated pharmacophore type. This compound given intracerebroventricularly (i.c.v.) produced a potent antinociceptive effect in the rat tail-flick test with 3-fold higher potency as compared to morphine. DIPP-$NH_2$[Ψ] chronically infused i.c.v. at high doses produced no physical dependence and less acute analgesic tolerance than morphine. Thus, this compound provided a “proof-of-concept” for the development of mixed μ agonist/δ antagonists as opioid analgesics with low propensity to produce dependence and tolerance. However, DIPP-$NH_2$[Ψ] showed a very limited ability to cross the BBB. A number of di- and tripeptide derivatives containing the Dmt-Tic pharmacophore were also synthesized and pharmacologically characterized in vitro. The dipeptide analogue H-Dmt-Tic-NH-$(CH_2)_3$-Ph is a potent μ agonist/δ antagonist with subnanomolar binding affinity for both μ and δ opioid receptors (Schiller et al. 1995). Subsequently, the dipeptide derivative Dmt ($NMe_2$)-Tic-NH-1- adamantane (Salvadori et al. 1999) and the tripeptide analogue H-Dmt-Tic-Gly-NH-$CH_2$-Ph (Balboni et al. 2002) were also reported to be potent μ agonist/δ antagonists.

Structural modifications of β-casomorphin and the endomorphins also led to mixed μ agonist/δ antagonists. The cyclic β-casomorphin analogue H-Tyr-c[-D-Orn-2-Nal-D-Pro-Gly-] was shown to be a moderately potent μ agonist/δ antagonist (Schmidt et al. 1994), and substitution of $Tyr^1$ with Dmt in the latter peptide produced a compound with a balanced μ agonist/δ antagonist profile and subnanomolar μ and δ receptor binding affinities (Schiller et al. 1995). Several analogues of endomorphin-1 and endomorphin-2 with mixed μ agonist/δ antagonist properties have been described. The endomorphin-2 analogue H-Dmt-Pro-Phe-NH-$C_2H_4$-Ph (Fujita et al. 2004) and the endomorphin-1 analogue H-Dmt-Pro-Trp-D-1-Nal-$NH_2$ (Fichna et al. 2007) both showed high μ agonist potency and moderate δ antagonist activity in vitro, whereas an endomorphin-2 analogue containing 2′,′,6′-trimethylphenylalanine (Tmp), H-Dmt-Pro-Tmp-Phe-$NH_2$, was reported to be a potent μ agonist/δ antagonist with high binding affinity for both receptors (Li et al. 2007).

In an effort to develop μ agonist/δ antagonists capable of crossing the BBB, a bifunctional peptide containing [$Dmt^1$]DALDA and the potent and selective δ antagonist (inverse δ agonist) TICP[Ψ] (H-Tyr-TicΨ[$CH_2$NH]Cha-Phe-OH; Cha = cyclohexylalanine) (Schiller et al. 1999b) connected “tail-to-tail” via a short linker was synthesized (Weltrowska et al. 2004). It was expected that this peptide, H-Dmt→D-Arg→Phe→Lys-NH-$CH_2$-$CH_2$-NH-Phe←Cha[$NHCH_2$]ΨTic←Tyr-H ([$Dmt^1$]DALDA→$CH_2CH_2NH$←TICP[Ψ]), would cross the BBB because its [$Dmt^1$]DALDA component, capable by itself to effectively penetrate into the CNS, would confer BBB crossing ability to the entire bifunctional peptide construct. In vitro,

[Dmt$^1$]DALDA→$CH_2$ $CH_2$NH←TICP[Ψ] showed the expected μ agonist/δ antagonist profile with μ and δ receptor binding affinities in the low nanomolar range. In the mouse tail-flick test, this compound given s.c. showed analgesic potency ($ED_{50}$ = 5.73 (3.14–10.45) μmol/kg) similar to that of morphine ($ED_{50}$ = 5.70 (2.81–10.32) μmol/kg), indicating that it does cross the BBB (Szeto and Schiller 2008, unpublished results). The duration of the antinoniceptive effect of this compound (8 hours) was twice as long as that of morphine (4 hours) when the two drugs were given s.c. at equipotent doses (5 × $ED_{50}$). Furthermore, this mixed μ agonist/δ antagonist was found to produce substantially less analgesic tolerance than morphine. More recently, a bifunctional compound consisting of the δ antagonist TIPP (H-Tyr-Tic-Phe-Phe-OH) (Schiller et al. 1999b) attached to the Lys$^4$ side chain of the μ agonist [Dmt$^1$]DALDA was synthesized (Nguyen and Schiller 2008, unpublished results). This peptide showed subnanomolar binding affinity for μ and δ opioid receptors, potent μ agonist activity in the guinea pig ileum (GPI) assay and, surprisingly, also potent δ *agonist* activity in the mouse vas deferens (MVD) assay. This result indicates that the efficacy of a component may change upon incorporation into a bifunctional construct, depending on the site of attachment to the other component. Using the same strategy, a bifunctional peptide containing endomorphin-2 as μ agonist component and the dipeptide Dmt-Tic as δ antagonist component was prepared (Salvadori et al. 2007). The resulting compound, H-Tyr-Pro-Phe-Phe-NH-$CH_2CH_2$-NH←Tic-Dmt-H, displayed the expected μ agonist/δ antagonist profile in vitro.

A number of non-peptide compounds with μ agonist/δ antagonist properties have been described in the literature. Among a series of prepared pyridomorphinans, the compound SoRI 9409 was characterized as a partial μ agonist/δ antagonist which did not produce significant analgesic tolerance upon repeated i.c.v. injection (Ananthan et al. 1999; Wells et al. 2001). The hydromorphone-derived pyridomorphinan SoRI 20411 developed by the same group turned out to be a μ agonist/δ antagonist with about 10-fold lower potency than morphine in the mouse tail-withdrawal assay (i.c.v. administration) and with low propensity to produce analgesic tolerance (Ananthan et al. 2004). A recently reported Dmt-pyrazinone derivative was shown to be a μ agonist with weak δ antagonist activity (Shiotani et al. 2007). A bifunctional compound containing the morphinan agonist MCL 101 linked to the δ antagonist Dmt-Tic via a short spacer showed μ agonist/δ antagonist properties but, as expected, was also a potent κ agonist (Neumeyer et al. 2006). In an effort to develop bivalent μ agonist/δ antagonist ligands capable of simultaneously binding to the μ and δ receptor binding sites in a putative μ-δ receptor heterodimer, compounds containing the μ agonist oxymorphone and the δ antagonist naltrindole connected via long spacers (16–21 atoms) were synthesized (Daniels et al. 2005b). However, these compounds were not shown to have the desired μ agonist/δ antagonist profile in in vitro assays. As discussed above, incorporation of a δ antagonist component into a bifunctional compound could alter its efficacy. Furthermore, an in vitro evaluation would have revealed whether these compounds show indeed the drastic binding affinity enhancement expected in the case of a bivalent binding mode. In fact, all these “bivalent” compounds administered i.c.v. showed lower antinociceptive potencies in the mouse tail-flick assay than the corresponding monovalent μ agonist compounds. It was found that the compounds with a spacer length of 19 atoms or greater elicited no tolerance and dependence and, interestingly, produced a potent antinociceptive effect when given i.v.

## Compounds with other mixed opioid agonist/opioid antagonist profiles

There is considerable evidence to indicate that the abuse-related effects of cocaine are mediated in part by increases in extracellular dopamine levels in the mesolimbic dopamine system (Johanson and Fischman 1989; Kuhar et al. 1991). Observations that both κ opioid agonists and μ opioid antagonists inhibit dopamine release in the nucleus accumbens (Maisonneuve et al. 1994) led to the suggestion that opioid compounds with a mixed κ agonist/μ antagonist profile might have therapeutic potential for the treatment of cocaine abuse (Archer et al.

1996). Non- peptide κ agonist/μ antagonists and κ agonist/μ agonists decreased cocaine self-administration with fewer side effects than highly selective κ agonists (Bowen et al. 2003). A number of non-peptide compounds with a mixed-action κ/μ profile, including κ agonist/partial μ agonists and partial κ agonist/μ antagonists, have been synthesized and characterized in vitro. Most of these compounds were obtained by connecting two opioid pharmacophores via a linker and were generally described as "bivalent ligands" (Neumeyer et al. 2003; Mathews et al. 2005; Peng et al. 2006, 2007b). However, because these compounds do not show the drastic binding affinity enhancement expected for simultaneous binding to two binding sites in a receptor heterodimer and because of the short spacer length used in most cases, a bivalent binding mode seems unlikely. These compounds might bind monovalently to κ and μ receptors and, therefore, it seems more appropriate to refer to them as bifunctional ligands rather than as bivalent ligands. An interesting series of carbamate analogues of morphinan with κ agonist/partial μ agonist properties has also been reported (Peng et al. 2007a). The cyclic peptide (3*R*)-Idp-c[D-Cys-Gly- Phe(*p*$NO_2$)-D-Cys]$NH_2$ containing (3*R*)-3-isopropyl-3-(2,6-dimethyl-4- hydroxyphenyl)propanoic acid [(3*R*)-Idp] in the 1-position was characterized in vitro as a potent κ opioid agonist with neutral μ antagonist activity and weak neutral δ antagonist activity (Schiller et al. 2005). This compound is also of considerable interest because it represents the first opioid peptide-derived *agonist* lacking a positive charge. Bivalent ligands containing δ antagonist and κ agonist pharmacophores were prepared as pharmacological tools to study δ and κ opioid receptor phenotypes (Daniels et al. 2005a).

## Compounds with an opioid agonist/non-opioid antagonist profile

There have been numerous studies on the antiopioid effects of cholecystokinin (CCK). CCK2 receptor antagonists have been shown to reverse tolerance to morphine in rats (Hoffmann and Wiesenfeld-Hallin 1994) and to attenuate the development of morphine dependence (Maldonado et al. 1995). These findings prompted attempts to develop bifunctional peptides containing an opioid agonist component and a CCK antagonist component. The heptapeptide H-Tyr-D-Phe-Gly-D-Trp-NMeNle-Asp-Phe-$NH_2$, containing overlapping opioid and CCK pharmacophores, showed quite good μ and δ opioid agonist potencies in the GPI and MVD assays, high CCK-2 receptor binding affinity, high selectivity for CCK-2 vs. CCK-1 receptors and CCK antagonist activity in the unstimulated GPI/LMMP (LMPP = longitudinal muscle with mesenteric plexus) assay (Hruby et al. 2003). Bifunctional peptides containing an opioid peptide pharmacophore and a CCK pharmacophore, linked via a hydrazide moiety, displayed high μ and δ receptor binding affinities, weak (μmolar) CCK receptor binding affinities and apparently marked CCK antagonist activity in the GPI/LMMP assay (Lee et al. 2006). The design of further heptapeptides with overlapping pharmacophores at opioid and CCK receptors led to a compound, H-Tyr-D-Ala-Gly-D-Trp-NMeNle-Asp-Phe-$NH_2$, with high δ receptor binding affinity, 15-fold δ vs. μ receptor selectivity, weak CCK-1 receptor binding affinity and high CCK-2 receptor binding affinity (Agnes et al. 2006). However, it was not demonstrated that this compound behaved as an antagonist against CCK. Partial retro-inverso, retro and inverso bifunctional peptides containing opioid peptide and CCK pharmacophores linked via a hydrazide moiety showed high δ and μ receptor binding affinities but weak CCK-1- and CCK-2 receptor binding affinities (Lee et al. 2007). Antagonism of these peptides at CCK receptors was not demonstrated. In summary, interesting bifunctional peptides with binding affinities for both opioid and CCK receptors and potent opioid agonist activities in vitro were prepared. However, a number of these compounds were not examined directly for CCK antagonist activity. Analgesic potency and tolerance/dependence studies have not been reported for these bifunctional compounds. Because these hepta- and octapeptide constructs do not seem to have structural characteristics that would promote crossing of the BBB, it is unlikely that they would be systemically active to produce centrally mediated analgesia.

It has been demonstrated that treatment with an NK1 receptor antagonist can prevent and reverse the development of morphine tolerance (Powell et al. 2003). Furthermore, it has been shown that blockade of NK1 receptors in rats induced a decrease in the expression of naloxone-precipitated morphine abstinence (Maldonado et al. 1993). On the basis of this evidence it has been suggested that combining a NK1 receptor antagonist with a μ opioid agonist could improve the effectiveness of the treatment of neuropathic pain and eliminate adverse effects such as tolerance and physical dependence (Kalso 2005). This provided the rationale for the development of mixed μ opioid agonist/NK1 antagonists. An attempt has been made to develop a chimeric peptide (AA501) with this profile (Bonney et al. 2004). However, this compound showed modest μ opioid receptor binding affinity and very weak NK1 receptor binding affinity. Furthermore, it was not demonstrated in vitro that AA501 has indeed the expected μ opioid agonist/NK1 antagonist profile. In the rat tail-flick test, this compound given i.th. did produce an analgesic effect; however, an $ED_{50}$ was not determined and a comparison with morphine in terms of antinociceptive potency was not provided. Upon repeated administration for five days AA501 produced analgesic tolerance. In another effort, octapeptides containing fused opioid peptide agonist and NK1 receptor antagonist sequences were prepared (Yamamoto et al. 2007). Two of these compounds showed good δ and μ agonist potencies in vitro with some selectivity for δ over μ opioid receptors. They displayed subnanomolar binding affinity for NK1 receptors and quite high antagonist activity against substance P in the GPI assay. Similarly, a C-terminally modified heptapeptide (H-Tyr-D-Ala-Gly-Phe-Pro-Leu-Trp-NH-Bzl), containing overlapping opioid agonist and substance P antagonist pharmacophore sequences, showed high δ and μ opioid agonist potencies in vitro with 15-fold δ vs. μ selectivity, as well as good antagonist activity against substance P in the GPI assay (Yamamoto et al. 2008). Analgesic activities of these bifunctional hepta- and octapeptides have not yet been reported and it remains to be seen whether they will be systemically active to produce a centrally mediated antinociceptive effect.

## Concluding remarks

Efforts to develop bi- or multifunctional non-peptide analgesics have met with limited success to date. The rational design of peptide drugs acting at two or more targets may often be easier than the development of corresponding nonpeptide drugs because peptides in general offer more latitude for structural modification than nonpeptide compounds. For example, it may be possible to attach a second pharmacophore moiety to several different sites of a peptide molecule, whereas this may not be possible with a relatively small nonpeptide drug without a drastic effect on potency in some cases. Connecting two pharmacophores directly or via a linker may not only affect the potency but also the efficacy of each component. This point is illustrated with the attachment of a δ antagonist component to the μ agonist [$Dmt^1$]DALDA, where δ antagonism was retained with C-terminal attachment, whereas linking to the $Lys^4$ side chain resulted in *δ agonism* (*vide supra*). It should be emphasized that a bifunctional construct containing an agonist and an antagonist component should always first be characterized in in vitro assays to verify that it does indeed have the desired agonist/antagonist profile. Bifunctional peptide drugs may have poor bioavailability because of their relatively large molecular weight, polarity and lack of stability. Stabilization against enzymatic degradation is often achieved by appropriate structural modifications. Furthermore, poor drug-like properties may be overcome if one of the peptide components has excellent ability to cross biological barriers such as the BBB and, thus, may confer satisfactory bioavailability on the entire bifunctional construct. This is illustrated with the mixed μ agonist/δ antagonist [$Dmt^1$] DALDA→$H_2CH_2NH$←TICP[Ψ] which produced a potent centrally mediated antinociceptive effect of long duration with s.c. administration. In future work to be performed in the author's laboratory the same approach will be used to prepare bifunctional compounds containing CB1-, NK1-, ORL1-, CCK- or other antagonists attached to various sites of the [$Dmt^1$]DALDA

peptide molecule in an effort to develop systemically active, centrally acting opioid analgesics with low propensity to produce tolerance and physical dependence.

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