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Ligands for the common allosteric site of acetylcholine M_2-receptors: development and application

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Abstract

Ligands for the allosteric site of acetylcholine M_2 receptors are able to retard the dissociation of simultaneously bound ligands for the orthosteric site. This effect promotes receptor occupation by the orthosteric ligand. The allosteric effect opens various therapeutic perspectives, e.g., in organophosphorus poisoning. The aim of our studies was to optimize the affinity of the modulators for the common allosteric binding site of muscarinic M_2 receptors, the orthosteric site of which was liganded with the *N*-methylscolopamine. The phthalimido substituted hexane-bisammonium compound W84 served as a starting point. Previous molecular modelling studies revealed two positive charges and two aromatic imides in a sandwich-like arrangement to be essential for a high allosteric potency. A three-dimensional quantitative structure activity relationship (3D QSAR) analysis predicted compounds with substituents of increasing size on the lateral imide moieties to enhance the affinity for the allosteric binding site. Thus, we synthesized and pharmacologically evaluated compounds bearing ''saturated'' phthalimide moieties as well as phthalimidines with substituents of systematically increasing size in position 3 or on the aromatic ring at one or both ends of the molecule. Within each series, QSAR could be derived: 1. ''Saturation'' of the aromatic ring of the phthalimide moiety results in less potent compounds. 2. Increasing the size of the substituents in position 3 of the phthalimide enhances the potency. 3. Putting substituents on the aromatic part of the phthalimide increases the potency more effectively: the introduction of a methyl group in position 5 gave a compound with a potency in the nanomolar concentration range which was subsequently developed as the first radioligand for the allosteric binding site.

Keywords: Allosteric site; Acetylcholine M_2 receptor; 3D QSAR analysis; N-methylscopolamine; W84; Organophosphorus poisoning; Allosteric modulation; Pain; Dementia

1. Introduction

Whereas the concept of allosteric modulation for enzymes (Koolman and Röhm, 1996) and for ion channel receptors is well-established, this phenomenon is hardly recognized in the case of G-protein coupled receptors. As a paradigm we consider the interaction at acetylcholine M_2 receptors between structurally heterogeneous allosteric modulators and orthosteric antagonists, such as *N*-methylscopolamine (NMS), (Christopoulos et al., 1998; Holzgrabe and Mohr, 1998) or agonists (Gharagozloo et al., 1999). The allosteric modulators can influence both the ligand association and dissociation resulting either in a reduction or in an elevation of ligand equilibrium binding. In combination with antagonists such as atropine, the therapy of organophosphorus poisoning can take advantage

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of the retardation of the dissociation. The allosteric elevation of endogenous acetylcholine binding (Birdsall et al., 1999) might be beneficial in the treatment of pain and dementia.

The goal of the investigations, presented here, was to optimize the affinity of the modulators for the common allosteric binding site of muscarinic M_2 receptors, the orthosteric site of which was liganded with the antagonist *N*-methylscolopamine. The phthalimido substituted alkane-bisammonium compound W84 (Fig. 1) was taken as a starting point.

Previous molecular modelling studies (Holzgrabe et al., 1996) revealed two positively charged nitrogens and two aromatic imides in a sandwich-like arrangement to be essential for a high allosteric potency (see Fig. 2). An additionally performed three-dimensional quantitative structure activity relationship (3D QSAR) analysis (Holzgrabe and Hopfinger, 1996) predicted compounds with substituents of increasing size on the lateral imide moieties to enhance the affinity for the allosteric binding site. This pharmacophore hypothesis was supported by the result of a study in which the length of the spacers between the pharmacophoric elements was varied (Nassif-Makki et al., 1999). The distance between the positive charges was determined to be at least 10 Å, which is equivalent to about seven methylene groups; the spacer between the positive charge and the lateral imide moiety has to be long enough to allow the phthalimides to properly reach the aromatic positions. In the case of the W84 compounds a length of three methylene groups is necessary.

Taking the findings of the theoretical studies into account, three projects were initiated:

(1) In order to check whether aromatic imides in lateral positions are necessary for a high allosteric potency the phthalimide moieties were replaced with corresponding ''saturated'' imides and, additionally, the hexahydrophthalimide was stepwise reduced to a succimide ring.

(2) Considering the QSAR analysis the allosteric potency should increase with increasing steric size of the substituent on the phthalimide. Thus, various substituents were introduced in position 3 and 5 of the phthalimide.

(3) Since it is unlikely that the allosteric binding site is symmetrical the structural variations mentioned above were performed at one end and at both ends of the symmetrical parent compound W84.

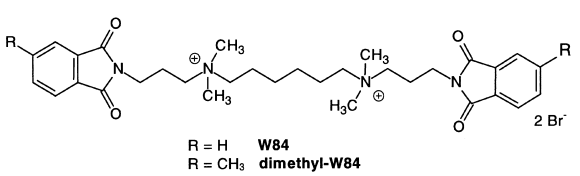

Fig. 1. Structural formula of the parent compound W84 and dimethyl-W84.

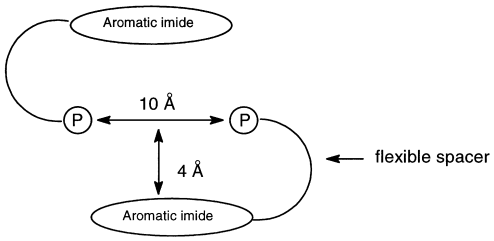

Fig. 2. Pharmacophore model.

In each project it was tried to built series of systematically varied compounds which can be used to establish QSAR analyses.

2. Common allosteric site

QSAR analyses of series of compounds are only valid when all compounds bind in the same mode to the same binding site. Generally, the allosteric action is evaluated as the quantification of an effect; the $EC_{50,diss}$ value is the concentration of the modulator at which the rate of radioligand dissociation is reduced by 50% (Jepsen et al., 1988). However, experiments to antagonize allosteric effects revealed that structurally closely related allosteric agents may nevertheless reveal a divergent mode of allosteric action (Tränkle and Mohr, 1997). In order to gain a more direct insight into the binding events, a high affinity radioligand for the allosteric binding site was developed. Introduction of a methyl group in position 5 of the phthalimide ring (Tränkle et al., 1998) yielded a compound, designated as dimethyl-W84 (see Fig. 1), with particularly high allosteric potency (for comparison with other compounds see Tränkle et al., 1996). Tritiation of dimethyl-W84 gave the first radioalloster suitable for direct binding measurements at the allosteric site (Tränkle et al., 1998). Competition binding experiments with the prototype modulators alcuronium, W84 and gallamine went parallel with the inhibition of the [^{3}H]NMS dissociation which indicated that the high affinity binding of [^{3}H]dimethyl-W84 reflects the occupation of the common allosteric site of the M_2 receptor.

3. Replacement of the phthalimide with corresponding ''saturated'' moieties

In previous studies, the unilateral replacement of the phthalimide with a morpholino or hydroxy substituents (Holzgrabe et al., 1997) or shortening of the lead compound W84 down to monoquaternary compounds (Kostenis

et al., 1994) resulted in a considerable reduction of the allosteric potency indicating the importance of the lateral aromatic rings. However, the question remains whether an aromatic annelation of the imide is necessary or whether a hydrophobic moiety is sufficient for the interaction with the receptor protein. Thus, a cis and *trans*-annelated cyclohexane and a *trans*-cyclohexene imide compound **1**–**3** in addition to a dimethyl- (**4**), a monomethyl- (**5**) and an unsubstituted (**6**) succimide derivative were synthesized by conversion of the corresponding 1,2-dicarbonic acids to the anhydrides, condensation of the N',N'-dimethylaminopropanamine with the anhydrides giving the imides and connecting two imide units with dibromoheptane (see Fig. 3). The allosteric potencies of these new compounds were compared to the $C_7/3'$-phth compound (Choo and Mitchelson, 1989) which was previously reported to have a higher potency than the corresponding hexane compound (Tränkle et al., 1996) (EC_{50} value of 390 nM vs. 1300 nM). In comparison to $C_7/3'$-phth all concentration–effect-curves except with the cyclohexene derivative compound **3** were shifted in a parallel fashion to the right indicating the same mode of action but a lower potency (see Fig. 3). Interestingly, the decrease in potency resembles the diminution of the size of the lateral moiety in going from the saturated cyclohexane-1,2-dicarbonic acid imide through the dimethyl- and monomethyl- to the non-substituted succimide.

A positive correlation between the allosteric potency and the lipophilicity of the lateral moiety was found suggesting that with increasing size of the hydrophobic imides the contact area with the receptor protein is enlarged.

$$\log(1/EC_{50}) = 1.412(\pm 0.61)\log P + 4.195(\pm 0.57)$$

$$n = 6;\ r^2 = 0.91;\ s = 0.33;\ F = 40.1;\ Q^2 = 0.83;$$

$$s_{PRESS} = 0.452 \qquad (1)$$

where n is the number of compounds, r^2 is the square of the correlation coefficient, s the standard deviation, F the ratio of explained to unexplained variance, Q^2 is the cross validated r^2 by using the leave-one-out procedure (Q^2 can adopt values between 1 and zero) and s_{PRESS} is the standard deviation from the predictive residual sum of squares. Since the cyclohexene derivative shows a steeper concentration–effect-curve, the data of this compound were omitted.

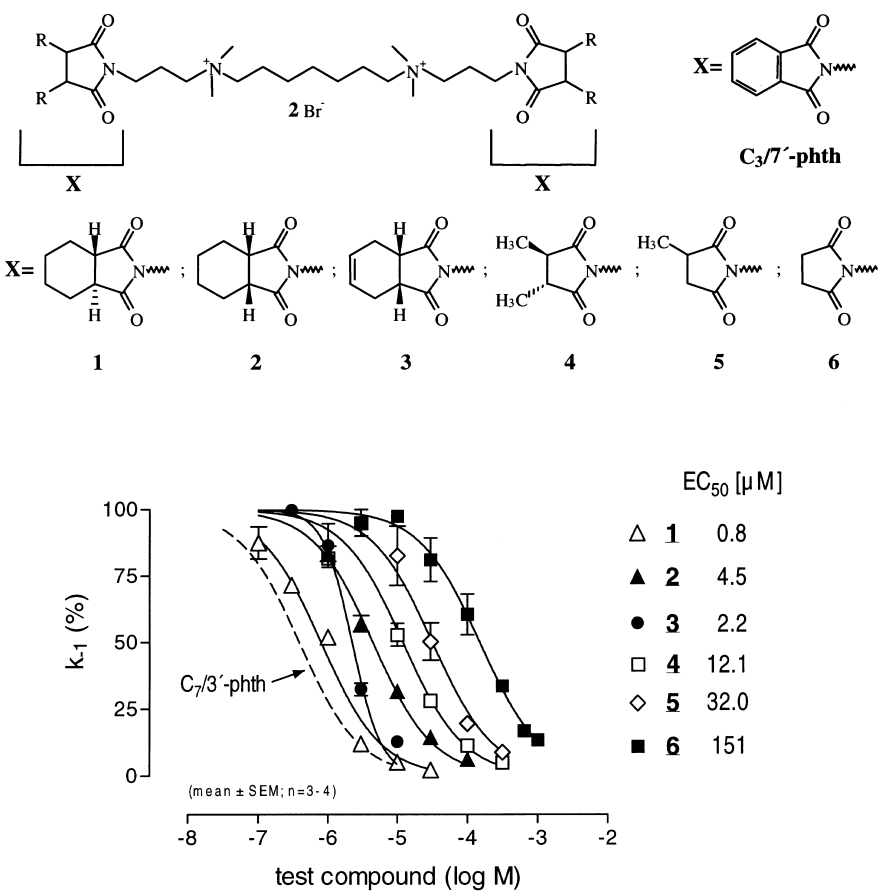

Fig. 3. Structural formula of the ''saturated'' imides and concentration–effect-curves for reducing the apparent rate constant of [^{3}H]NMS dissociation from M_2 receptors in porcine heart membranes.

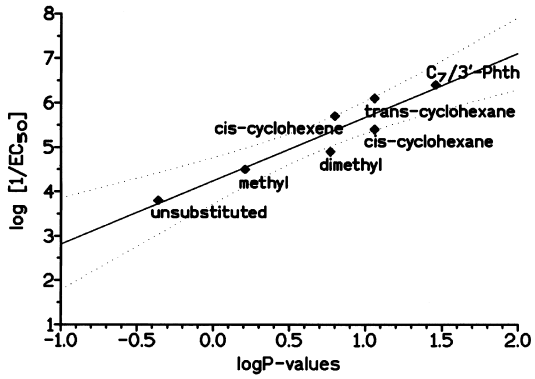

Fig. 4. Correlation between the allosteric potency of C_3/7′-phth and **1**, **2**, **4**–**6** and the lipophilicity.

Even though the correlation is found to be highly significant, the difference in potency between the *trans*- and *cis*-diastereomers of the cyclohexane compound cannot be explained by the calculated lipophilicity (see Fig. 4). Since the trans isomer of the cyclohexane-imide sterically resembles the phthalimide more than the cis isomer, the higher potency of the *trans*-isomer can be easily understood. However, even though no significant correlation of the potency with steric parameters was found within this series of compounds, this stereochemical finding is a hint that the phthalimide binds into a pocket at the entrance of the receptor which has steric restrictions. Thus, in the next step, it was aimed to probe the size of the hypothesized binding pocket.

4. Symmetrical substituent variations at the phthalimide skeleton

Since the preceding study had revealed a cycloalkane moiety to be disadvantageous as a lateral system, in the following the aromatic phthalimide skeleton was kept the same whilst one carbonyl functional was replaced with hydrogens (**7**), as well as hydroxy (**8**) and alkoxy groups (**9**) and with phenyl (**10**) and benzyl (**11**) and benzylidene rings (**12**) (see Fig. 5). Whereas the hydroxy and alkoxy derivatives can be easily obtained by reduction of the carbonyl group with $NaBH_4$ and conversion with the corresponding alcohol in acidic media, other synthesis pathways had to be developed for the other compounds. The synthesis of the phenyl substituted compound **10** started off with the reduction of phenylphthalazone with Zn/HCl to give the phthalimidine which was alkylated

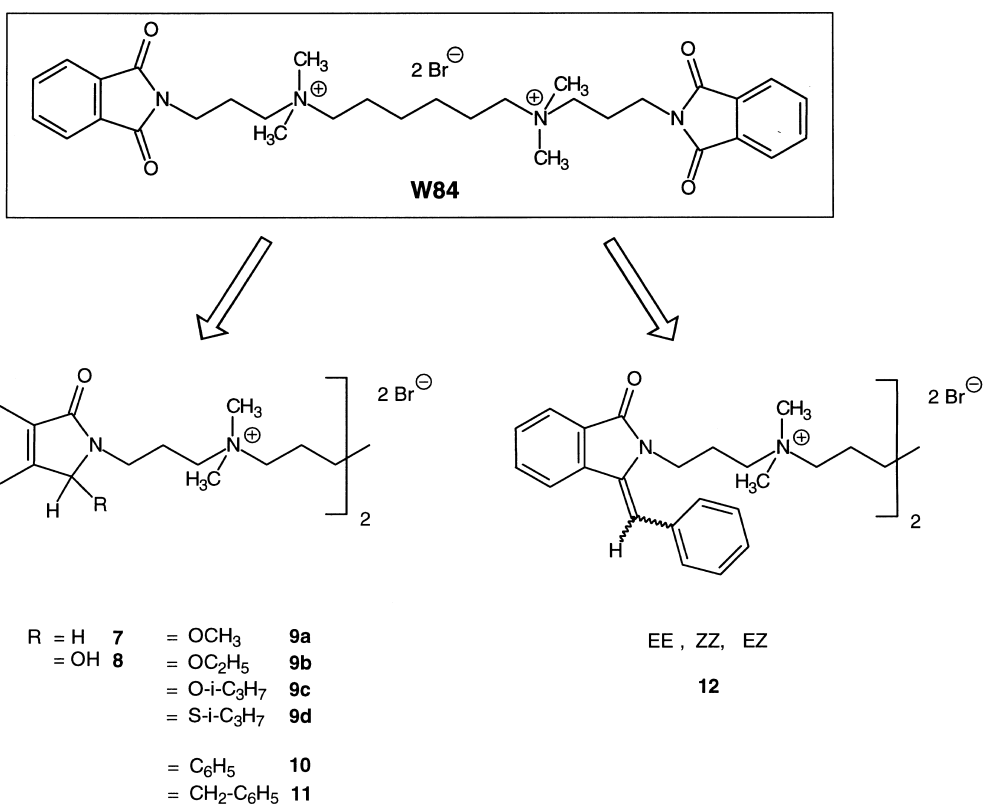

Fig. 5. Structural formula of the symmetrical phthalimido substituted allosteric modulators **7**–**12**.

with dibromoalkane in presence of NaH. Two of these molecules were connected with 1,6-bis(dimethylamino)hexane to obtain the bisammonium salt. The benzyl and benzylidene compounds **11** and **12**, resp., were achieved via the benzalphthalide which was converted to the imidine; this imidine can be either reduced to give the benzyl product **11** or the obtained E/Z isomers separated for the preparation of the benzylidene bisammonium salts **12** (Botero Cid et al., 1999).

The parallel concentration–effect-curves for these compounds exhibit an allosteric potency of the same order of magnitude as the parent compound W84. Whereas most of the alkoxy substituted compounds **9** are less potent than W84, the benzyl and phenyl substituted ones **10** and **11** show a slightly higher potency; interestingly, all benzylidene isomers **12** are more than one order of magnitude more potent than W84. Again, the correlation between the potency and the lipophilicity (π) was checked, but the regression analysis results in a poor correlation. The benzylidene derivatives turned out to be outliers. Omitting these compounds gave a significant, but still poor positive correlation:

$$\log(1(\mathrm{EC}_{50}) = 0.304(\pm 0.15)\pi + 5.464(\pm 0.19)$$

$$n = 8;\ r^2 = 0.80;\ s = 0.167;\ F = 23.4;\ Q^2 = 0.67;$$

$$s_{\mathrm{PRESS}} = 0.21 \qquad (2)$$

In addition, steric parameters, such as volume, surface and refractivity calculated by means of Hyperchem/Chemplus, were checked. Using a parabolic model a poor correlation with the volume was found wherein the benzyl compound was an outlier. Omitting this compound resulted in a striking correlation (Fig. 6):

$$\log(1/\mathrm{EC}_{50}) = +0.0000867(\pm 0.000026)\mathrm{vol}^2 - 0.0977(\pm 0.031)\mathrm{vol} + 32.76(\pm 9.26)$$

$$n = 11;\ r^2 = 0.95;\ s = 0.18;\ F = 80.4;\ Q^2 = 0.91;$$

$$s_{\mathrm{PRESS}} = 0.24 \qquad (3)$$

From this analysis the minimum potency was calculated to be related with a volumes in a range between 540 and 575 Å^3, which corresponds to the methoxy group. Groups of smaller and bigger size induced a higher potency. The increase in potency going along with the diminution of the substituent size might be caused by a different sort of interaction between this part of the phthalimide (carbonyl and hydroxy groups versus alkoxy groups) and the receptor protein. The increase in potency connected with the expansion of the alkane ether chain goes along with the elevation of the intermolecular hydrophobic contact surface with the receptor protein (Böhm and Klebe, 1996) and, therefore, with the enhancement of the affinity of the compounds to the receptor binding site. Interestingly, the benzyl substituted compound is one order of magnitude less potent than the benzylidene compounds although the volume of both substituents is almost the same. The striking difference between these compounds is the difference in rigidity caused by the double bond. In the case of the benzylidene compounds the smaller loss of entropy on binding to the receptor protein (Andrews, 1993) might be the reason for the high potency. Remarkably, the stereochemistry of the benzylidene groups attached to the phthalimide skeleton, EE, ZZ and EZ, does not influence the potency. Since the alkane bisammonium compounds were found to be highly flexible (Holzgrabe and Hopfinger, 1996), it is tempting to speculate that either the aromatic ring of the phthalimide or of the benzylidene ring can serve as the partner for interaction with the binding site. Molecular modelling investigations supported this hypothesis (Botero Cid et al., 1999).

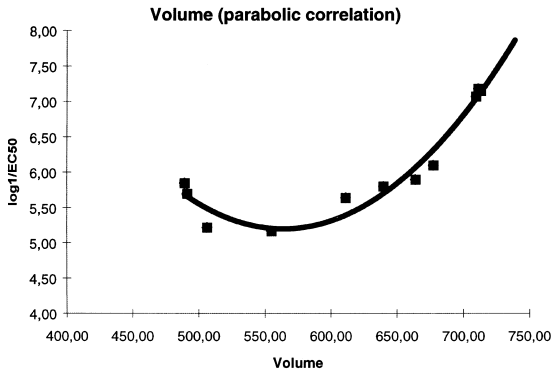

Fig. 6. Correlation between the allosteric potency and the volume of the lateral substituents of the compounds **7**–**10** and **12**.

Summarizing this part of the study: since no steric restriction and no stereoselectivity concerning the substituent in position 3 could be found the binding pocket is supposed to be rather large.

5. Asymmetrical substituent variations at the phthalimide skeleton

Due to the simplicity of the synthesis most of the newly synthesized allosteric modulators were symmetrical. However, the binding site of the modulators is unlikely to be symmetrical. In order to check whether both ends of the modulators have a different contribution to the potency we aimed to synthesize asymmetrical compounds. Due to the results of the 3D QSAR analysis is was predicted that enlargement of one phthalimide moiety should increase the potency even with the contralateral phthalimide kept unchanged. On the one hand the synthesis started off with the alkylation of dimethylaminopropylphthalimide with a huge excess of dibromohexane without any solvent in order to

avoid the bisamination of dibromohexane. On the other hand, a corresponding 1,2-dicarbonic acid was converted via the anhydride to the dimethylaminopropylimide which can be connected to the above obtained bromo-compound to give the asymmetrical compounds **13** (Fig. 7).

With exception of the ''pyridophthalimide'' all aromatic compounds were found to have a higher potency than the parent compound W84. Especially the naphthalimide compound shows a very high potency. In order to quantify the potency, it was checked whether a correlation between the potency and lipophilic or steric parameters could be found. Again, the correlation with the lipophilicity was poor in comparison with the volume of the lateral substitutent (Bender et al., 1999) (Fig. 8).

$$\log(1/EC_{50}) = -0.000089(\pm 0.000036)\mathrm{vol}^2 + 0.108(\pm 0.041)\mathrm{vol} - 25.85(\pm 12.2)$$

$$n = 8;\ r^2 = 0.93;\ s = 0.151;\ F = 41.6;\ Q^2 = 0.898;\ s_{PRESS} = 0.199 \quad (4)$$

In contrast to Eq. (3), the allosteric potency is running through an maximum volume which amounts to 600 to 650 Å^3. These findings clearly indicate that the hypothesised binding area has a defined shape: On the one hand, there is obviously much space for hydrophobic substituents at position 3 whereas on the other hand there is pocket which can perfectly take in and interact with the naphthalimide (or phenothiazine moiety (Holzgrabe et al., 1997)). The interactions with smaller and bigger substituents are less strong causing a lower potency. At this point, the questions still remains, whether the allosteric modulators have to be symmetrical or asymmetrical. Therefore, pairs of corresponding symmetrical and asymmetrical phthalimides will be compared in Section 6.

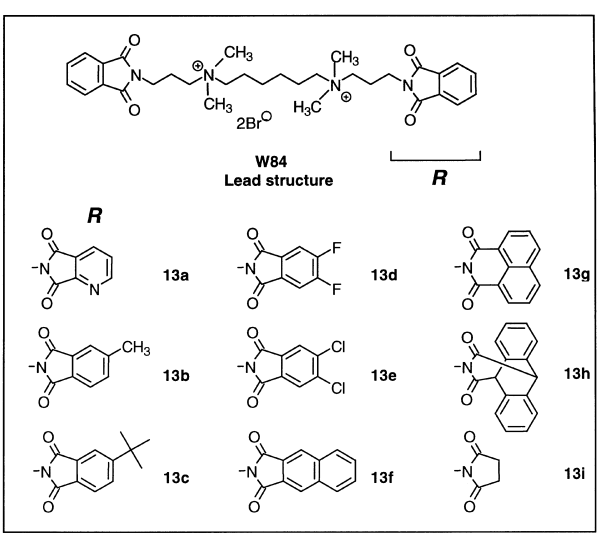

Fig. 7. Structural formulae of the asymmetrically substituted phthalimide compounds **13**.

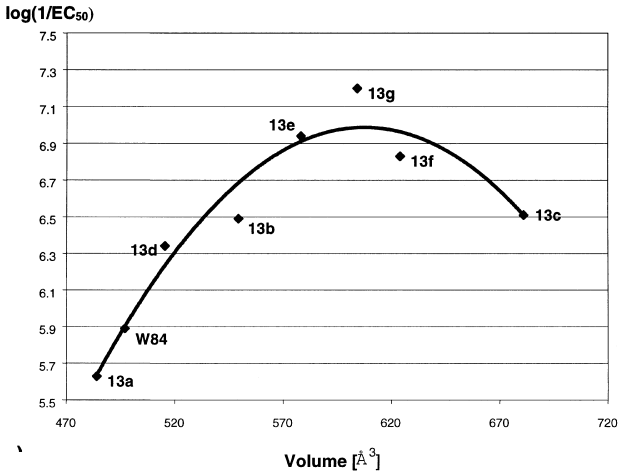

Fig. 8. Correlation between the allosteric potency and the volume of the lateral substituents of the compounds **13**.

6. Symmetrical versus asymmetrical substituent variations

6.1. Comparison of the differences in potency upon symmetrical and asymmetrical variations of lateral moieties

The diminution of one phthalimide to a succimide resulted in the series of W84 compounds in a loss of potency of $\Delta\log(1/EC_{50}) = 0.88$ (Bender et al., 1999). Replacement of both phthalimides with the succimide in the corresponding heptane series induced about twice the loss of potency: $\Delta\log(1/EC_{50}) = 2.54$. This clearly indicates that the phthalimide moiety is important in both places.

6.2. Stepwise replacement of the phthalimide with analogue imides

Preliminary investigations showed that the replacement of one phthalimide in W84 with the benzylidene phthalimidine moiety results in an increase in potency in comparison to W84 which could be further enhanced by the replacement of the second phthalimide with benzylidene phthalimidine moiety (Holzgrabe et al., 1997). Similar observations were made for the stepwise replacement of the phthalimide with the above discussed optimal naphthalimide substituent. The symmetrical compound appears to be about 100 times more potent than W84 and seems to be the most active compound reported till now. It is interesting to find out in the next step whether an asymmetrical compound composed of an hexane-bisammonium-chain connecting a benzylidene phthalimidine and a naphthalim-

ide will show a higher or lower potency. Investigations in this direction are in progress.

7. Conclusions

Taken together, it can be concluded that the 3D QSAR analysis, which predicted higher affinity to the allosteric binding site by enlargement of the non-overlap volume of the modulators, has led to highly potent compounds characterized by a rigid hydrophobic moiety in position 3 of the phthalimide and a large aromatic area annellated to the imide. These findings initiate the synthesis of new modulators of higher allosteric potency and may open the perspective to find compounds suitable for therapeutic purposes.

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References

Andrews, P.R., 1993. Drug-receptor interactions. In: Kubinyi, H. (Ed.), 3D QSAR in Drug Design — Theory Methods and Applications. ESCOM, Leiden, pp. 13–40.

Bender, W., Staudt, M., Tränkle, C., Mohr, K., Holzgrabe, U., 1999. Probing the size of a hydrophobic binding pocket within the allosteric site of muscarinic acetlylcholine M_2-receptors. Life Science, in press.

Birdsall, N.J.M., Farries, T., Gharagozloo, P., Kobayashi, S., Lazareno, S., Sugimoto, M., 1999. Subtype-selective positive cooperative interactions between brucine analogs and acetylcholine at muscarinic receptors: functional studies. Mol. Pharmacol. 4, 778–787.

Böhm, H.-J., Klebe, G., 1996. Was läßt sich aus der molekularen Erkennung in Protein–Ligand-Komplexen für das Drug-Design neuer Wirkstoffe lernen?. Angew. Chem. Int. Ed. Engl. 35, 2588–2614.

Botero Cid, H.M., Pick, R., Holzgrabe, U., Mies-Klomfaß, E., Kostenis, E., Mohr, K., 1999. Structure-activity relationships in a series of bisquaternary phthalimidine derivatives modulating the muscarinic M_2-receptor allosterically. J. Med. Chem., submitted.

Choo, L.K., Mitchelson, F., 1989. Characterization of the antimuscarinic effect of heptane-1,7-bis(dimethyl-3′-phthalimidopropyl ammonium bromide. Eur. J. Pharmacol. 162, 429–435.

Christopoulos, A., Lanzafame, A., Mitchelson, F., 1998. Allosteric interactions of muscarinic cholinoceptos. Clin. Exp. Pharmacol. Physiol. 25, 185–194.

Gharagozloo, P., Lazareno, S., Popham, A., Birdsall, N.J., 1999. Allosteric interactions of quaternary strychnine and brucine derivatives with muscarinic acetylcholine receptors. J. Med. Chem. 42, 438–445.

Holzgrabe, U., Hopfinger, A.J., 1996. Conformational analysis, molecular shape comparison and pharmacophore identification of different allosteric modulators of muscarinic receptors. J. Chem. Inf. Comp. Sci. 36, 1018–1024.

Holzgrabe, U., Mohr, K., 1998. Allosteric modulation of antagonist binding to muscarinic receptors. Drug Discovery Today 3, 214–222.

Holzgrabe, U., Wagener, M., Gasteiger, J., 1996. Variation of the oxime function in bispyridinium-type allosteric modulators of M_2-cholinoceptors. J. Mol. Graph. 14, 185–193.

Holzgrabe, U., Staudt, M., Bender, W., Mesagna, P., Pfletschinger, C., Tränkle, C., Mohr, K., 1997. Allosteric modulation of antagonist binding to the acetylcholine M_2-receptor: unilateral structural variations of alkane bisammonium-type modulators. Abstract DPhG and SGPhW Symposium, Zürich.

Jepsen, K., Lüllmann, H., Mohr, K., Pfeffer, J., 1988. Allosteric stabilization of ^{3}H-*N*-methylscopolamine binding in guinea-pig myocardium by an antidote against organophosphate intoxication. Pharmacol. Toxicol. 63, 163–168.

Koolman, J., Röhm, K.-H., 1996. Color Atlas of Biochemistry. Thieme Verlag, Stuttgart, pp. 107–108.

Kostenis, E., Holzgrabe, U., Mohr, K., 1994. Allosteric effect on M_2-cholinoceptors of derivatives of the alkane-bis-ammonium compound W84 — comparison with bispyridinium-type allosteric modulators. Eur. J. Med. Chem. 29, 947–953.

Nassif-Makki, T., Tränkle, C., Bejeuhr, G., Cambareri, A., Pfletschinger, C., Kostenis, E., Mohr, K., Holzgrabe, U., 1999. Bisquaternary ligands of the common allosteric site of M_2 acetylcholine receptors: optimization of the distances between the pharmacophoric elements. J. Med. Chem. 42, 849–858.

Tränkle, C., Mohr, K., 1997. Divergent modes of action among cationic allosteric modulators of muscarinic M_2 receptors. Mol. Pharmacol. 51, 674–682.

Tränkle, C., Kostenis, E., Burgmer, U., Mohr, K., 1996. Search for lead structures to develop new allosteric modulators of muscarinic receptors. J. Pharmacol. Exp. Ther. 279, 926–933.

Tränkle, C., Mies-Klomfaß, E., Botero Cid, M.H., Holzgrabe, U., Mohr, K., 1998. Identification of a [^{3}H]ligand for the common allosteric site of muscarinic acetylcholine M_2 receptors. Mol. Pharmacol. 54, 139–145.