Anthracene Based Compounds as New L-type Ca^{2+} Channel Blockers: Design, Synthesis, and Full Biological Profile

Sergio Bova,[†] Simona Saponara,[‡] Angela Rampa,[§] Silvia Gobbi,[§] Lorenzo Cima,[†] Fabio Fusi,[‡] Giampietro Sgaragli,[‡] Maurizio Cavalli,[†] Cristobal de los Rios,[||] Jörg Striessnig,[⊥] and Alessandra Bisi*[,§]

Department of Pharmacology and Anaesthesiology, University of Padova, Italy, Department of Neurosciences, University of Siena, Italy, Department of Pharmaceutical Sciences, University of Bologna, Italy, Department of Pharmacology, School of Medicine, University Autonoma of Madrid, Spain, and Department of Pharmacology and Toxicology, Institute of Pharmacy, Center of Molecular Biosciences, University of Innsbruck, Austria

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Abstract: L-Type Ca^{2+} channels (LTCCs) play a key role in the regulation of vascular smooth muscle contraction, and substances that interfere with their function (Ca^{2+} channel blockers) are widely used in the therapy of hypertension. Here, we report anthracene−maleimide derivatives as new LTCC blockers. Among these, **3**, lacking intracellular effects, was investigated in more detail. The results show that **3** binds preferentially to inactivated LTCCs, directly interacting with the pore-forming subunit of the channel.

L-Type Ca^{2+} channels (LTCCs[a]) are important regulators of Ca^{2+} influx in a number of cell types. Ca^{2+} entry through these channels activates a plethora of intracellular events, from the broad stimulation of gene expression and Ca^{2+}-dependent second messenger cascades to the specific release of neurotransmitters within the nervous system and contraction in smooth and cardiac muscle. The development of drugs that block Ca^{2+} channels has provided a valuable route for studying channel structure and function and a very successful group of therapeutic agents targeted at cardiovascular disorders, i.e. angina, hypertension, and arrhythmias.[1]

We have previously characterized the complex vasoconstrictor and vasorelaxant effects of norbormide, a rat-specific toxin.[2−5] Its species-specific vasoconstriction is responsible for the lethal effect in rats, and it is likely mediated by the interaction of the drug with a phospholipase C coupled receptor selectively expressed in rat vascular myocytes, such as those of the rat caudal artery;[3−5] in contrast, vasorelaxation is not species-specific and has been shown to be mediated by inhibition of LTCCs.[6,7]

Considering the complex structure of norbormide and with the aim of identifying its potential pharmacophoric fragments directed toward the Ca^{2+} antagonist effect, a series of rigid anthracenic derivatives was designed, synthesized, and tested in rat vascular smooth muscle (Chart 1). The new compounds **1**−**10**, whose structures are reported in Table 1, can be considered as simplified analogues in which the cyclic amidic function has been retained and the two aromatic rings could mimic two of the rings of norbormide. Moreover, the complex stereochemistry of the lead molecule, which is a mixture of eight isomers, was also simplified. To assess the role of the nitrogen, an important feature in several Ca^{2+} channel blocking agents, the N-alkylated derivative **11**, was also synthesized.

Chart 1. Design of Compounds **1**−**10**

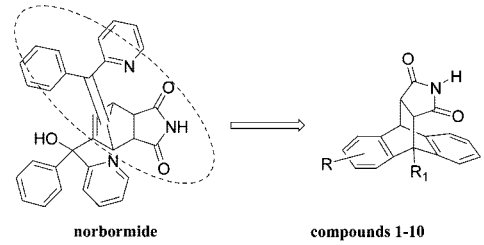
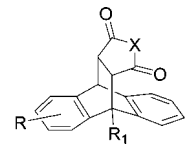
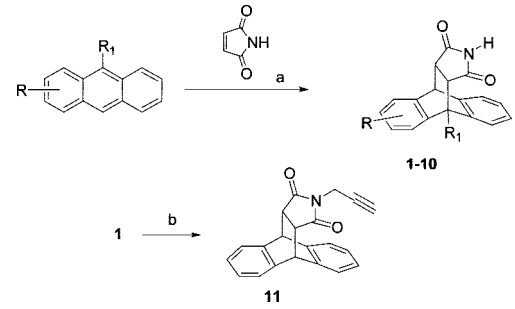


Table 1. Structures of the Studied Compounds



compd	R	R_1	X
1	H	H	NH
2	4-CH_3	H	NH
3	4-Cl	H	NH
4	3-Cl	H	NH
5	H	CHO	NH
6	H	CH_2OH	NH
7	H	Cl	NH
8	H	CH_3	NH
9	H	NO_2	NH
10	H	CN	NH
11	H	H	NCH_2—≡

Scheme 1. Synthesis of the Studied Compounds[a]



[a] Reagents and conditions: (a) toluene or xylene, reflux, 4−16 h; (b) potassium *tert*-butoxide, propargyl bromide, DMSO, room temp.

According to Scheme 1, **1**−**11** were prepared via Diels−Alder cycloaddition, starting from commercially available substituted anthracenes and maleimide. Compound **11** was obtained by alkylation of **1** with propargyl bromide.

The blood vessel activity of the newly synthesized compounds was studied using a classic pharmacological approach. As we expected, all the new compounds were devoid of contracting activity in rat caudal artery rings, even at the maximum concentration used in this study (50 μM; data not shown). In contrast, all the derivatives led to a reduction of the high KCl-

* To whom correspondence should be addressed. Phone: +39 0512099710. Fax: +39 0512099734. E-mail: alessandra.bisi@unibo.it.
[†] University of Padova.
[‡] University of Siena.
[§] University of Bologna.
[||] University Autonoma of Madrid.
[⊥] University of Innsbruck.
[a] Abbreviations: Phe, phenylefrine; LTCC, L-type Ca^{2+} channels.

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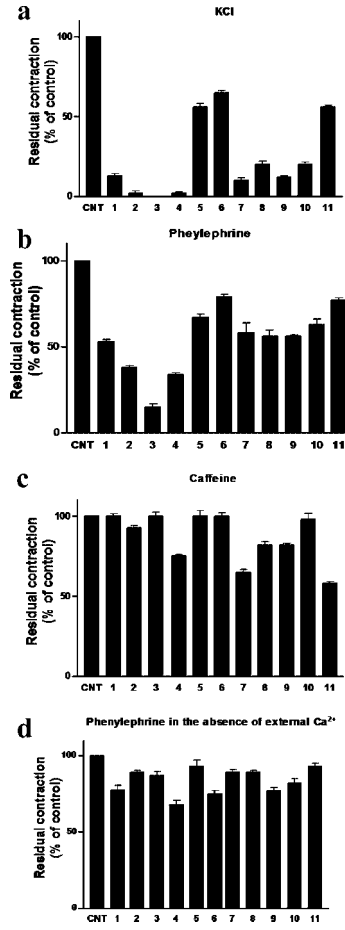

Figure 1. Relaxing effect of **1**−**11** (50 μM) on rat caudal artery rings contracted with 10 μM Phe, 90 mM KCl, 20 mM caffeine, or 10 μM Phe in Ca^{2+}-free PSS1. Data points are mean ± SEM ($n = 8$).

induced contraction, most of them by more than 78%. In particular, **2**−**4**, bearing a R substituent (see Chart 1), were able to reduce contractile activity by more than 90%, **3** (4-Cl) being the most active of the series (100%, Figure 1a). On the other hand, the introduction of a substituent in position 9 (R_1), appears not to be crucial, being the unsubstituted compound **1**, comparable with **7** (9-Cl). The N-alkylation (**11**) greatly reduced the activity.

Several studies indicate that LTCCs are also activated during agonist-induced contraction of vascular smooth muscle,[8−10] although the majority of the contractile response appears to be mediated by other mechanisms, such as Ca^{2+} release from the sarcoplasmic reticulum and Ca^{2+} entrance through non-LTCCs.[11] For this reason, agonist-induced contractions are less sensitive to LTCC blockers, compared to high KCl-induced one.[12] Accordingly, when phenylefrine (Phe), an α_1 adrenergic agonist, was used to contract vessels, the maximal inhibitory effect induced by **1**−**11** was lower compared to that observed with KCl (Figure 1b). Non-LTCC targets, e.g., reduction of Ca^{2+} release from the sarcoplasmic reticulum and/or reduced sensitivity of the contractile elements to Ca^{2+}, have been described for several Ca^{2+} channel blockers[13,14] and may contribute to their relaxing effect. This possibility was ruled out for the compounds that showed an overall marked reduction of their relaxant effect on both caffeine- (Figure 1c) and Phe-induced contraction in Ca^{2+} free solution (Figure 1d). Under these experimental conditions, in fact, the contractile response is due to the release of Ca^{2+} from the sarcoplasmic reticulum and therefore it cannot be modified by drugs that only reduce Ca^{2+} entrance. This

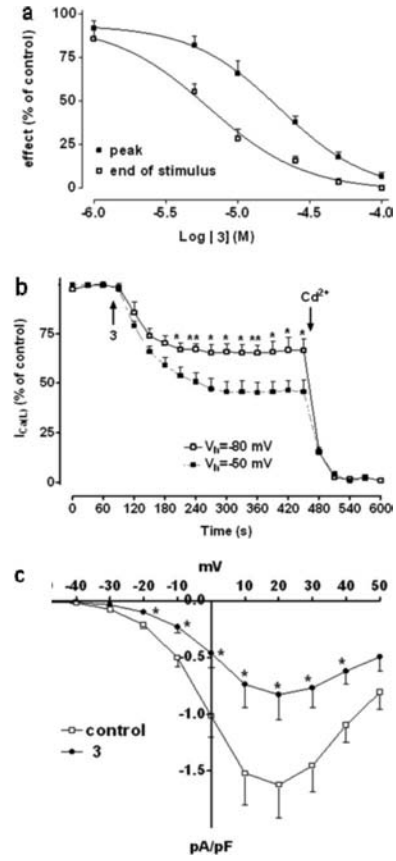

Figure 2. Inhibition by **3** of $I_{Ca(L)}$ in rat caudal artery myocytes: (a) concentration-dependent effect of **3** measured at the peak or at the end of $I_{Ca(L)}$ trace; (b) time course of $I_{Ca(L)}$ inhibition induced by 15 μM **3** at −50 or −80 mV; (c) current−voltage relationships constructed prior to the addition of **3** (control) and in the presence of 15 μM **3** at V_h of −50 mV. Data points are mean ± SEM [$n = 3$−4 (a), 8 (b), and 7 (c)]: (*) $P < 0.05$, (**) $P < 0.01$, Student's t test for unpaired or paired samples.

finding further supports the hypothesis that the compounds act preferentially at the plasmalemma level by blocking LTCCs. Noteworthy, in Ca^{2+} free solution neither caffeine- nor Phe-induced contraction was affected by **3**, the most active of the series on KCl-induced response. Therefore, **3** was selected and deeply investigated by means of patch-clamp analysis and binding assays to provide direct evidence of its LTCC blocking properties.

Compound **3** inhibited peak L-type Ca^{2+} current [$I_{Ca(L)}$] in a concentration-dependent manner with an IC_{50} of 16.5 ± 2.8 μM (Figure 2a). At the maximum concentration tested (100 μM), **3** inhibited peak $I_{Ca(L)}$ by about 93%. An even higher fraction of $I_{Ca(L)}$ was inhibited at the end of the depolarizing pulse, thereby significantly shifting the inhibition curve to lower concentrations (Figure 2a; IC_{50} of 5.5 ± 0.9 μM). A characteristic of **3**-induced inhibition of $I_{Ca(L)}$ was a noteworthy tonic effect, which developed independently of channel activation.[15] Indeed, at 0.033 Hz, a frequency allowing full recovery of LTCCs from inactivation between pulses in rat caudal artery myocytes,[16] the amplitude of $I_{Ca(L)}$ was decreased by **3** in a time-dependent manner. This suggested that the compound efficiently blocked the resting channel state. The block induced by **3** was clearly voltage-dependent and was significantly more pronounced at −50 mV than at −80 mV, as evident from the normalized curves illustrated in Figure 2b. Voltage-dependent block is typical of dihydropyridines and phenylalkylamines[17] and indicates that **3** binds preferentially to inactivated channels.

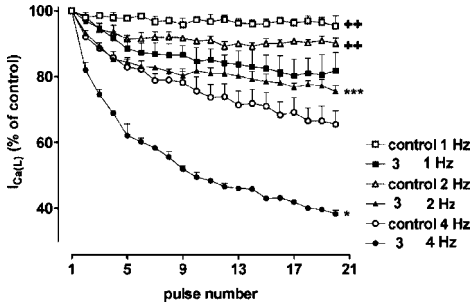

Figure 3. Frequency-dependent block of $I_{Ca(L)}$ by 15 μM **3**. Data points are mean ± SEM ($n = 3$): (*) $P < 0.05$, (***) $P < 0.001$ vs the 20th pulse recorded in the corresponding control; (++) $P < 0.01$ vs the 20th pulse recorded in control condition at 4 Hz; Student's t test for paired samples or ANOVA/Bonferroni post test, respectively.

Under control conditions, the kinetics of both current activation and inactivation showed time courses that could be fitted by monoexponential equations, with a τ of activation of 2.93 ± 0.17 ms ($n = 5$) at V_h of −50 mV or with 1.81 ± 0.19 ms ($n = 8$) at V_h of −90 mV and a τ of inactivation of 87.94 ± 7.86 or 82.46 ± 4.43 ms, respectively. In the presence of 15 μM **3**, activation remained unaffected (data not shown), whereas inactivation was markedly accelerated and significantly better fitted ($P < 0.05$) by two rather than by one exponential function (22.42 ± 0.97 and 68.16 ± 5.07 ms at V_h of −50 mV, and 24.52 ± 1.39 and 114.95 ± 14.57 ms at V_h of −80 mV, respectively), likely indicating that the compound unmasks a second inactivation state of the channel. This phenomenon might also reflect a progressive increase in channel block, during the depolarizing step, due to the voltage-dependency of the **3**-induced effect. Decrease in the rate constant of inactivation has also been reported for other voltage-dependent Ca^{2+} channel blockers, such as dihydropyridines and phenylalkylamines.[18] The current−voltage relationship recorded at V_h of −50 mV (Figure 2c) showed that **3** inhibited significantly the peak inward current at all potentials tested in the range −20 to 40 mV, without modifying the threshold or shifting the maximum of the relationship.

To test the possibility of a frequency-dependence of the action of **3**, we studied its inhibitory effect during 20 depolarizing pulses applied at stimulation frequencies of 1, 2, and 4 Hz. In control conditions recorded in the absence of **3**, the peak amplitude of $I_{Ca(L)}$ evoked by the 20th pulse decreased significantly as the frequency of stimulation increased, thus suggesting an accumulation of the channel in the inactivated state (Figure 3). Compound **3** produced a frequency-dependent block of $I_{Ca(L)}$; this was significantly lower than that observed under the corresponding control condition at 2 and 4 Hz. Frequency-dependent block of $I_{Ca(L)}$ is generally observed with drugs possessing a high affinity for the open and/or inactivated channel. Such state-dependent action can also explain the higher extent of block observed at the end of a depolarizing pulse compared to the inhibition of peak current (Figure 3b).

Drugs possessing Ca^{2+} channel blocking activity can act directly at the channel protein or exert their effect via diffusible intracellular components. The electrophysiological data obtained with the conventional whole-cell patch-clamp method indirectly support the former mechanism, since the cell cytoplasm underwent extensive dialysis, subsequently removing any cytoplasmic messenger during the first few minutes of current recordings in the whole-cell configuration.[19] However, direct evidence for the interaction of **3** with LTCCs came from our radioligand binding studies employing (+)-[^{3}H]isradipine and

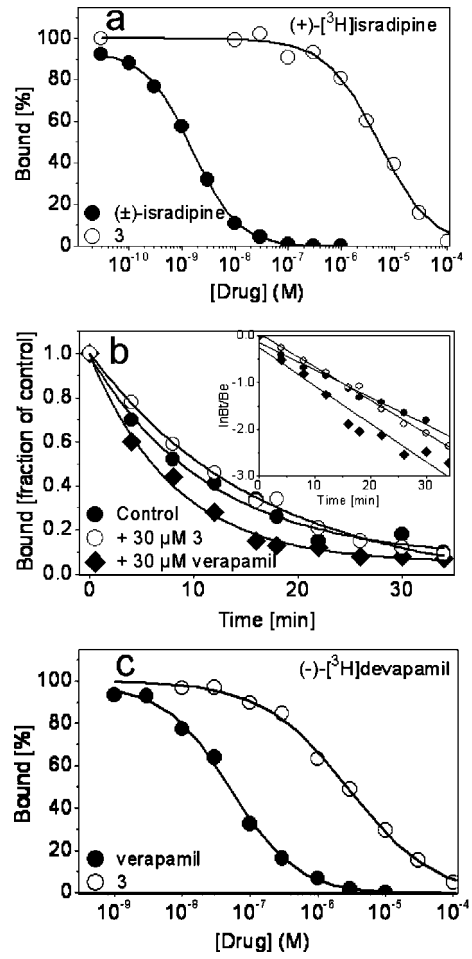

Figure 4. Radioligand binding studies: (a) inhibition of (+)-[^{3}H]isradipine binding to rabbit skeletal muscle membranes by (±)isradipine and **3**. One representative experiment is shown. (b) Dissociation kinetics. Dissociation of (+)-[^{3}H]isradipine from Ca^{2+} channels was induced by the addition of 1 μM (±)-isradipine (control, ●), 1 μM (±)-isradipine + 30 μM **3** (○), or 1 μM (±)-isradipine + 30 μM verapamil (◆). (c) Inhibition of (−)-[^{3}H]devapamil binding to rabbit skeletal muscle membranes by **3** and verapamil. One representative experiment is shown.

(−)-[^{3}H]devapamil, which reversibly labeled the dihydropyridines and phenylalkylamine binding domains of LTCCs, respectively, with similar affinities in different tissues.[20] These binding domains are located close to each other at the channel pore and display noncompetitive interaction.[21] **3** completely inhibited (+)-[^{3}H]isradipine binding to skeletal muscle (Figure 4a, $IC_{50} = 6.3 \pm 0.9$ μM, slope 1.02 ± 0.06) similarly to (±)isradipine (1.4 ± 0.2 nM and 1.07 ± 0.09, respectively). **3** also inhibited brain LTCCs ($IC_{50} = 19.4 \pm 2.8$ μM, $n = 3$; not illustrated) as well as (−)-[^{3}H]devapamil binding to skeletal muscle Ca^{2+} channels (Figure 4c, $IC_{50} = 5.6 \pm 1.6$ μM, $n = 3$). Unlike verapamil, which is a well-known allosteric inhibitor of dihydropyridine binding and significantly accelerates (+)-[^{3}H]isradipine dissociation from its binding domain (Figure 4b), 30 μM **3** did not accelerate dissociation. Together, the observed slope factor for inhibition of (+)-[^{3}H]isradipine binding close to one and the observed complete inhibition of binding suggested competitive interaction of **3** with the dihydropyridine binding domain. Assuming competitive inhibition and a dissociation constant for (+)-[^{3}H]isradipine in rabbit skeletal muscle Ca^{2+} channel of 0.43 nM[22] and a mean radioligand concentration of 0.5 nM, the K_i for **3** can be calculated from the IC_{50} (6.3 μM) to be 3.4 μM.[23] This corresponds well to the highest apparent

affinity determined in the electrophysiological study (IC_{50} = 5.5 μM for end of stimulus current inhibition).

Together, the electrophysiological and binding data prove the direct interaction of **3** with the pore-forming subunit of LTCCs and explain why **3**, in the presence of extracellular Ca^{2+}, did not inhibit completely Phe-induced contraction that results from membrane depolarization and Ca^{2+} mobilization from the intracellular stores,[15] whereas it was able to fully antagonize KCl-induced contraction that is totally mediated by the opening of LTCCs.

In conclusion, we have reported a new series of anthracene derivatives as prototypes of a new class of LTCC blockers. A promising hit proved to be **3**, with a very interesting profile and apparently lacking intracellular effects in vascular smooth muscle.

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Supporting Information Available: Full experimental procedures of syntheses and biological evaluation of target compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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