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Polyphenolic Antioxidants Mimic the Effects of 1, 4-Dihydropyridines on Neurotensin Receptor Function in PC3 Cells

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NT, neurotensin; NTR1, NT receptor subtype 1; VGCC, voltage-gated Ca^{2+}-channel; SOCC, store-operated Ca^{2+}-channel; DHP, 1,4-dihydropyridine; PLC, phosphatidylinositol-specific phospholipase C; IP, inositol phosphates; G-protein, GTP-binding protein; ROS, reactive oxygen species; UTP, uridine triphosphate; BAPTA-AM, 1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid tetra(acetoxymethyl) ester; EDTA, ethylenediaminetetraacetic acid; DTT, dithiothreitol; DMSO, dimethylsulfoxide; BHA, butylated hydroxy anisole; NAD, nicotinamide adenine dinucleotide; PIP2, phosphatidylinositol-(4, 5)-bisphosphate.

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ABSTRACT

This study aimed to determine the mechanism(s) by which 1,4-dihydropyridine Ca^{2+}-channel blockers (DHPs) enhance the binding of neurotensin (NT) to prostate cancer PC3 cells and inhibit NT-induced inositol phosphate formation. Earlier work indicated that these effects, which involved the G-protein-coupled NT receptor NTR1, were indirect and required cellular metabolism or architecture. At the micromolar concentrations used, DHPs can block voltage-sensitive and store-operated Ca^{2+}-channels, K^{+}-channels and Na^{+}-channels, and can inhibit lipid peroxidation. By varying [Ca^{2+}] and testing the effects of stimulators and inhibitors of Ca^{2+}-influx and internal Ca^{2+}-release, we determined that while DHPs may have inhibited inositol phosphate formation partly by blocking Ca^{2+}-influx, the effect on NT binding was Ca^{2+}-independent. By varying [K^{+}] and [Na^{+}], we showed that these ions did not contribute to either effect. For a series of DHPs, the activity order for effects on NTR1 function followed that for antioxidant ability. Antioxidant polyphenols (luteolin and resveratrol) mimicked the effects of DHPs and showed structural similarity to DHPs. Antioxidants with equal redox ability, but without structural similarity to DHPs (such as α-tocopherol, riboflavin and N-acetyl-cysteine) were without effect. A flavoprotein oxidase inhibitor (DPI) and an hydroxy radical scavenger (BHA) also displayed the effects of DHPs. In conclusion, DHPs indirectly alter NTR1 function in live cells by a mechanism that depends on the drug's ability to donate hydrogen but does not simply involve sulfhydryl reduction.

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Neurotensin (NT) [1], a regulatory peptide found in brain and intestine (Carraway and Leeman, 1976), exerts a number of biologic effects by way of its G-protein-coupled receptor NTR1 (Vincent et al, 1999) located on neurons, endocrine cells and smooth muscle cells (Ferris, 1989; Rostene and Alexander, 1997). Since NTR1 is often coupled to $G_{q/11}$, one of the pathways activated by NT involves phosphatidylinositol-specific phospholipase C (PLC)-mediated formation of inositol phosphates (IP) and the release of intracellular Ca^{2+} (Hermans and Maloteaux, 1998). NT also stimulates an influx of Ca^{2+} into excitable (Trudeau, 2000) and non-excitable cells (Gailly, 1998), and as a consequence, some of its effects are inhibited by Ca^{2+}-channel blockers (CCBs). For example, the contractile effect of NT on intestinal smooth muscle (Mule and Serio, 1997) is antagonized by nifedipine, a blocker of voltage-gated Ca^{2+}-channels (VGCC). However, the effects of CCBs in these systems are not well defined and some results are controversial. In guinea pig atria, nifedipine alters the ionotropic response to NT but investigators question whether the effect depends on Ca^{2+}-influx (Golba et al, 1995). Also inconsistent is the fact that NT inhibits, rather than stimulates, VGCC-currents in frog melanotrophs (Belmeguenai et al, 2002). These contradictory findings have led us to hypothesize that CCBs can alter NT signaling by exerting effects that do not involve Ca^{2+}-channels.

It is well established that CCBs in the 1,4-dihydropyridine (DHP) class, such as nifedipine and nimodipine, can affect multiple targets (Triggle, 2003). Used in the sub-micromolar range, DHPs are relatively specific VGCC blockers; however, in the micromolar range, they block store-operated Ca^{2+}-channels (SOCC) (Harper et al, 2003), voltage-dependent K^+-channels (Hatano et al, 2003), Na^+-channels (Yatani et al, 1988) and ligand-gated ion channels (Lopez et al, 1993). In addition, DHPs are powerful antioxidants which can inhibit lipid peroxidation (Diaz-Araya et al, 1998) and protect cells against oxidative injury (Mak et al, 2002).

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In the first paper of this series (Carraway et al, 2003), we reported that a variety of CCBs dose-responsively enhanced the binding of ^{125}I-NT to NTR1 in prostate cancer PC3 cells, whereas they inhibited NT-induced IP formation. The effects were drug specific, receptor specific and indirect, suggesting an involvement of selective cellular mediators and a requirement for cellular metabolism or architecture. Implicating Ca^{2+}-channel(s) and/or Ca^{2+}-dependent step(s) was the fact that IP formation required Ca^{2+} in the medium and that NT caused an influx of Ca^{2+} into the cells. The most potent agents were VGCC blockers (nifedipine and nimodipine); however, their effects on NTR1 function occurred in a dose range shown by others to block SOCC. Since NT was shown to release Ca^{2+} from internal stores and to stimulate SOCC-mediated store refilling, this suggested that CCBs altered NT binding and NT-induced IP formation by blocking SOCC function. Although a number of relatively selective SOCC blockers were also able to alter NTR1 function, the rank order of potency (nifedipine > SKF-96365 > miconazole > trifluoperazine) did not agree with published results for inhibition of SOCC conductance. Furthermore, the effect of nifedipine on NT binding occurred without Ca^{2+} in the medium. We concluded that a number of possible explanations should be considered: a)- Ca^{2+}-channel occupation per se, not Ca^{2+}-influx, might have mediated the effects of DHPs on NTR1 function; b)- the effects of DHPs could have involved effects on Na^{+}- or K^{+}-channels, since NT binding is modulated by these ions (Carraway et al, 1993); and c)- the antioxidative property of DHPs might have been the basis for these actions, since NT binding is sulfhydryl-dependent (Mitra and Carraway, 1993).

To clarify this issue, we have now performed studies to elucidate the mechanism(s) by which DHPs alter NTR1 function in PC3 cells. The role of Ca^{2+} and Ca^{2+}-channels was examined by testing the effects of agents that altered cellular [Ca^{2+}] and/or perturbed Ca^{2+}-channel structure. We tested the possibility that Na^{+}- and/or K^{+}-channels participated by varying the concentrations of these ions. The importance of redox activity was assessed by comparing the effects of a series of DHPs with known

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antioxidative ability and by testing other antioxidants. Our results support the hypothesis that DHPs alter NTR1 function via an indirect, redox-sensitive mechanism that does not appear to involve reduction of sulfhydryl groups.

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METHODS

Materials- Radiochemicals, [^{125}I]-sodium iodide (2000 Ci/mmol) and [1,2-^{3}H(N)]-myo-inositol (60mCi/mmol) were obtained from Dupont New England Nuclear (Boston, MA). Ionomycin, thapsigargin, UTP, PMA, resveratrol and luteolin were from Calbiochem (San Diego, CA). FPL-64176 was from Biomol (Plymouth Meeting, PA). NT, nifedipine, nimodipine, (-)BayK-8644, felodipine, nicardipine, BHA, DTT, EGTA, BAPTA-AM, α-tocopherol, β-carotene, ascorbic acid, riboflavin, thiamine, pyridoxine, menadione and all other chemicals were from Sigma (St. Louis, MO). Compound-1, 1-ethyl-1,4-dihydro-2,6-dimethyl-4-(4-methoxyphenyl)-3,5-pyridinedicarboxylic acid dimethyl ester, was a generous gift from Dr. Juan Arturo Squella, University of Chile, Santiago 1, Chile.

Binding to PC3 Cells- PC3 cells, obtained from American Type Culture Collection (Rockville, MD), were maintained by our tissue culture facility (Seethalakshmi et al, 1997). Cells were grown to 95% confluency in 24-well culture plates. HPLC-purified monoiodinated NT (^{125}I-NT) at 2000 Ci/mmol was prepared and binding was performed as described by us (Carraway et al, 2003). In brief, cells were washed with 2ml/well of hepes-buffered Locke-BSA (Locke): 148mM NaCl; 5.6mM KCl, 6.3mM hepes; 2.4mM $NaHCO_3$; 1.0mM $CaCl_2$; 0.8mM $MgCl_2$; 5.6 mM glucose; 0.1% BSA; pH 7.4. Stock solutions of each agent in Locke or in DMSO (10mM) were prepared just before use and were diluted to give ≤1% DMSO final. Equilibrium binding at 37°C was performed for 25 min using 10^5 cpm/ml ^{125}I-NT in 1.0ml Locke with varying amounts of NT. The reaction was stopped on ice for 15min, the medium was aspirated and the cells were washed twice with 2ml and once with 4ml ice-cold saline. Total cellular binding was assessed by measuring radioactivity (Packard 10-well γ-counter) and protein (Bio-Rad assay; BSA standard) in cells extracted in 0.6ml 0.2M NaOH. Specific binding, defined as that displaceable by 1μM NT, was 95%. Binding displacement curves were constructed for

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each set of treatments and binding parameters were determined by Scatchard analysis and by using the Cheng-Prusoff equation {Ki = IC50 / 1+[L] / Kd} where Kd and [L] are the dissociation constant and the concentration of the ligand, respectively. The sucrose buffer used in some experiments was identical to Locke except that 296mM sucrose was substituted for the NaCl and $NaHCO_3$. The K^+-depolarization buffer used in some experiments was identical to Locke except that 60mM KCl was substituted for 60mM NaCl.

Binding to PC3 Cell Membranes- PC3 cell membranes were prepared and collected by centrifugation at 30,000g as described by us (Seethalakshmi et al, 1997). Binding of ^{125}I-NT (10^5cpm) to membranes (10-50μg) was performed at 20°C for 60min in 10mM Tris-HCl (pH 7.5), containing 1mM $MgCl_2$, 1mM dithiothreitol, 0.1% BSA and protease inhibitors as described. Membranes were collected and washed onto glass fiber (GF-B) filters using a Brandel cell harvester, and the filters were counted (Carraway et al, 1993).

Measurement of inositol phosphate (IP) formation- Formation of [^{3}H]-IP in response to NT was measured as described (Carraway et al, 2003). Briefly, PC3 cells in 24-well plates were incubated 48 hrs with myo-[^{3}H]-inositol (2.5μCi/ml) in medium 199, 5% fetal calf serum. After washing in Locke, cells were preincubated 10 min with varying concentrations of test agent in Locke, 15mM LiCl. After aspiration, fresh Locke with test agent was added, and reactions were started by adding NT or control. After 30 min at 37°C, medium was aspirated, ice-cold 0.1M formic acid in methanol (1ml) was added and plates were placed at –20°C overnight. Samples were adsorbed to AG-1X8 (formate form, Bio-Rad), which was washed five times in 5mM myo-inositol and eluted in 1.5M ammonium formate, 0.1M formic acid. Scintillation counting was performed in Ecoscint (National Diagnostics).

Statistics- Statistical comparisons were made using the Student t-test. Data were calculated as mean±SEM and $p<0.05$ was considered significant.

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RESULTS

Dependence on Extracellular [Ca^{2+}]- Previously (Carraway et al, 2003), we reported that CCBs, particularly those in the DHP class, dose-responsively enhanced the binding of ^{125}I-NT to PC3 cells (as much as ≅3-fold) and inhibited NT-induced IP formation (as much as ≅70%). The fact that NT caused an influx of $^{45}Ca^{2+}$ in PC3 cells that was inhibited by nifedipine suggested that DHPs might alter NTR1 function by blocking Ca^{2+} movement. To test this hypothesis, we examined the effects of Ca^{2+}-chelators and Ca^{2+}-ionophores on NT binding to PC3 cells, and on the ability of nifedipine to enhance NT binding. Blocking Ca^{2+}-influx with 2mM EGTA enhanced NT binding (38±6% increase; $p<0.05$) but the effect was small relative to the 200% increase by nifedipine. In addition, the ability of nifedipine to enhance NT binding persisted in the absence of extracellular Ca^{2+}, was not reversed by 20μM ionomycin, and was not altered by chelation of intracellular Ca^{2+} using 50μM BAPTA-AM (Fig 1).

These results indicated that the enhancement of NT binding by DHPs was not due to a change in Ca^{2+}-influx. In contrast, the inhibition of NT-induced IP formation by DHPs might have involved an effect on Ca^{2+}, since our earlier work showed that this response was Ca^{2+}-dependent (Carraway et al, 2003).

Ca^{2+}-Channel Agonists vs Antagonists– The VGCC agonist (-) BayK-8644 and the antagonist nifedipine enhanced NT binding to a similar extent (Fig 2A). Both compounds enhanced binding by increasing NTR1 affinity, not by altering receptor number (Fig 2B; Table 1). Another VGCC agonist FPL-64176, known to act at a unique non-DHP site (Zheng et al, 1991), was also active, although less potent (Fig 2A). In addition, the agonists (-) BayK-8644 and FPL-64176 shared with the antagonist nifedipine an ability to inhibit NT-induced IP formation (Fig 2C). For each agent, the NT dose-

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response relationship was shifted downward, indicating that the efficacy of NT was decreased, not its potency (Fig 2D).

These results were consistent with the possible involvement of SOCC, but not VGCC, in the effects of DHPs on NTR1 function. Since IP formation was Ca^{2+}-dependent, the fact that these agents decreased the efficacy of NT was in keeping with their known ability to diminish SOCC conductance.

Agents in Combination Gave Additive Effects- We wondered how combinations of CCBs would interact with NTR1, especially in regards to agonist/antagonist combinations and mixtures of CCBs that bind at different sites on Ca^{2+}-channels. To test this, dose-response studies were performed using combinations of antagonist nimodipine and agonist FPL-64176, which are known to bind at discrete sites. Whereas NT binding was enhanced in an additive manner at low concentrations of each drug, the results were less than additive at high concentrations (Fig 3A). NT-induced IP formation was inhibited in an analogous manner and at high concentrations, it reached a limit at ≅70% inhibition (Fig 3B). Similar studies were performed using various combinations of nifedipine, verapamil and diltiazem (all antagonists). Again, when low doses of these drugs were combined, additive effects were observed for the enhancement of NT binding and for the inhibition of NT-induced IP formation, while at high doses the effects were less than additive (data not shown). No potentiative or antagonistic effects were observed. Taken together, these results indicated that the drugs tested, whether Ca^{2+}-channel agonists or antagonists, appeared to act in a similar manner to alter NTR1 function.

Effects of Ca^{2+}-channel Pertubation- Since evidence for direct "conformational coupling" of Ca^{2+}-channels with some receptors existed (Grazzini et al, 1996), it was conceivable that Ca^{2+}-channels might interact directly with NTR1. To examine this hypothesis, we tested treatments expected to

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perturb Ca^{2+}-channel structure for effects on NTR1 function. VGCC can respond to membrane depolarization and Ca^{2+}-feedback (Catterall, 2000). Agents that elevated cellular [Ca^{2+}], either via release of internal Ca^{2+}-stores (thapsigargin, UTP) or by enhancing Ca^{2+}-influx (ionomycin), increased NT binding by ≅30% (Fig 4A). BAPTA-AM, which would decrease cellular [Ca^{2+}], had little effect. Since thapsigargin, ionomycin and UTP did not change NT binding to PC3 cell membranes (results not shown), their effects in intact cells were indirect. Cell membrane K^+-depolarization also increased NT binding (increment, ≅15%) and this was associated with an inhibition of NT-induced IP formation (Fig 4B). The dose-response relationship was shifted downward by K^+-depolarization, indicating that the efficacy of NT was decreased by ≅35% (Fig 4B).

The inhibition of NT-induced IP formation by treatments expected to perturb Ca^{2+}-channels implicated VGCC and/or SOCC in this response, and was consistent with the Ca^{2+}-dependence of PLC (Carraway et al, 2003). However, these effects were relatively small (≅1/3 that of DHP), as were the effects of these treatments on NT binding (≅1/5 that of DHP). Thus, we were drawn to the idea that the effects of DHPs were, to a large degree (as much as 80%), attributable to some other property that was not necessarily related to the ability to alter Ca^{2+}-channel behavior.

DHP Effects were not Na^+-dependent- Since DHPs can inhibit Na^+-channels and since NT binding to PC3 cell membranes is decreased by Na^+ (Seethalakshmi et al, 1997), we considered the hypothesis that DHPs enhanced cellular binding of NT by interfering with the inhibitory effect of Na^+. Substituting sucrose for NaCl in the Locke buffer increased NT binding and shifted the NT displacement curve to the left; however, nifedipine caused a further shift to the left, even in the absence of Na^+ (Fig 5A). Scatchard analyses showed that each of these effects was due to an increase in NTR1 affinity, without a change in NTR1 number (Table 2). Careful comparison showed that the effect of 50μM nifedipine on NT binding (≅2-fold increase) and on the Ki for displacement of NT

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binding (≅2-fold decrease) was unaffected by removal of Na^+ (Table 2). Similarly and in accordance with the binding data, NT was ≅2 fold more potent in stimulating IP formation in the absence of Na^+ than in its presence (EC50: Locke, 1.1±0.1nM; sucrose 0.5±0.1nM; 3 experiments; $p<0.05$). However, the ability of nifedipine to inhibit NT-induced IP formation was independent of Na^+ (Fig 5B). These results indicated that nifedipine altered NTR1 function by mechanism(s) that did not require Na^+ in the buffer.

DHP Effects were not K^+-dependent- Since DHPs can inhibit K^+-channels and since K^+ inhibits NT binding to cell membranes, we performed experiments similar to those described above to assess the K^+-dependence of the effects on NTR1 function. Substitution of 60mM KCl for NaCl in the Locke buffer did not alter the ability of nifedipine to enhance NT binding (EC50: control, 22±3 μM; 60mM K^+, 24±3 μM; 3 experiments) and to inhibit NT-induced IP formation (IC50: control, 15±2 μM; 60mM K^+, 14±2 μM; 3 experiments). Thus, these effects of nifedipine were not K^+-dependent.

Relationship to Antioxidant Activity- Since DHPs exhibit antioxidant ability (Mak et al, 2002), we wondered how this related to the effects on NTR1 function. DHPs inhibited Fe^{3+}/ascorbate stimulated lipid peroxidation in rat brain slices with activity order: nicardipine > nimodipine > nifedipine (Diaz-Araya et al, 1998). The same activity order was found when these agents were compared for ability to enhance NT binding (Fig 2A) and to inhibit NT-induced IP formation (Fig 2C). In both systems, nicardipine was 2- to 4-fold more potent than nifedipine (Table 3). We also tested felodipine, which was 2-to 4-fold more active than nicardipine (Table 3). While felodipine was reported to be inactive in the rat brain assay mentioned above, it was more active than nicardipine in a similar assay using myocardial membranes (Janero and Burghardt, 1989). In addition, the relative chemical reactivity of DHPs with superoxide anion was reported to be: felodipine > nimodipine > nifedipine > compound-1

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(Ortiz et al, 2003). For these substances, the potency to alter NT binding correlated to antioxidant activity, giving r^2=0.89 (Table 3). Compound-1, a DHP analog with N-ethyl in place of the NH moiety, was reported by Ortiz et al (2003) to have a greatly reduced reactivity with superoxide (<10% that of felodipine). Here, we found that it displayed ≅4% the activity of felodipine and 20-50% the activity of nifedipine in altering NTR function (Table 3). These results suggested that DHPs might act by some reaction(s) involving hydrogen donation.

Effects of Various Antioxidants- ROS scavengers include vitamin-like antioxidants, flavonoids and polyphenols (Rice-Evans et al, 1996). Testing vitamin-like antioxidants on NT binding in PC3 cells, we found β-carotene, thiamine, riboflavin, pyridoxine, ascorbic acid, α-tocoferol and tetrahydro-biopterin to be ineffective (used at 20-180μM; n=3), while vitamin K (menadione) had a small effect at 180μM (% control: 168±8; n=3; $p<0.05$). Other antioxidants without effect included N-acetyl cysteine, glutathione and sodium borohydride (used at 1-3mM; n=3); trolox, ellagic acid, (+)-catechin, (-)-epigallocatechin gallate and rutin (used at 10-100μM; n=3).

In striking contrast were the results for the polyphenolic antioxidants luteolin (a flavonoid) and resveratrol, which displayed effects that were indistinguishable from those of DHPs. Luteolin and resveratrol enhanced NT binding (Fig 6A), and the effect involved an increase in NTR1 affinity without a significant change in NTR1 number (Fig 6B; Table 1). These antioxidants also inhibited NT-induced IP formation (Fig 6C), and the effect involved a dose-dependent decrease in NT efficacy (Fig 6D). When tested together for effects on NT binding, the response to luteolin plus nimodipine and resveratrol plus nimodipine were additive at low doses of each agent, while they were less than additive at high doses (results not shown). Thus, polyphenolic antioxidants mimicked the effects of DHPs and appeared to act via the same pathway.

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Involvement of Sulfhydryl Groups- Since some antioxidants act by reducing sulfhydryl groups on proteins and since NT binding requires sulfhydryl groups associated with NTR1 (Mitra and Carraway, 1993), we tested the hypothesis that DHPs increase NT binding by maintaining sulfhydryl-group(s) in a reduced state. Confirming the importance of sulfhydryl groups, we showed that sulfhydryl chelators, Ni^{3+} (IC_{50}, ≅50μM) and Cd^{2+} (IC_{50}, ≅600μM), inhibited NT binding to PC3 cells, and that their effects were inhibited by 2mM DTT (data not shown).

However, in the basal state, the sulfhydryl group(s) required for NT binding to PC3 cells were primarily reduced, since NT-binding was increased only slightly by 1mM ascorbic acid (114±7%, n=4), 2mM DTT (125±8%, n=4) and 5mM N-acetyl-cysteine (107±6%, n=4). In addition, 2mM DTT did not alter the NT displacement curve (Fig 5A) and did not inhibit the effects of nifedipine. NT binding was enhanced similarly by 50μM nifedipine in the presence and absence of 2mM DTT (control, 246±10%; DTT, 231±11%; n=4). NT-induced IP-formation was inhibited similarly by nifedipine in the presence and absence of DTT (Fig 5B). Thus, DHPs acted by an antioxidant mechanism that did not involve the reduction of sulfhydryl groups in NTR1.

Involvement of flavoprotein dehydrogenase(s)- Hypothesizing that DHPs might act by scavenging ROS produced by cellular flavoprotein dehydrogenases, we tested the effects of diphenylene iodonium (DPI), an inhibitor of these enzymes. DPI mimicked the effects of DHPs on NT binding (Fig 6A) and NT-induced IP formation (Fig 6C). The hydroxy radical scavenger butylated hydroxy anisole (BHA) was also effective (Table 3). These results suggest that flavoprotein dehydrogenases and/or ROS species produced by these enzymes participate in the effects of DHPs on NTR1 function.

Comparisons of Chemical Structures- The chemical structures of the DHPs and polyphenols were similar, each possessing aromatic ring structures with redox capability (Fig 7). The order of potency

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(Table 3) for ability to alter NTR1 function (felodipine > nitrendipine ≅ nicardipine > nimodipine > nifedipine > luteolin > resveratrol) appeared to relate to donor group acidity (NH > OH) and to the number of conjugated double bonds. For DHPs, chloro substituents in the adjacent phenyl ring gave the highest activity (felodipine), while nitro in the meta position was less effective (nitrendipine, nicardipine, nimodipine) and nitro in the ortho position was least effective (nifedipine). Luteolin and resveratrol contained conjugated π-bonded rings which could potentially support the stability of radicals and cations (Solomons, 1994). By donating hydrogen(s), DHPs could conceivably form pyridinium or pyridine analogs with an even greater number of conjugated double bonds and potential to support radical and cation formation. The very high membrane partition coefficients displayed by DHPs (Mason et al, 1999) could determine their ability to accumulate at target site(s).

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DISCUSSION

This study investigated the mechanism(s) by which DHPs enhance NT binding and inhibit NT signaling in PC3 cells. We explored various hypotheses and our results indicated that the effects of DHPs on NTR1 function correlated to their antioxidant activity and were mimicked by polyphenolic antioxidants. Since IP formation was Ca^{2+}-dependent, DHPs could have inhibited NT-induced IP formation partly by blocking NT-induced Ca^{2+}-influx (Carraway et al, 2003). However, this effect may have been made unimportant by the overriding effects of DHPs on NT binding which were clearly derived from the antioxidant property. Some of the DHP effect on NT binding (≅20%) was reproduced by treatments aimed to perturb Ca^{2+}-channel structure (elevation of cellular [Ca^{2+}] and membrane depolarization). Although this might have indicated that Ca^{2+}-channels interact with NTR1, the simplest explanation was that these manipulations also acted along the antioxidant pathway.

Based on our finding that the enhancement of NT binding by nifedipine did not require Ca^{2+} and was not reversed by ionomycin, we concluded that DHPs did not act by diminishing Ca^{2+}-influx. The direction of the Ca^{2+}-flux was also unimportant, since DHP Ca^{2+}-channel agonists and antagonists had similar effects. A second hypothesis considered was that Ca^{2+}-channel occupation per se was sufficient to promote these effects, since Ca^{2+}-channels were known to interact with G-proteins (De Waard et al, 1997) and receptors (Grazzini et al, 1996). Postulating that pertubation of VGCC (Catterall, 2000) might alter NTR1 function, we tested the effects of K^+-depolarization and agents known to alter cellular [Ca^{2+}]. The effects observed were relatively small and it was unlikely that the far more robust responses to DHPs could be explained on this basis. Therefore, we hypothesized that DHPs altered NTR1 function by mechanism(s) not necessarily involving Ca^{2+}-channels.

DHPs are commonly used at concentrations as high as 10μM to block VGCC, although they are specific for this purpose only in the nanomolar range (Triggle, 2003). Above 1μM, DHPs disrupt SOCC (Harper et al, 2003), Na^+-channels (Yatani et al, 1988) and K^+-channels (Hatano et al, 2003),

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and inhibit lipid peroxidation (Diaz-Araya et al, 1998). All of these were possible targets for the effects observed here, given that the IC50 for inhibition of NT-induced IP formation ranged from ≅1μM (felodipine) to ≅15μM (nifedipine). Since Na^+ was known to inhibit NT binding to cell membranes (Carraway et al, 1993), we tested the hypothesis that DHPs enhanced NT binding by blocking Na^+-channels. When sucrose was substituted for NaCl, NT binding was enhanced but the effects of nifedipine persisted. In agreement with the binding data, NT was more potent in promoting IP formation in the absence of Na^+; however, this had no effect on the ability of nifedipine to inhibit the response to NT. This work and similar studies with K^+ indicated that Na^+ and K^+ were not involved in the effects of nifedipine on NTR1 function, although a conformational coupling involving Na^+- or K^+-channels was still possible.

DHPs are antioxidants that inhibit lipid peroxidation and impart cytoprotective effects (Mak et al, 2002). Testing a series of DHPs with known antioxidant ability, we found that the activity order for ability to alter NTR1 function (felodipine > nicardipine > nimodipine > nifedipine) was similar to that reported for inhibition of lipid peroxidation (nicardipine ≅ nimodipine > nifedipine; Diaz-Araya et al, 1998) and for chemical reactivity with superoxide (felodipine > nimodipine > nifedipine; Ortiz et al, 2003). In addition, the effects of DHPs were mimicked by antioxidant polyphenols (luteolin and resveratrol), a hydroxy radical scavenger (BHA) and an inhibitor of flavoprotein oxidases (DPI). The IC50 's determined for these agents (Table 3) were in good agreement with values for antioxidant effects in other systems, e.g., luteolin (Hendricks et al, 2003), resveratrol (Leonard et al, 2003) and DPI (Brar et al, 2002). These findings support the hypothesis that DHPs act on NTR1 by a redox-sensitive mechanism, although the target(s) remain to be identified.

For each DHP and antioxidant that enhanced NT binding, there was an associated ability to inhibit NT-induced IP formation. The potency order for these drugs was the same in the two assays (Table 3) and the potency values were correlated (r^2=0.58), indicating that NT binding and IP

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formation were similarly sensitive to the chemical properties of these drugs. DHPs might exert two separate effects (one to increase NT binding and another to inhibit IP formation), each having the same drug dependence. Alternatively, they could exert one effect (e.g., altering the state of NTR1) which determines the ability to bind NT and to activate signaling. It is also possible that by inhibiting IP formation, DHPs produce feedback effects on NT binding. Given that DHPs altered NTR1 function at concentrations near to the blood levels (≅0.2μM) in patients receiving these drugs therapeutically (Palma-Aguirre, 1995) and below those used for *in vitro* work (Lopez et al, 1993; Triggle, 2003), these effects could be of clinical and pharmacological importance.

There are multiple mechanisms by which antioxidants might alter NTR1 function. Since NT binding to cell membranes is sulfhydryl-dependent, antioxidants might enhance NT binding by maintaining essential sulfhydryl groups in a reduced state. However, this hypothesis is not supported by our finding that cell permeable sulfhydryl reducing agents (DTT and N-acetyl-cysteine) have little effect on NTR1 function, and do not interfere with responses to nifedipine. A second possibility is that antioxidants disrupt signaling cascades that modulate NTR1 activity, for example by protein phosphorylation. Phosphorylation of NTR1 can desensitize the receptor (Hermans and Maloteaux, 1998), and inhibition of this process might give enhanced binding. In some systems, phosphorylation of $G\alpha_q$ is required for activation of PLC (Umemori et al, 1997). Inhibition of this process might diminish NT-induced IP formation. A third idea is that antioxidants might disrupt mitochondrial ATP production, causing secondary effects on GTP/GDP exchange or on kinases involved in NTR1 action.

Although the targets for the actions of DHPs on NTR1 function are not known, the scavenging of ROS is one possibility. Major sources of ROS include mitochondrial enzymes involved in oxidative metabolism (Kamata and Hirata, 1998) and plasma membrane NAD(P)H oxidases involved in signaling by tyrosine kinase receptors (Bae et al, 1997) and G-protein-coupled receptors (Seshiah et al, 2002). Thus, ROS scavengers not only protect against oxidative injury but they inhibit signal

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transduction involved in inflammation and cell growth (Lassegue and Clempus, 2003). Since NT can induce inflammation and regulate cell growth (Seethalakshmi et al, 1997), it is possible that NT signaling involves ROS. Preliminary data shows that DHPs and polyphenols inhibit NT-induced DNA synthesis[2]. It also may be important that PKC, which mediates some NT effects (Vincent et al, 1999), is implicated in the activation of NAD(P)H oxidases (Brodie and Blumberg, 2003). Since polyphenolic antioxidants can inhibit PKC (Ferriola et al, 1989), one can imagine multiple mechanisms by which these drugs might disrupt NTR1 function.

The chemical structures of DHPs and polyphenols contain two aromatic ring systems with a number of conjugated double bonds and redox reactive NH or OH group(s) (Fig 7). These features appear to be essential for this activity, given that equally powerful antioxidants lacking these structures are totally ineffective. Notable is the inactivity of α-tocoferol, which is a standard for many assays (Mitchell et al, 1998). In general, DHPs are more potent than polyphenols in altering NTR1 function and this may be related to the acidity of the hydrogen donor (NH > OH). The activity of compounds within each group also vary (Table 3) and this might be due to the influence of phenyl ring substituents on donor acidity and on resonance stabilization of reaction intermediates (Solomons, 1994). Another important determinant of reactivity could be the membrane partition coefficient (Mason et al, 1999).

The chemical structures of DHPs are reminiscent of NADH. The reaction scheme whereby NAD-linked dehydrogenases donate hydrogen atoms to substrates is shown in Fig 8A. One hydrogen is transferred from NADH as a hydride ion (H^-) and another is taken as H^+ from the medium (Lehninger, 1982). It is tempting to speculate that DHPs can react analogously, transferring hydrogen atoms to superoxide by way of cationic (Fig 8B) or radical intermediates (Fig 8C) to generate pyridine derivatives and water. DHPs are known to form pyridine adducts when reacted with alkyl radicals (Nunez-Vergara et al, 2003). Since the stability of the intermediates in Fig 8 is negatively affected by electron withdrawal, this predicts that nitro groups in the phenyl ring (especially ortho) would diminish

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reactivity. The order derived from such considerations (felodipine > nitrendipine ≅ nicardipine ≅ nimodipine > nifedipine) is in fair agreement with that measured by Ortiz et al (2003) and that found here for altering NTR1 function. Since nitrendipine, nicardipine and nimodipine each have nitro in the meta position, a near equal reactivity with superoxide is expected. The differences in their activity in our system might be attributed to the effects of other ring substituents on lipophilicity (Fig 7), which could affect their ability to enter cells and partition into membranes.

DHPs inhibit cardiac contractility and relax vascular smooth muscle, and their relative abilities to do so vary >10-fold. Felodipine is ≅10-times more vascular-selective than nifedipine (Triggle, 2003). Although this might be due to differential expression of various Ca^{2+}-channels, it is tempting to speculate that the antioxidative effects of DHPs also contribute. For example, if the relaxant effects of DHPs on vascular smooth muscle involve ROS signaling, this might explain the enhanced activity of felodipine relative to nifedipine.

In conclusion, DHPs enhance NT binding and inhibit NT-induced IP formation by an indirect mechanism that appears to require an aromatic structure and functional groups to facilitate hydrogen atom donation. For a series of DHPs, the ability to alter NTR1 function correlates to the ability to scavenge superoxide anion. Polyphenolic antioxidants and an inhibitor of flavoprotein oxidases mimic the effects of DHPs. We propose that DHPs disrupt NTR1 function by inhibiting cellular oxidative reaction(s) or by scavenging ROS involved in receptor regulation and signal transduction.

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FOOTNOTES

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FIGURE LEGENDS

Fig 1- Ca^{2+}-chelators (EGTA and BAPTA-AM) and Ca^{2+}-ionophore (ionomycin) did not prevent the enhancing effect of nifedipine on NT binding. PC3 cells were pretreated 10min with indicated agents or with vehicle control, and NT binding was measured. Plot shows that 2mM EGTA and 20μM ionomycin increased NT binding by 25-40% but did not prevent the response to nifedipine (NIF). 50μM BAPTA-AM did not alter NT binding or the response to nifedipine. Results were from 3 experiments. * indicates result was significantly different ($p<0.05$) from appropriate control.

Fig 2- Ca^{2+}-channel agonists, like antagonists, enhanced NT binding (A) by increasing NTR affinity, not NTR number (B). Ca^{2+}-channel agonists and antagonists inhibited NT-induced IP formation (C) by decreasing NT efficacy (D). A- Cells were pretreated 10 min with indicated agents and NT binding was measured. The minimum dose that significantly ($p<0.05$) elevated NT binding was 0.3μM (felodipine), 0.7μM (nicardipine), 2μM (nifedipine), 3μM ((-)BayK-8644) and 9μM (FPL-64176). B- Scatchard plots show that NTR affinity was increased by 50μM nifedipine and by 50μM (-) BayK-8644. The Ki for NT was (nM): control, 0.84; nifedipine, 0.29; (-) BayK-8644, 0.24. The agents did not alter NTR number (fmol/mg): control, 147; nifedipine, 140; (-) BayK-8644, 160. C- PC3 cells were pretreated 10min with indicated agents and IP formation in response to 30nM NT was measured. For control, NT increased IP formation ≅5-fold. Each agent inhibited the response by 50-75%. The minimum dose that significantly ($p<0.05$) decreased IP formation was 0.3μM (felodipine), 1μM (nicardipine), 5μM (nifedipine), 7μM ((-)BayK8644) and 20μM (FPL-64176). D- PC3 cells were pretreated 10min with agents indicated, and the dose-response for NT-induced IP formation was measured. Note that nifedipine and (-) BayK-8644 decreased efficacy, not potency. At [NT]≥0.3nM,

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IP formation for each treatment was significantly different from the control. In A, C and D, results were from at least 3 experiments for each plot. B shows a typical result for an experiment that was repeated twice.

Fig 3. Non-additivity for effects of nimodipine (NIM) and FPL-64176 on NT binding (A) and NT-inducd IP formation (B)- In A, PC3 cells were pretreated 10 min with combinations of indicated agents, and NT binding was measured. Plots show that low doses of agents in combination gave additive increases in NT binding, while high doses gave non-additive responses. In B, PC3 cells were pretreated 10 min with combinations of indicated agents, and IP formation was measured in response to 30 nM NT. Plots show that low doses of agents in combination gave additive decreases in IP formation, while high doses gave non-additive effects. Results in A and B were from at least 3 experiments for each plot.

Fig 4- Thapsigargin, ionomycin and UTP enhanced NT binding to PC3 cells (A). Cell membrane depolarization inhibited NT-induced IP formation (B). A- PC3 cells were pretreated 10 min with agents indicated and NT binding was measured. NT binding was significantly ($p<0.05$) elevated by thapsigargin (>0.3μM), ionomycin (>3μM) and UTP (>30μM). BAPTA-AM had no effect. B- PC3 cells, labeled with ^{3}H-inositol, were placed in Locke or isotonic K^+-depolarization buffer (60mM K^+). IP formation was measured in response to indicated amounts of NT. At each [NT], IP formation for the K^+-depolarized set was significantly different from control ($p<0.05$). In A and B, results are from 3 experiments for each plot.

Fig 5- Nifedipine shifted the NT binding displacement curve to the left (A) and inhibited NT-induced IP formation (B) in the presence (Locke) or absence (sucrose) of Na^+. The effects of

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substituting sucrose for NaCl in the buffer were determined for NT binding and NT-induced IP formation. A- Maximal NT binding was higher in Locke plus nifedipine (2.2-fold), sucrose (1.7-fold) and in sucrose plus 50μM nifedipine (3.2-fold) than in Locke or Locke plus 2mM DTT. Plots show displacement of ^{125}I-NT binding by NT, in which NT binding was expressed as % maximal. Note that the curves were shifted to the left by nifedipine and by sucrose. The IC50 for NT was: 1.1nM (Locke and Locke plus DTT), 0.4nM (nifedipine), 0.5nM (sucrose), 0.2nM (sucrose plus nifedipine). Results are from typical experiment that was repeated twice. B- PC3 cells were pretreated 10min in Locke, Locke plus 2mM DTT or sucrose containing the indicated amounts of nifedipine. IP formation in response to 30nM NT was measured. Plots show that nifedipine inhibited NT-induced IP formation with a similar IC50 (13-16μM) for each condition. Results are from 3 experiments.

Fig 6- Antioxidants enhanced NT binding (A) by increasing NTR affinity, not NTR number (B), and they inhibited NT-induced IP formation (C) by decreasing NT efficacy (D). A- PC3 cells were pretreated 10min with agents indicated and NT binding to PC3 cells was measured. NT binding was significantly ($p<0.05$) elevated above control for: luteolin (>10μM); resveratrol (>20μM); diphenylene iodonium (>3μM). B- Scatchard plots show that 60μM luteolin and 150μM resveratrol increased NTR affinity. The Ki for NT was (nM): 1.07 (control); 0.28 (luteolin); 0.36 (resveratrol). There was little effect on NTR number (fmol/mg): 170 (control); 175 (luteolin); 153 (resveratrol). C- PC3 cells were pretreated 10min with agents indicated, and IP formation in response to 30nM NT was measured. For the control, NT increased IP formation ≅4-fold. IP formation was significantly different from control for: luteolin (>10μM); resveratrol (>30μM); diphenylene iodonium (>30μM). D- PC3 cells were pretreated 10min with agents indicated, and the dose-response for NT-induced IP formation was measured. Luteolin and resveratrol decreases efficacy, ie., shifted the curves downward. At [NT]>0.3nM, IP formation was significantly different from the control for each treatment. Results in

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A, C and D were from 3 experiments each. Results in C were from typical experiment that was repeated twice.

Fig 7- Chemical structures of DHPs and polyphenols- The basic structure of DHP is shown at the top with the specific substituents for each Ca^{2+}-channel blocker. Hydrogens at the 1 and 4 positions (bold) are potential donors. The activity order for effects on NTR function (felodipine > nicardipine ≅ nitrendipine > nimodipine > nifedipine) may relate to the ability of R1 substituents to support hydrogen donation: Cl_2 (felodipine) > meta NO_2 (nitrendipine, nicardipine, nimodipine) > ortho NO_2 (nifedipine). Polyphenols (lower left) display less activity than DHPs, due to the less acidic nature of OH versus NH. The activity order (luteolin > resveratrol) may relate to the number of OH-groups (4 versus 3) and conjugated double bonds (8 versus 7). Ca^{2+}-channel agonist BayK-8644 (ortho CF_3 at R1) displays activity like nifedipine (ortho NO_2 at R1). Compound-1 (N-ethyl instead of NH) has only one hydrogen donor and displays 20-50% activity relative to nifedipine. Ca^{2+}-channel agonist FPL-64176 (pyrrole instead of DHP) has only one hydrogen donor and displays ≅40% activity relative to nifedipine.

Fig 8- Reaction scheme for NAD-linked dehydrogenase action (A) compared to those proposed for reaction of DHPs with superoxide anion (B, C). In A, NADH is oxidized to NAD^+ as it donates a hydride ion (H^-) to acceptor (R), which reacts with H^+ to give RH_2. In B, we propose an analagous two electron transfer reaction, whereby DHP donates H^- to superoxide anion ($O2^{\cdot-}$) forming a pyridinium cation intermediate that further donates H^+ to the peroxide anion. In C, a single electron transfer is shown forming a neutral radical intermediate. In both cases, a pyridine derivative and water are the final products. For simplicity, we show the dihydropyridine ring with the attached phenyl group (Ph) but the various substituents are omitted.

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Table 1. Effects of CCBs and Antioxidants on NT Binding Parameters in PC3 Cells

Agent [a]	Classification	Bmax[b] (fmol/mg)	Ki[b] (nM)
none	control	155±11	1.0±0.07
50μM nifedipine	VGCC antagonist	152±10	0.51±0.05[c]
50μM BayK-8644	VGCC agonist	162±12	0.56±0.06[c]
60μM luteolin	flavonoid antioxidant	164±10	0.62±0.05[c]
150μM resveratrol	polyphenol antioxidant	171±11	0.36±0.04[c]

[a] PC3 cells were pretreated 10 min with indicated concentrations of each agent or vehicle control. ^{125}I-NT (10^5 cpm, 50pM) was added and specific binding was measured at 37°C.

[b] Scatchard analyses were performed using 12 concentrations of NT. The results for Bmax and Ki (mean±SEM) were from at least 3 experiments per agent.

[c] Indicates significant difference ($p<0.05$) as compared to control.

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Table 2. Effects of Nifedipine on NT Binding Parameters in Locke and Sucrose Buffer

Buffer	Agent[a]	Zero Binding[b] (cpm/μg)	Bmax[c] (fmol/mg)	Ki[c] (nM)
Locke	control	20.4±1.1	165±13	1.0±0.07
Locke	nifedipine	46.3±3.2[d]	168±11	0.51±0.05[d]
Sucrose	control	33.7±1.8	165±10	0.61±0.05
Sucrose	nifedipine	65.8±4.1[d]	175±12	0.35±0.04[d]

[a] PC3 cells were pretreated 10 min with 50μM nifedipine or vehicle control. ^{125}I-NT (10^5 cpm, 50pM) was added and specific binding was measured at 37°C in Locke or sucrose buffer.

[b] Zero binding was defined as specific binding measured at equilibrium in absence of competitor. The results, given as cpm/μg protein, are mean ± SEM from 3 to 6 experiments.

[c] Ki was calculated from NT displacement curves generated using 12 concentrations of NT. The results are mean ± SEM for at least 3 experiments.

[d] Indicates significant difference ($p<0.05$) as compared to appropriate control.

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Table 3. Activity of DHPs and Polyphenols on NT Binding and NT-induced IP Formation

Classification	Agent	NT Binding [a] EC50 (μM)	IP Formation[b] IC50 (μM)	Antioxidant [c] Activity (relative)
VGCC antagonist	felodipine	3	1	2.86
	nitrendipine	7	2	1.34
	nicardipine	7	3	
	nimodipine	7	6	2.12
	nifedipine	15	15	0.73
	compound-1 [d]	75	28	<0.30
VGCC agonist	BayK-8644	16	15	
	FPL-64176	29	27	
Antioxidant	luteolin	40	38	
	resveratrol	80	48	
	Diphenylene iodonium	61	35	
	BHA	110	nd	

[a] EC50 was defined as the [agent] giving 75% increase in NT binding. The data are means determined in at least 3 experiments.

[b] IC50 was defined as the [agent] giving 50% decrease in IP formation. The data are means from at least 3 experiments. nd, not determined.

[c] Relative activity coefficient for reactivity to superoxide ion. Data from Ortiz et al, 2003.

[d] Compound-1 (Ortiz et al, 2003) is an N-ethyl DHP (structure in Fig 7) that displays reduced reactivity to superoxide. Its effects on calcium channels are not known.

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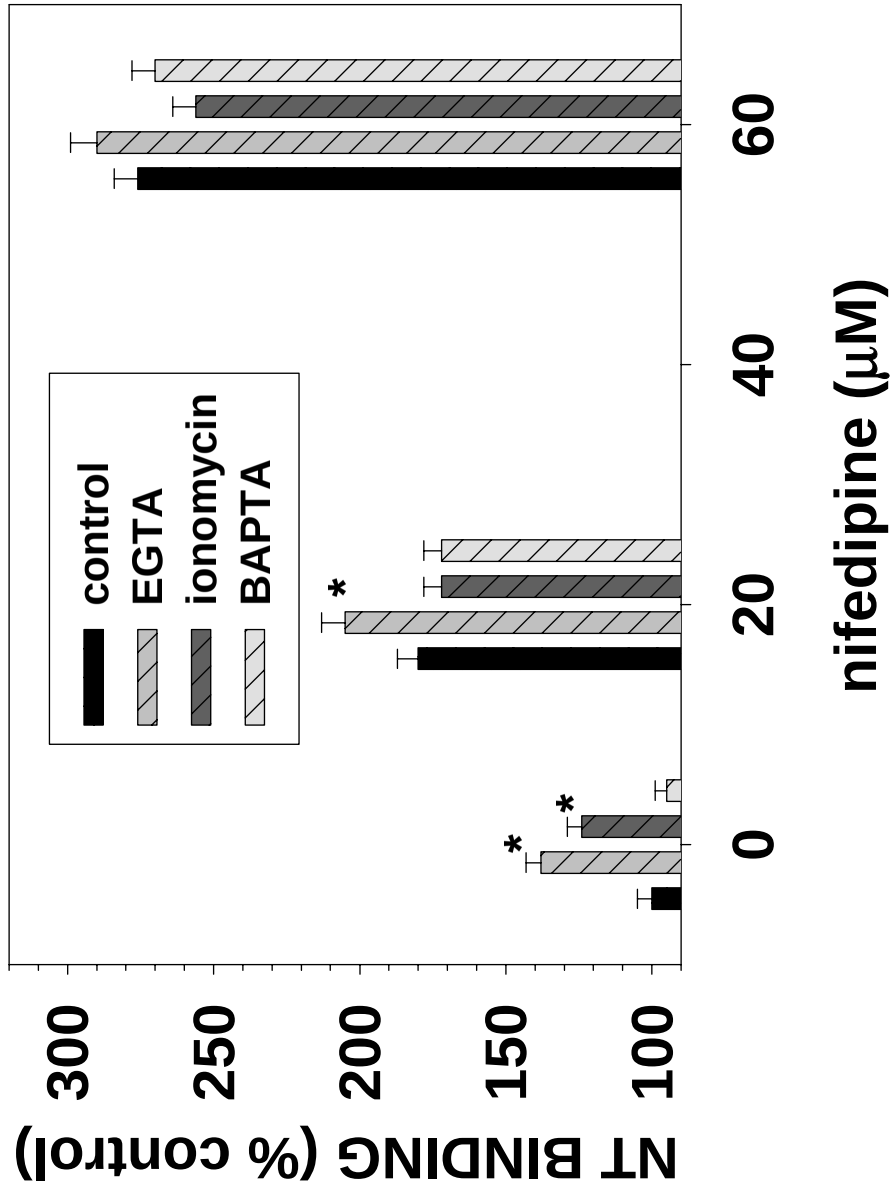

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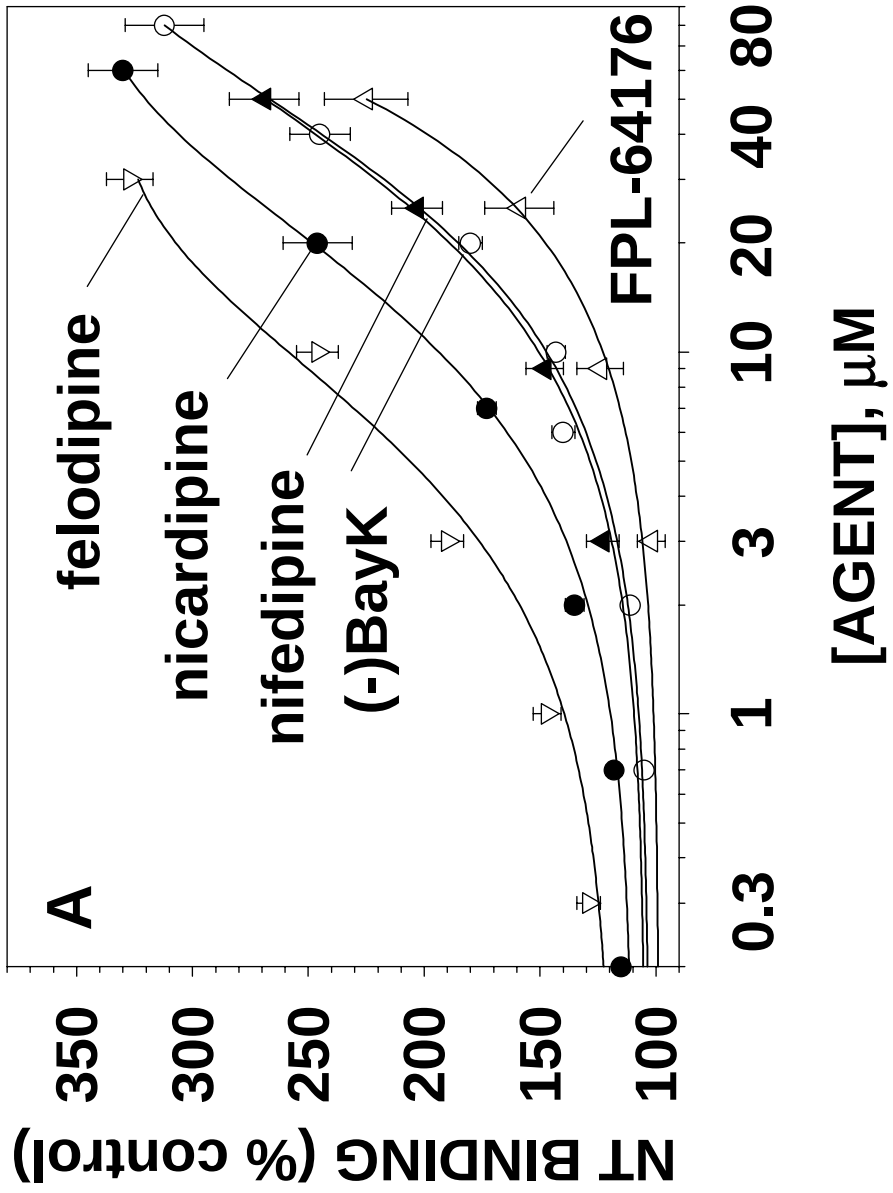

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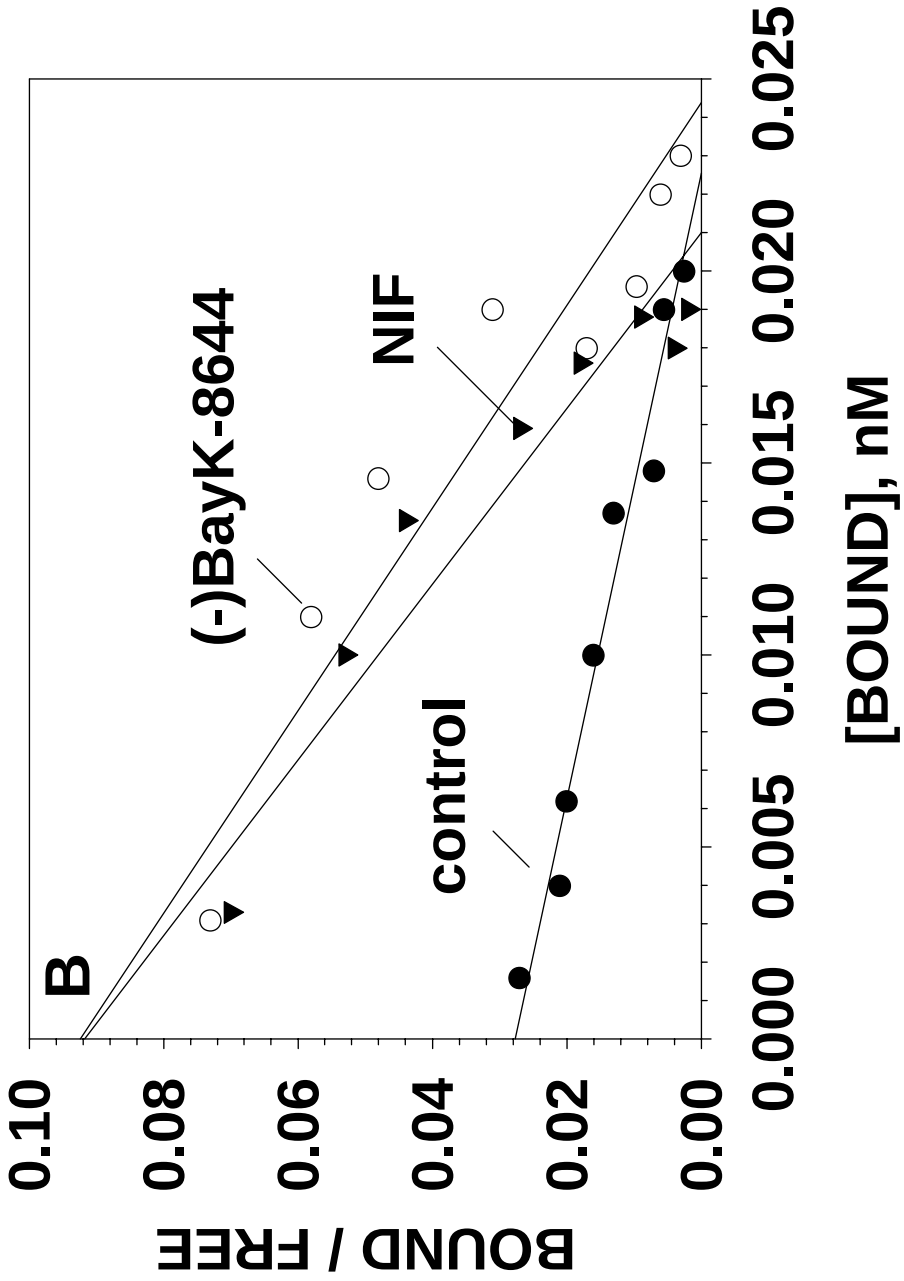

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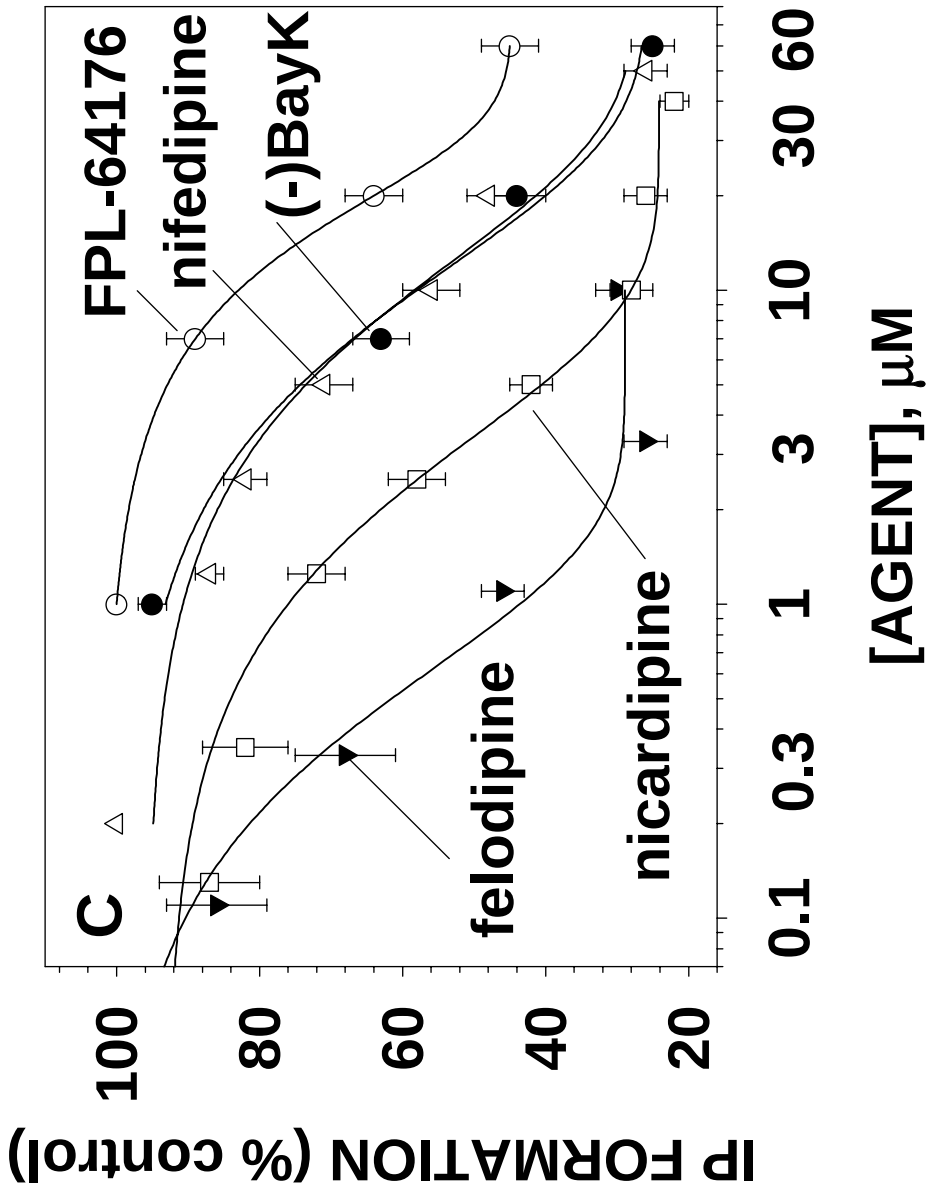

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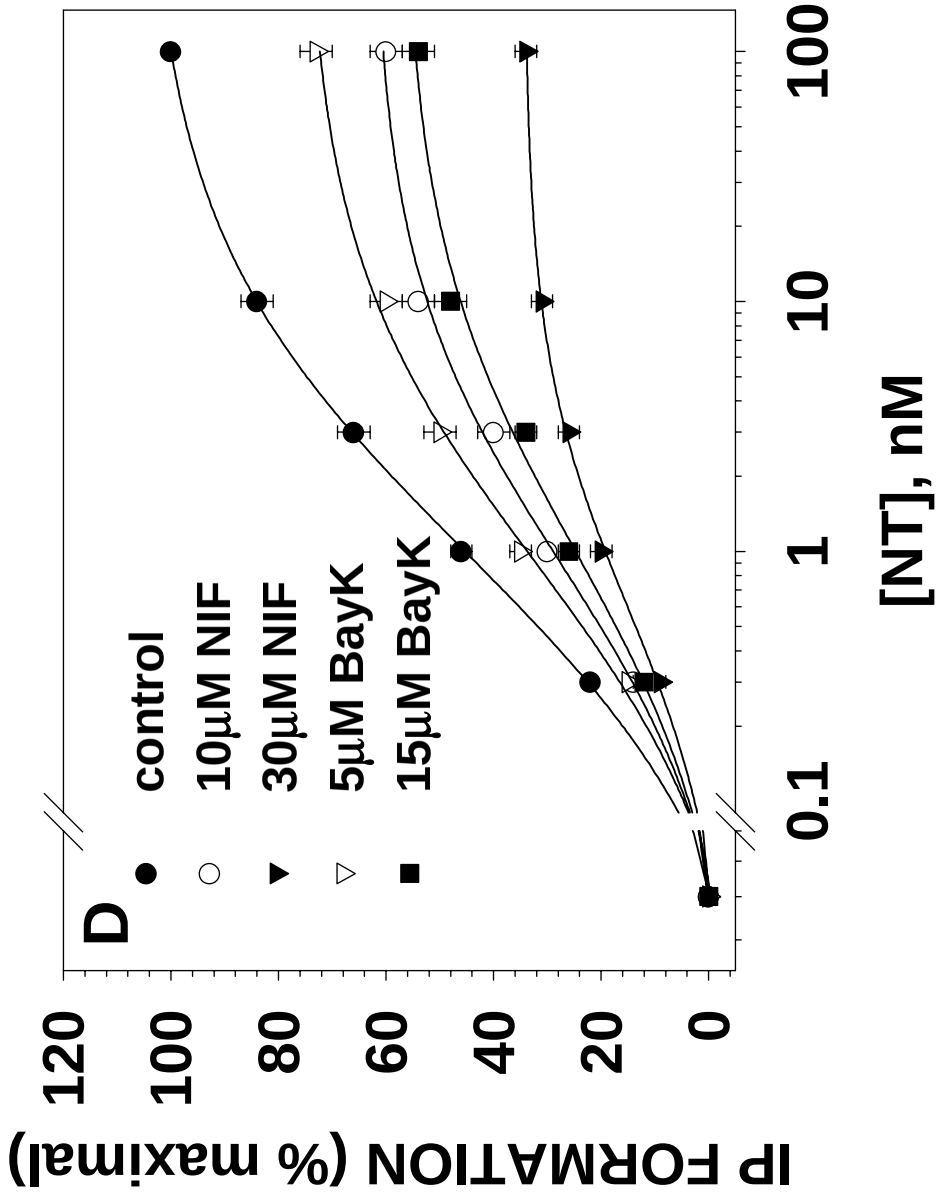

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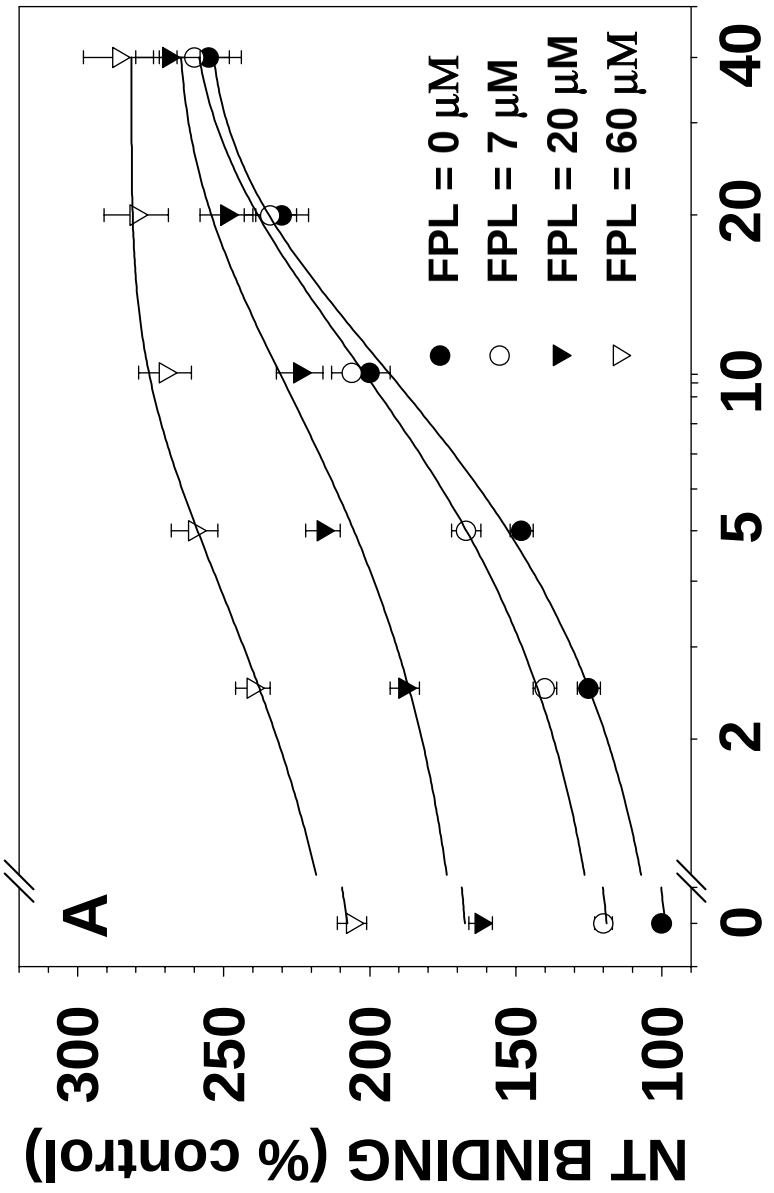

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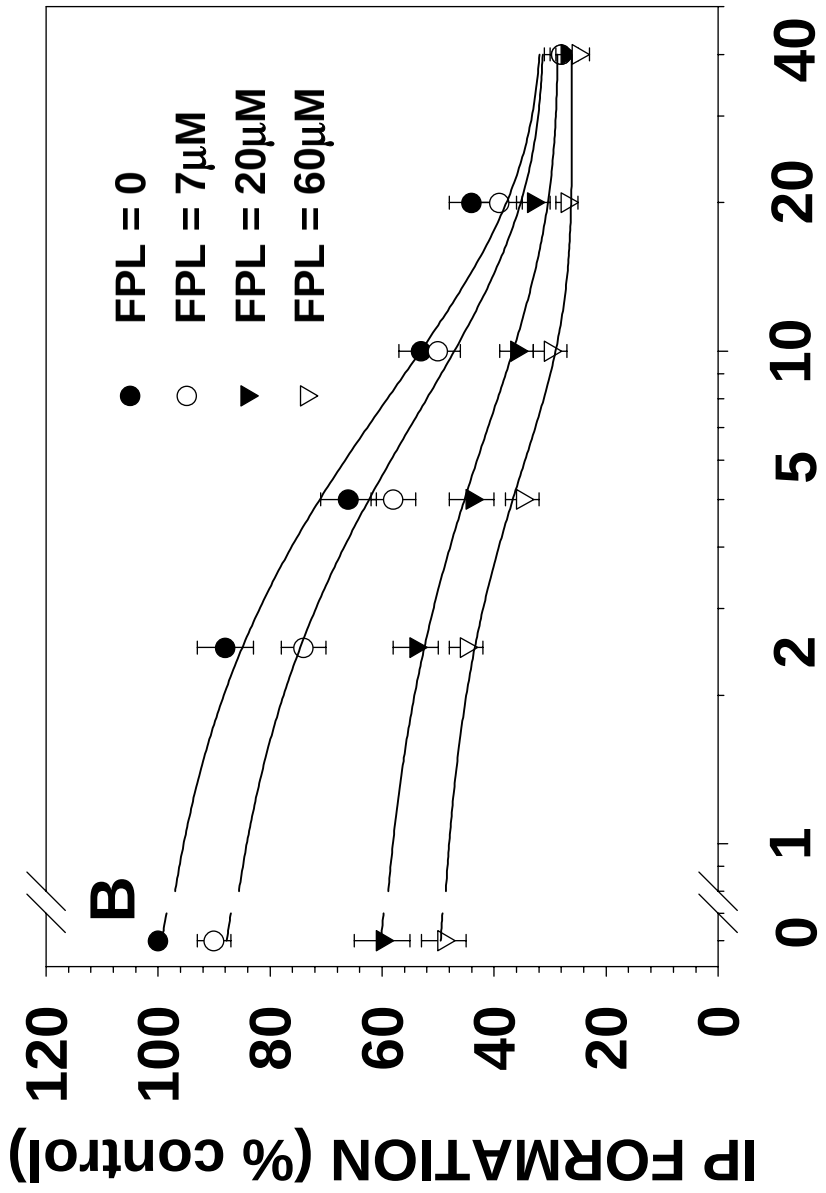

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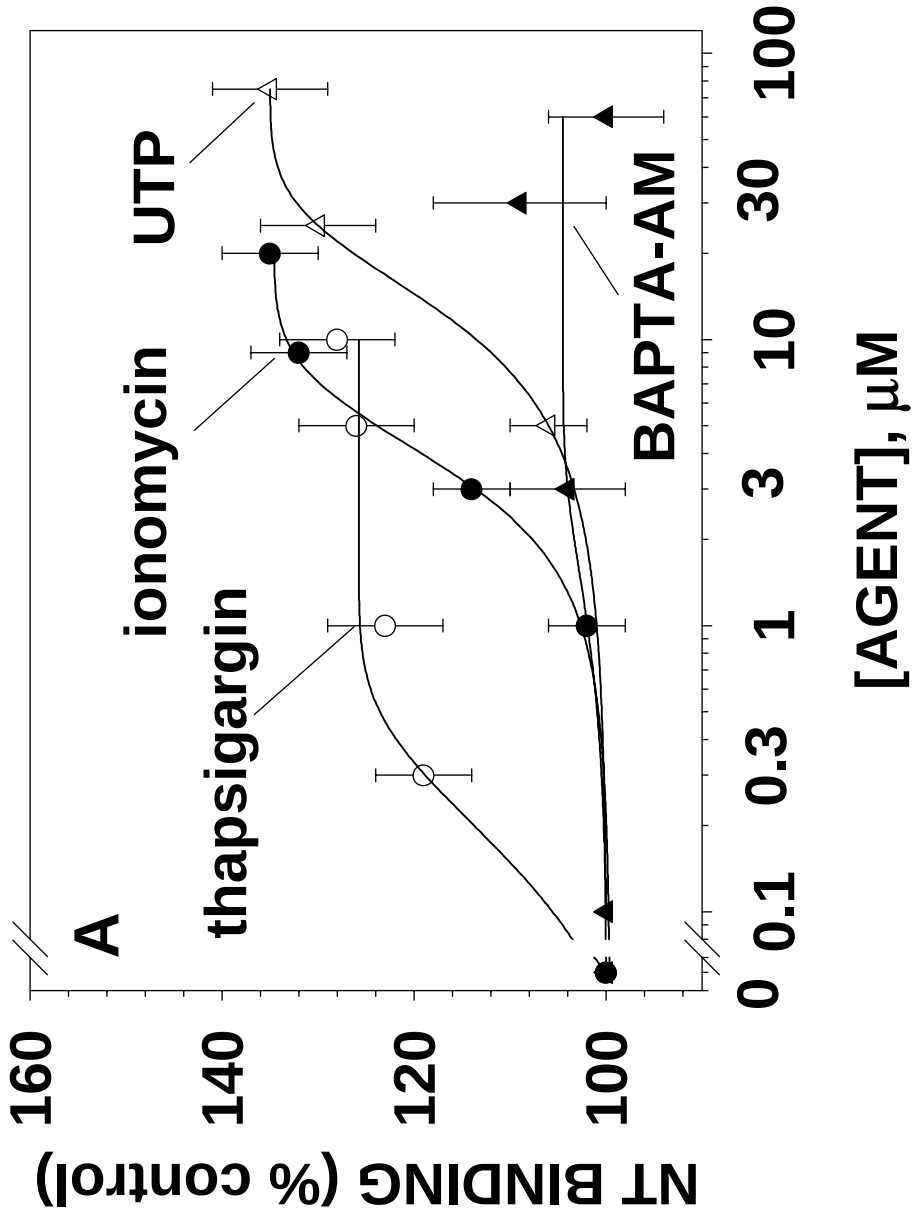

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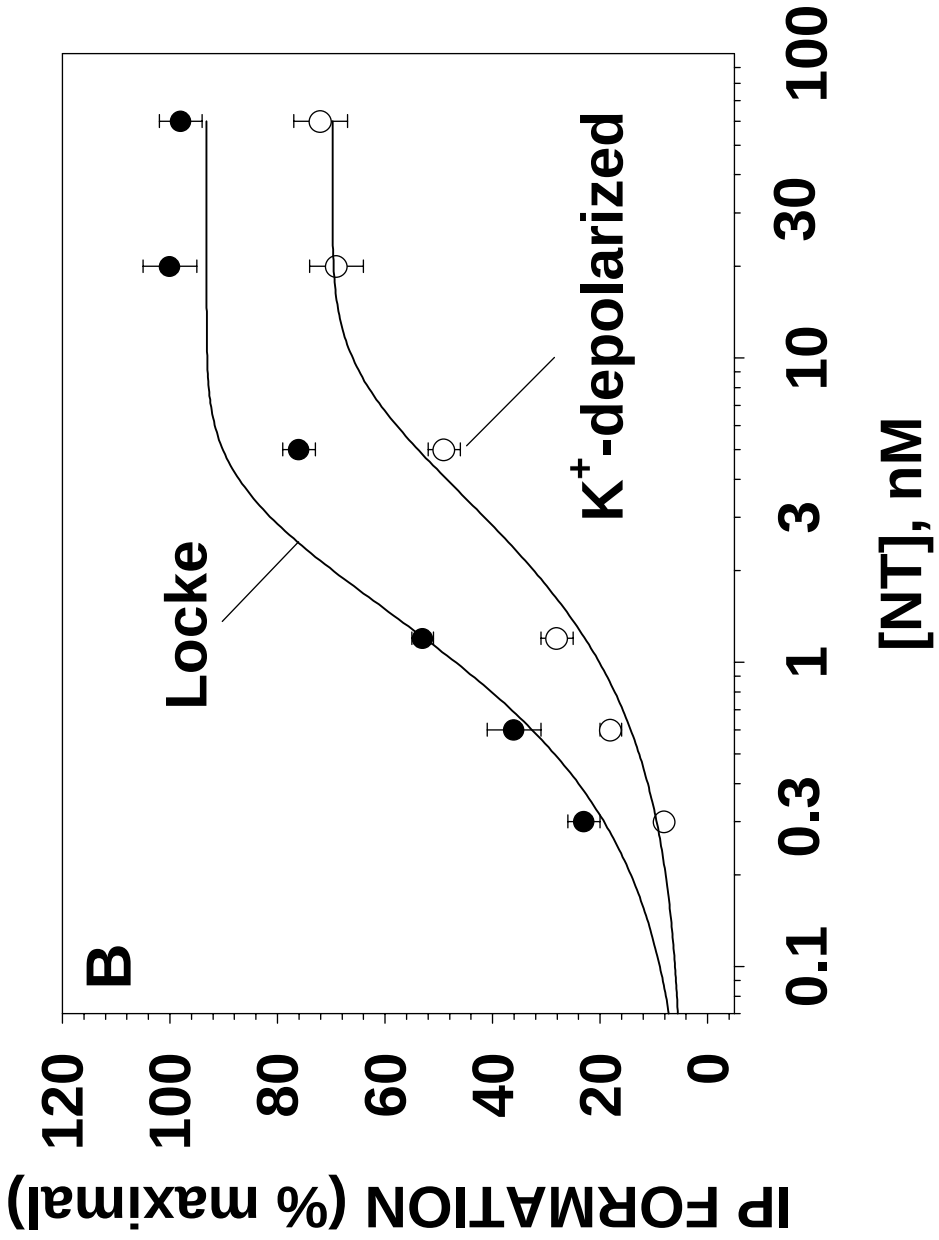

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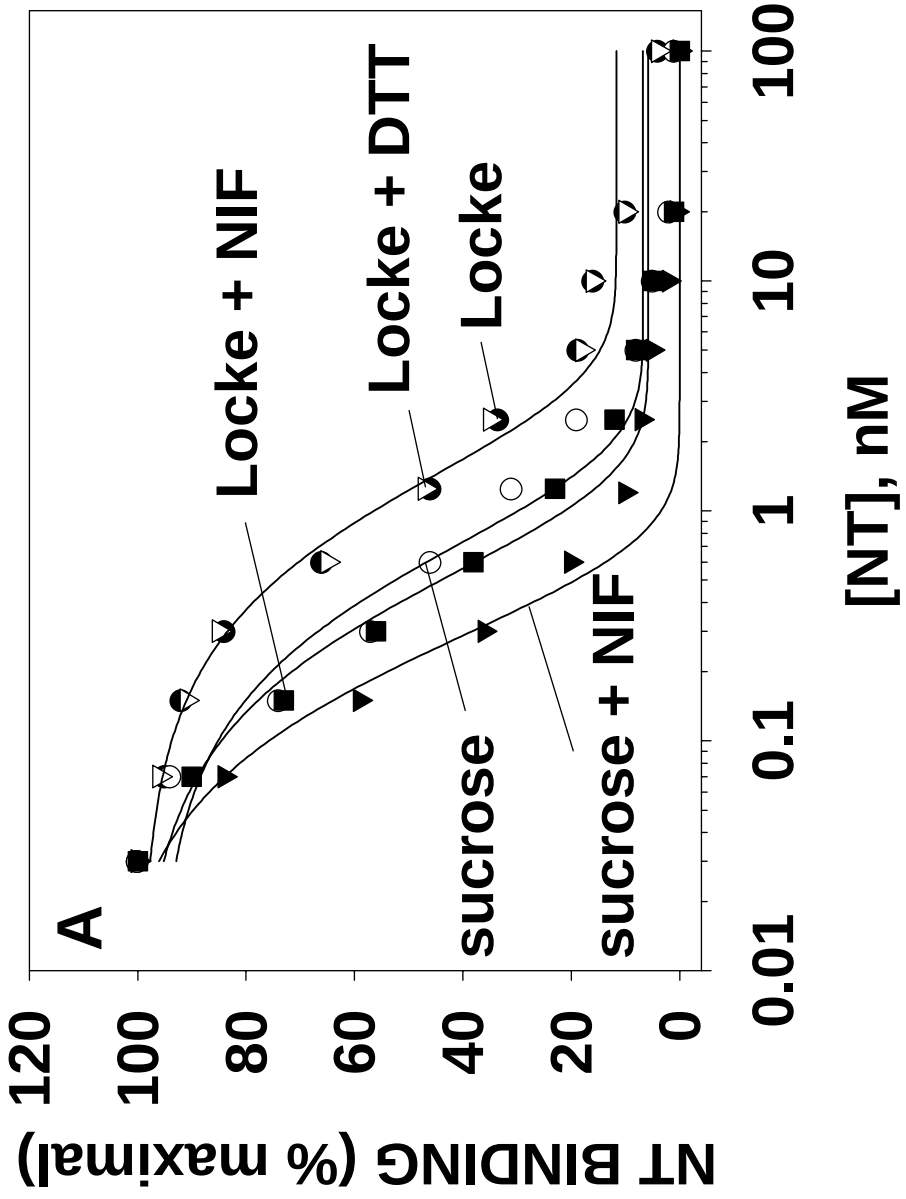

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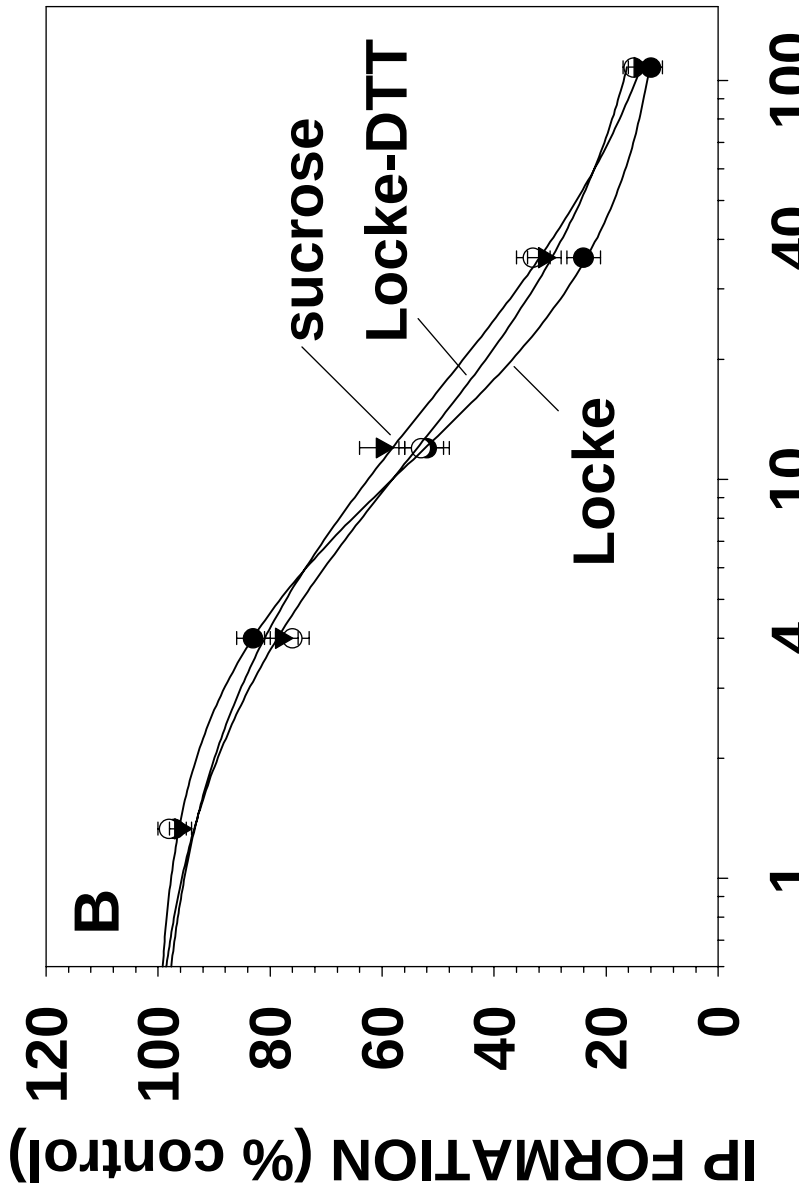

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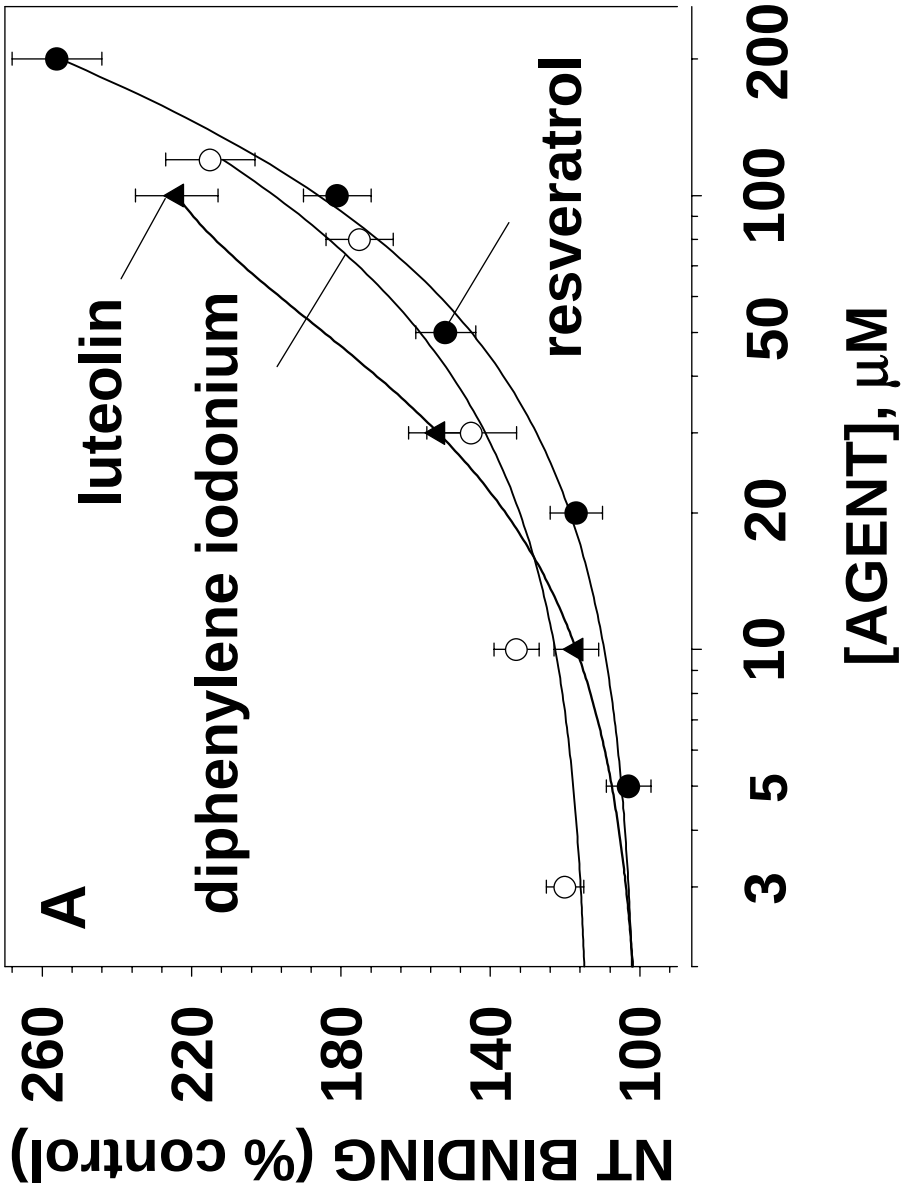

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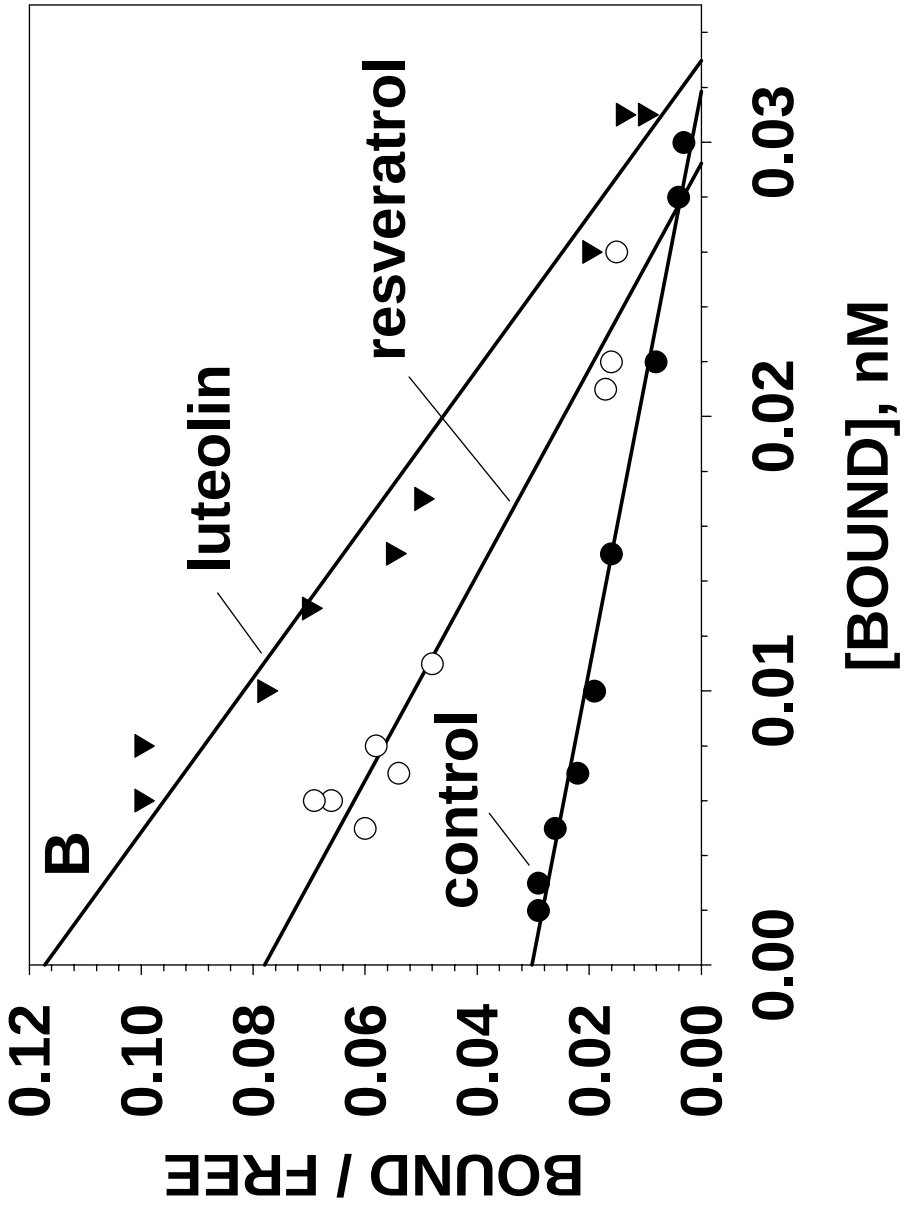

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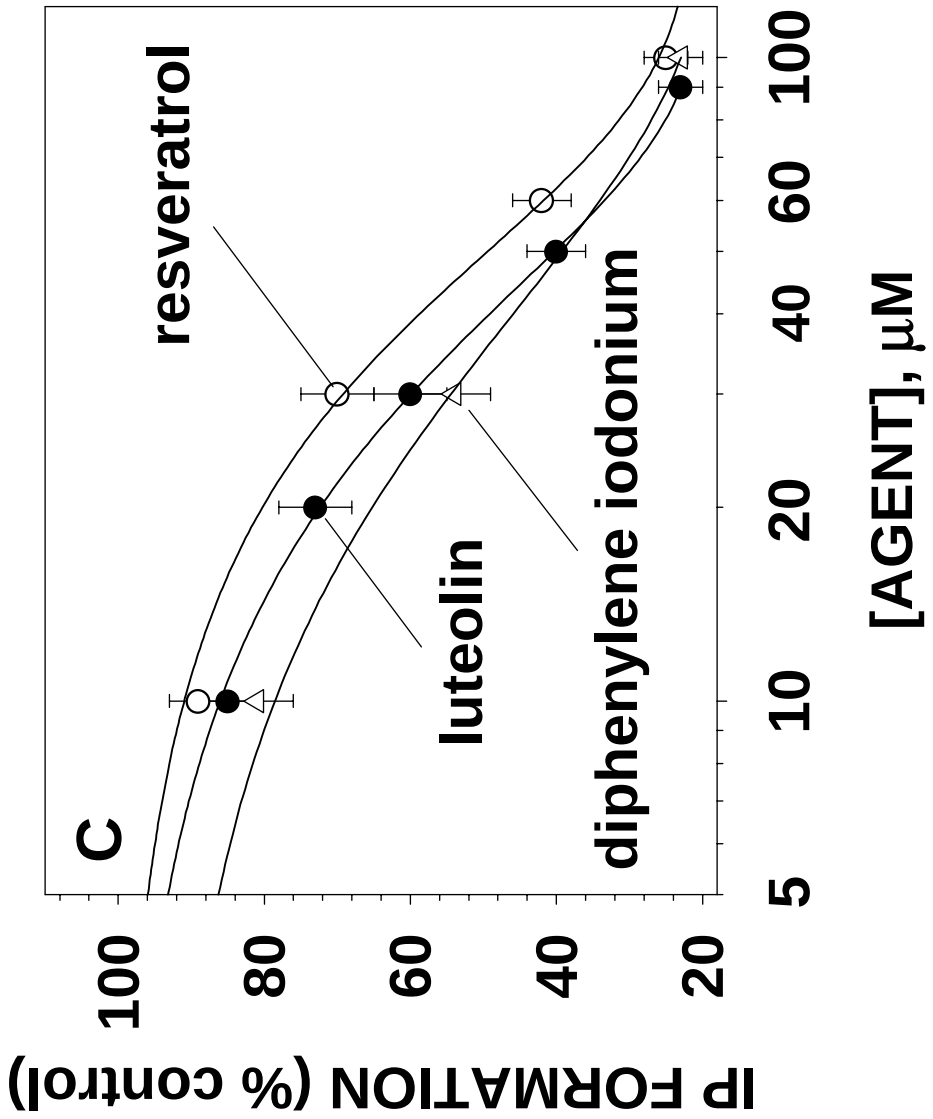

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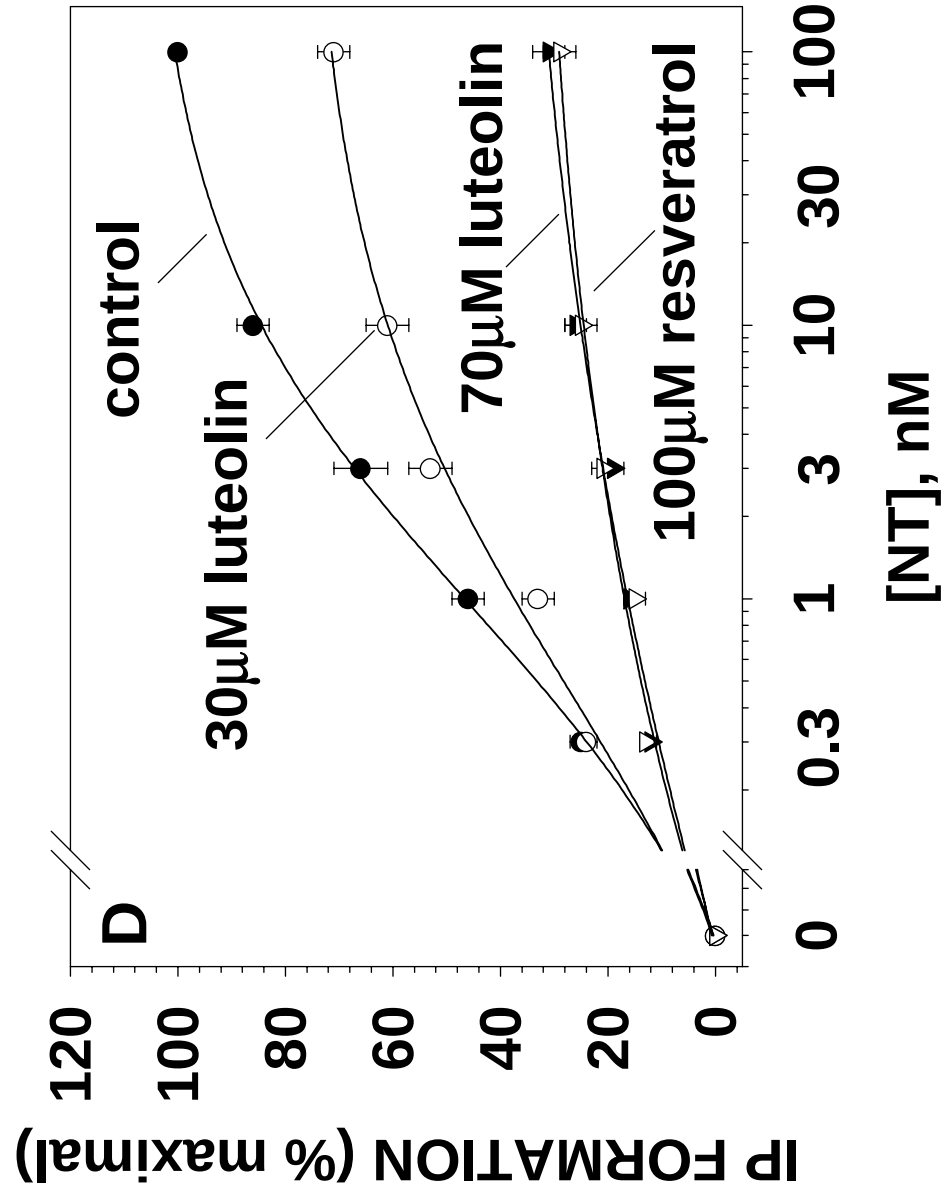
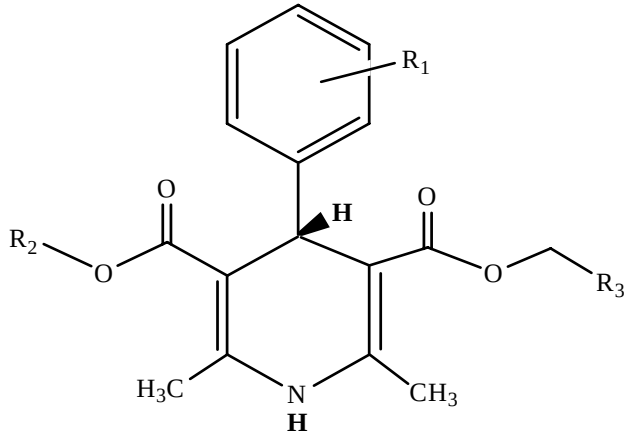
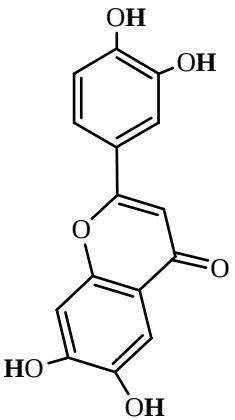
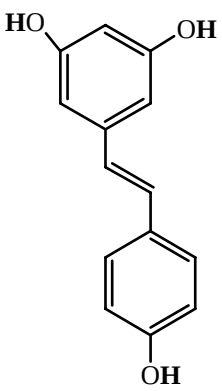




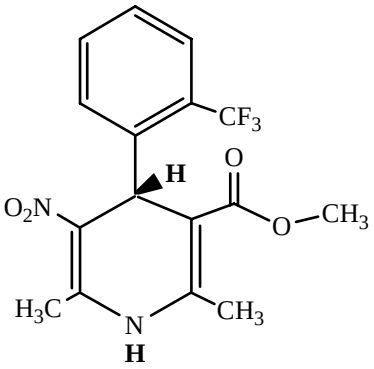
	R_1	R_2	R_3
FELODIPINE	(o,m)-Cl_2	-CH_2-CH_3	-CH_3
NICARDIPINE	(m)-NO_2	-$(CH_2)_2$-N-CH_3-CH_2-Ph	-CH_3
NITRENDIPINE	(m)-NO_2	-CH_2-CH_3	-CH_3
NIMODIPINE	(m)-NO_2	-$(CH_2)_2$-O-CH_3	-CH-$(CH_3)_2$
NIFEDIPINE	(o)-NO_2	-CH_3	-CH_3



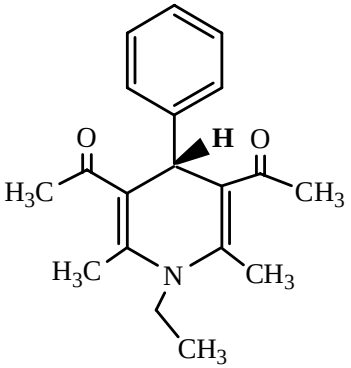
Luteolin



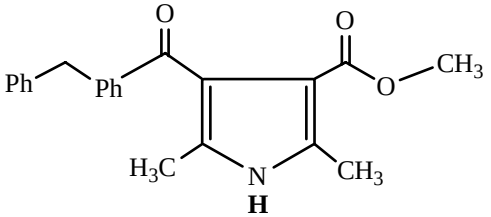
Resveratrol



Bay K-8644



Compound-1



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