JPP Journal of Pharmacy and Pharmacology

JPP 2002, 54: 283–288

Received May 18, 2001
Accepted September 21, 2001
ISSN 0022-3573

Comparative inhibitory effects of niflumic acid and novel synthetic derivatives on the rat isolated stomach fundus

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Abstract

Novel derivatives of 2-[3-(trifluoromethyl)-analino]nicotinic acid (niflumic acid) were synthesized. The compounds were compared for their inhibitory effects on 5-hydroxytryptamine (5-HT)- and KCl-induced contraction of the rat fundus. The aim was to assess structure–activity relationships regarding the selectivity and potency of these compounds. Niflumic acid (1–100 μM) concentration-dependently inhibited 5-HT-induced tonic contractions with an IC50 value (concentration reducing the control contractile response by 50%, calculated from semi-log graphs) of 0.24×10^{-4} M (n = 9). In contrast, it was significantly less potent at inhibiting KCl-induced responses (IC50 = 1.49×10^{-4} M, n = 9). The methyl ester (NFAme) and amido (NFAm) analogues showed no selectivity between 5-HT- and KCl-induced contractions with IC50 values of 1.64×10^{-4} M (n = 8) and 1.87×10^{-4} M (n = 9) for 5-HT responses, and 2.61×10^{-4} M (n = 8) and 2.55×10^{-4} M (n = 7) for KCl-induced responses, respectively. Our results suggest that alteration of the carboxylic acid moiety of niflumic acid reduces the selectivity and potency of its inhibitory action on 5-HT-induced contractile responses of the rat fundus, possibly via a reduced interaction with calcium-activated chloride channels.

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Funding: This study was supported by the CNPq and FUNCAP.

Introduction

The putative excitatory role of calcium-activated chloride ($Cl_{(Ca)}$) channels in the contractile action of various neurotransmitters on vascular and non-vascular smooth muscle has received growing interest (Large & Wang 1996; Chipperfield & Harper 2000). This has largely been due to the development of relatively selective inhibitors of these channels, such as the fenamates, typified by niflumic acid. This compound has been shown to inhibit calcium-activated chloride currents ($I_{Cl(Ca)}$) in isolated smooth muscle cells (Pacaud et al 1989; Akbarali & Giles 1993; Hogg et al 1994; Lamb et al 1994), although the exact mechanism of interaction of niflumic acid with the ion channel remains unclear. Nevertheless, niflumic acid has been increasingly utilized as a pharmacological tool for investigation of $Cl_{(Ca)}$ channels in functional studies in isolated tissues. We have previously demonstrated that niflumic acid inhibits noradrenaline (norepinephrine)-induced contraction of isolated rat aorta and, on the basis of comparative data with nifedipine, suggested that activation of $Cl_{(Ca)}$ channels may lead to a depolarization-induced opening of voltage-dependent calcium channels (VDCCs) causing extracellular calcium entry and consequent contraction (Criddle et al 1996). Subsequent studies have provided supportive data for this hypothesis in a number of smooth muscle preparations

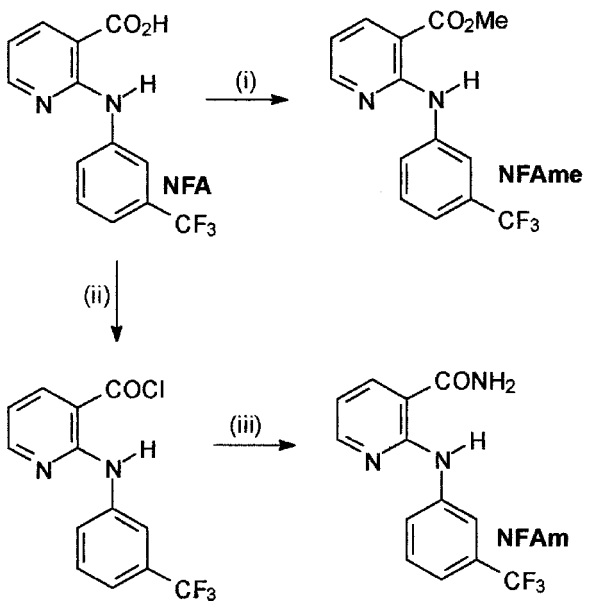

Figure 1 Synthesis of methyl 2-[3-(trifluoromethyl)-analino]-nicotinate (NFAme) and 2-[3-(trifluoromethyl)-analino]nicotinamide (NFAm). Reagents and conditions: (i) $(MeO)_2SO_2$, K_2CO_3, MeOH, rt, 2 h; (ii) $SOCl_2$, THF, rt, 16 h; (iii) NH_4OH (conc.), EtOAc, rt, 2 h.

(Criddle et al 1997; Yuan 1997; Hyvelin et al 1998; Lamb & Barna 1998). More recently, we have demonstrated a potent and selective inhibition of 5-HT-induced contraction of rat isolated stomach fundus by niflumic acid (Scarparo et al 2000), and this tissue may provide a simple and convenient preparation for the functional assay of analogues of niflumic acid. Methyl 2-[3-(trifluoromethyl)-analino]nicotinate (NFAme) and 2-[3-(trifluoromethyl)-analino]nicotinamide (NFAm) were synthesized to ascertain the importance of the carboxylic acid moiety that is present in niflumic acid but not in these derivatives (for synthetic scheme, see Figure 1). The inhibitory effects of these compounds on 5-HT- and KCl-induced contraction of the rat fundus were assessed. The preliminary results of this study have recently been communicated to the British Pharmacology Society (Birmingham BPS Meeting, December 2000; Criddle et al 2001).

Materials and Methods

Chemical procedures

Melting points were determined using a Gallenkamp melting point apparatus and are uncorrected. The ^{1}H NMR data were recorded on a Jeol JNMR-EX270 270 MHz spectrometer. Chemical shifts (δ ppm) and coupling constants (J Hz) were measured using trimethylsilane as the internal standard. Microanalytical data were carried out at the Department of Chemistry, University of Manchester.

Methyl 2-[3-(trifluoromethyl)-analino]nicotinate (NFAme)

To a solution of niflumic acid (500 mg, 1.77 mmol) in methanol (30 mL) was added K_2CO_3 (450 mg, 3.26 mmol), and dimethyl sulfate (0.5 mL, 670 mg, 5.3 mmol). The mixture was stirred at room temperature for 2 h. The resulting yellow suspension was filtered. The filtrate was extracted with ethyl acetate (2 × 75 mL), washed with water (2 × 25 mL) and brine (50 mL), dried over $MgSO_4$, and condensed at the pump to give a yellow oil which was chromatographed (eluting with ethyl acetate) to give NFAme (380 mg, 73%) as white solid: m.p. 74–75 °C, ^{1}H NMR ($CDCl_3$): δ 10.38 (1H, s, N*H*), 8.40 (1H, m, Ar-*H*), 8.29 (1H, dd, J = 6.0, 9.0 Hz, Ar-*H*), 8.10 (1H, t, J = 6.0 Hz, Ar-*H*), 7.89 (1H, m, Ar-*H*), 7.41 (1H, t, J = 6.0 Hz, Ar-*H*), 7.17 (1H, m, Ar-*H*), 6.79 (1H, dd, J = 6.0, 9.0 Hz, Ar-*H*), 3.95 (3H, s, CO_2CH_3). Calculated for $C_{14}H_{11}F_3N_2O_2$: C, 56.76; N, 9.46; H, 3.74; found: C, 56.40; N, 9.31; H, 3.97%.

Niflumic acid chloride

To a solution of niflumic acid (500 mg, 1.77 mmol) in anhydrous tetrahydrofuran (10 mL) was added excess thionyl chloride (1 mL). The mixture was stirred under N_2 for 16 h. The resulting yellow solution was condensed at the pump and chromatographed (eluting with 1:1, hexane–ethyl acetate) to give a yellow solid (420 mg, 79%), m.p. 215–216°C, ^{1}H NMR (DMSO-d_6): δ 10.67 (1H, s, N*H*), 8.47 (1H, m, Ar-*H*), 8.13 (2H, m, 2 × Ar-*H*), 7.87 (1H, m, Ar-*H*), 7.58 (1H, t, J = 6.0 Hz, Ar-*H*), 7.36 (1H, m, Ar-*H*), 6.99 (1H, m, Ar-*H*).

2-[3-(Trifluoromethyl)-analino]nicotinamide (NFAm)

To a solution of niflumic acid chloride (400 mg, 1.33 mmol) in ethyl acetate (20 mL) was added ammonia solution (conc., 20 mL). The mixture was stirred for 4 h at room temperature and the product was extracted with ethyl acetate (2 × 150 mL), washed with water (2 × 50 mL) and brine (50 mL), dried over $MgSO_4$, and condensed at the pump. The crude product was recrystallized from ethyl acetate to afford NFAm (220 mg, 59%) as white crystals: m.p. 284–285°C, ^{1}H NMR (DMSO-d_6): 10.73 (1H, s, N*H*), 8.37 (2H, m, CONH_2),

Table 1 Comparison of IC50 values for inhibition of 5-HT- (10 μM) and KCl-(60 mM) induced contractions of the rat isolated fundus by niflumic acid, NFAme and NFAm.

Compound	5-HT-induced contraction	IC_{50} (M) KCl-induced contraction	Relative potency ratio[a]
Niflumic acid	0.24×10^{-4} (0.11–0.37)	1.49×10^{-4} (0.76–2.22)#	6.2
NFAme	1.64×10^{-4} (0.51–2.77)*	2.61×10^{-4} (0.46–4.76)	1.6
NFAm	1.87×10^{-4} (0.56–3.18)*	2.55×10^{-4} (1.30–3.80)	1.4

Values are expressed as the mean of 7–9 observations, with 95% confidence limits included in brackets. [a]KCl-induced contraction divided by 5-HT-induced contraction. *$P < 0.05$, compared with NFA-induced inhibition of contraction. #$P < 0.05$, comparing 5-HT- and KCl-induced contraction.

8.31 (1H, m, Ar-*H*), 8.21 (1H, dd, J = 5.9, 9.0 Hz, Ar-*H*), 7.80 (1H, m, Ar-*H*), 7.54 (1H, t, J = 5.9 Hz, Ar-*H*), 7.30 (1H, m, Ar-*H*), 6.95 (1H, dd, J = 5.9, 9.0 Hz, Ar-*H*). Calculated for $C_{13}H_{10}F_3N_3O$: C, 55.52; N, 14.94; H, 3.58; found: C, 55.81; N, 14.52; H, 3.30%.

Inhibition assays

Institutional approval for the protocols used for animal experimentation was obtained. Male Wistar rats (250–350 g) were killed by stunning and cervical dislocation. The abdomen was opened and the stomach removed. The fundus was dissected and washed in fresh Tyrode's solution. Longitudinal strips (2 cm long) were carefully prepared and mounted vertically in an organ bath (10 mL capacity) containing Tyrode's solution bubbled with air (37°C, pH 7.4). Tissues were mounted under an initial resting tension of 2 g and left to equilibrate for a period of 1 h, following which a further 2 g was applied before starting the experimental protocol. Tension changes were recorded using isometric force transducers connected to a computerized data acquisition system (WINDAQ). The effects of niflumic acid (Sigma Chemical Company), and its analogues, NFAme and NFAm, were assessed on contractions induced by KCl (60 mM) and 5-HT (10 μM) with each agonist being evaluated in a separate experimental group. Following stable contractions to the contractile agent, niflumic acid (or analogue) was applied in increasing concentrations and further responses obtained (contact time of 15 min). Since dimethyl sulfoxide (DMSO) was used as a solvent for stock solutions of niflumic acid, the effects of this solvent at equivalent concentrations were assessed as time-matched controls, on 5-HT-induced contraction of the fundus.

Niflumic acid and analogues were prepared as stock solutions (10 mM) in DMSO and diluted on the day of the experiment in Tyrode's solution. The bathing solution was a standard Tyrode's solution of the following composition (mM): NaCl 136, KCl 5, $MgCl_2$ 0.98, $CaCl_2$ 2, NaH_2PO_4 0.36, $NaHCO_3$ 11.9, glucose 5.5. In solutions in which the potassium concentration was raised (60 mM), the NaCl concentration was concomitantly reduced to maintain osmolarity. The pH was maintained at 7.4 throughout the experimental period.

Data are expressed graphically as the mean of n observations $\pm$s.e.m. and curves were fitted using Microcal Origin. Inhibitory effects are expressed as % of control responses in the absence of the drug. IC50 values (concentration producing 50% of control response in absence of drug) were calculated by interpolation from semi-logarithmic plots and are expressed as mean values with 95% confidence limits. Statistical analysis was performed using analysis of variance and values were taken to be significantly different when $P < 0.05$.

Results

Inhibitory effects of niflumic acid on fundus contractions

Application of 5-HT (10 μM) to the rat fundus induced stable tonic contractions (1.67 ± 0.16 g, n = 26). In the presence of niflumic acid (1–100 μM) these tonic contractions were completely inhibited in a concentration-dependent manner, with an IC50 value of 20 μM (Table 1 and Figure 2). This inhibitory effect of niflumic acid was fully reversible on washout of the drug from the tissue. In control-paired tissues exposed only to the solvent DMSO at equivalent concentrations, the response to 5-HT did not diminish over the experimental period (n = 8). Application of KCl (60 mM) to the rat fundus induced reproducible tonic contractions (2.61 ± 0.34 g, n = 24), which were inhibited less potently by

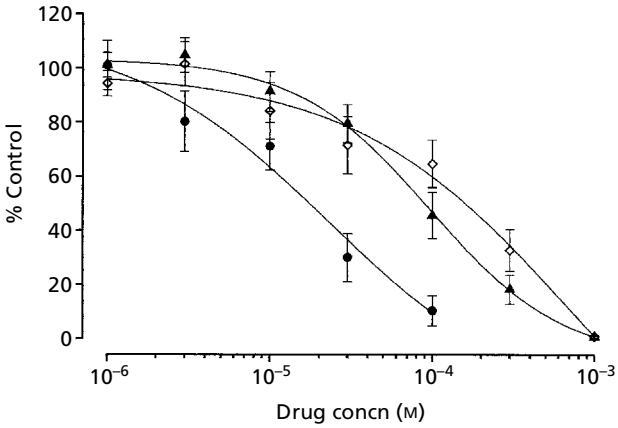

Figure 2 Inhibitory effects of niflumic acid (○), NFAme (▲) and NFAm (◇) on contractions induced by 5-HT (10 μM) in the rat isolated stomach fundus. Values are shown as means±s.e.m.

niflumic acid (IC50 value of 120 μM; Figure 3 and Table 1) as previously demonstrated (Scarparo et al 2000).

Inhibitory effects of niflumic acid analogues on fundus contractions

Analogues NFAme and NFAm were significantly less potent inhibitors of 5-HT-induced contractions than niflumic acid (6–8 fold order of magnitude; Figures 2 and 3), producing inhibition of both 5-HT- and KCl-induced responses with similar IC50 values (164–261 μM; Table 1 and Figure 3).

Discussion

This study has shown that alteration of the carboxylic acid moiety of niflumic acid changes the pharmacological profile of this class of compounds, reducing both its inhibitory selectivity and potency on 5-HT-induced contractions of the rat fundus. 5-HT-induced contraction in this tissue, mediated via the 5-HT_{2B} subtype of the receptor (Foguet et al 1992; Baxter et al 1994; Hoyer et al 1994; Kursar et al 1994), is likely to involve the activation of $I_{Cl(Ca)}$ as an integral part of the pharmacological transduction mechanism. Foguet et al (1992) have shown that $Cl_{(Ca)}$ channels are activated by stimulation of rat fundus 5-HT_{2B} receptors expressed in *Xenopus* oocytes, while 5-HT-induced activation of $I_{Cl(Ca)}$ has been reported in epithelial cells (Garner et al 1993). In addition, a more recent study in pulmonary artery has shown that both 5-HT-induced contraction and depolarization are inhibited by niflumic acid (Yuan 1997). In this respect the contractile pathway utilized by 5-HT is likely to be fundamentally different to that involved in KCl-induced contraction, which appears to be exclusively mediated by calcium influx through VDCCs (Bolton 1972) and is completely blocked by nifedipine in the rat fundus (Smaili et al 1991; Scarparo et al 2000). Therefore the bulk of current evidence suggests that the inhibitory actions of niflumic acid on 5-HT-induced contraction in this tissue are mediated via blockade of $Cl_{(Ca)}$ channels, leading to a closure of VDCCs. Previous work in the rat mesenteric vascular bed has shown that the inhibitory effects of niflumic acid on 5-HT-induced pressor responses are not mediated via a direct antagonistic action at plasmalemmal receptors, since no blockade was observed under calcium-free conditions (Criddle et al 1997), further strengthening the argument that the site of action of niflumic acid is distal to agonist-induced intracellular calcium release.

These data show that analogues NFAme and NFAm are less potent than the parent compound in inhibiting 5-HT-induced contraction in this tissue. This indicates that the interaction of NFAme and NFAm with $Cl_{(Ca)}$ channels may be weaker, as a result of the derivatisation of the carboxylic acid function of the molecule. Previous studies in smooth muscle have suggested that niflumic acid induces open-channel block by binding to a site within the chloride channel (Hogg et al 1994). It may be that an ionic interaction with a functional group of the ion-channel protein has been impaired by these structural modifications, or alternatively may reflect a more general change due to alteration of the net charge of the molecule. For example, niflumic acid would be predominantly in its zwitterionic form, whereas analogues NFAme and NFAm do not exist in this state.

As would be predicted by both of these hypotheses, we found no difference between the pharmacological profiles of the analogues in this study. Thus both inhibited 5-HT- and KCl-induced contractions with a similar potency, in contrast to niflumic acid, which was significantly more potent at inhibiting 5-HT-induced contractions than KCl-induced changes in the rat fundus (Scarparo et al 2000; this study). The inhibitory effects of niflumic acid and synthetic analogues on KCl-induced contraction may occur as a result of actions unrelated to inhibition of $I_{Cl(Ca)}$. For example, a recent study in vascular smooth muscle has demonstrated relaxant effects of niflumic acid that appear to be independent of chloride-channel blockade (Kato et al 1999), while niflumic acid ($\geqslant$ 50 μM) has been shown to directly modulate a variety of ion channels in addition to chlor-

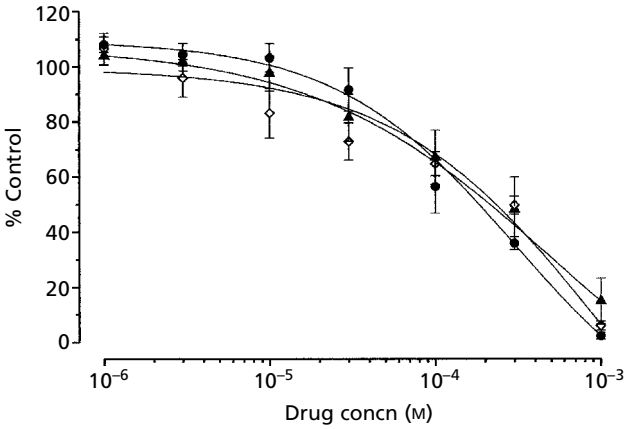

Figure 3 Inhibitory effects of niflumic acid (○), NFAme (▲) and NFAm (◇) on contractions induced by KCl (60 mM) in the rat isolated stomach fundus. Values are shown as means±s.e.m.

ide channels (Greenwood & Large 1995; Kirkup et al 1996) which may contribute to its relaxant effects in smooth muscle.

Conclusions

Our current results suggest that alteration of the carboxylic acid moiety of niflumic acid reduces the selectivity and potency of its inhibitory action on 5-HT-induced contractile responses of the rat fundus, possibly via a reduced interaction with $Cl_{(Ca)}$ channels. Based on these findings, it is possible that more potent or selective novel niflumic acid analogues may arise from the synthesis of novel niflumic acid derivatives which retain the carboxylic acid function of the parent molecule and this forms the basis of an on-going study. Since there is a certain controversy regarding the selectivity of chloride-channel blockers currently available commercially (e.g. see Kato et al 1999), the evaluation of novel structurally-related compounds is fundamental for the advancement of current knowledge of chloride channels in smooth muscle and may also lead to the discovery of potential therapeutic agents.

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