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GENISTEIN AND FLUORINATED ANALOGS SUPPRESS AGONIST-INDUCED AIRWAY SMOOTH MUSCLE CONTRACTION

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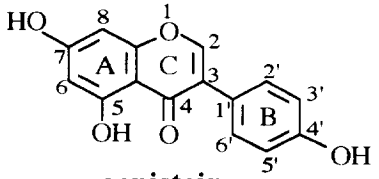
Abstract 4'-fluoro-5,7-dihydroxyisoflavone (DHIF), 2',4'-difluoro-5,7-DHIF and 3',4'-difluoro-5,7-DHIF were synthesized as analogs of the naturally occuring 5,7,4'-trihydroxyisoflavone (genistein). On guinea-pig trachea, genistein exhibited potent relaxant effect on strips precontracted with acetylcholine, KCl and histamine and a potent inhibitory effect on KCl-induced contraction. Fluorinated compounds showed less activity.

Smooth muscle contains high levels of tyrosine kinase activity [1] and evidence indicates that tyrosine kinase activity influences force development [2] by acting on mechanisms which regulate both intracellular free calcium levels and the effect of calcium on the contractile apparatus [3]. Genistein (5,7,4'-trihydroxy-isoflavone) belongs to the flavonoid family and has been identified as a potent inhibitor of tyrosine kinases [4]. Genistein has been also shown to block voltage-dependent Ca^{2+} channels (VOC) and to inhibit cyclic AMP and cyclic GMP phosphodiesterases (PDE) [5]. These latter effects may not be ascribable to inhibition of tyrosine kinases [6]. Therefore, genistein merits further attention as a prototype of novel therapeutic agent for the treatment of smooth-muscle-related lung, cardiovascular and gastrointestinal disorders. Genistein effects on smooth muscle have been extensively studied on vascular and gastrointestinal preparations but, to our knoweldge, there is no data on airway smooth muscle. We have shown on guinea pig trachea (manuscript in preparation) that genistein blocks VOC and inhibits cyclic AMP-PDE explaining its inhibitory activity on this preparation. We report in the present study, for the first time, the effects of genistein on airway smooth muscle and the effects of fluorinated analogs.



genistein

Structure-activity relationship and comparative studies have concluded that 5 and 7 hydroxyl groups are necessary for isoflavonoids activity [7]. We raised the question as to whether, conserving the 5,7 hydroxyl groups and substitution of B ring hydroxy group might possess inhibitory activity on airway smooth or, in a more general sense, what types of structural changes can be tolerated on the B-ring without loss of activity. It has been well known that fluorine can block the metabolic site of organic molecules, while maintaining the biological

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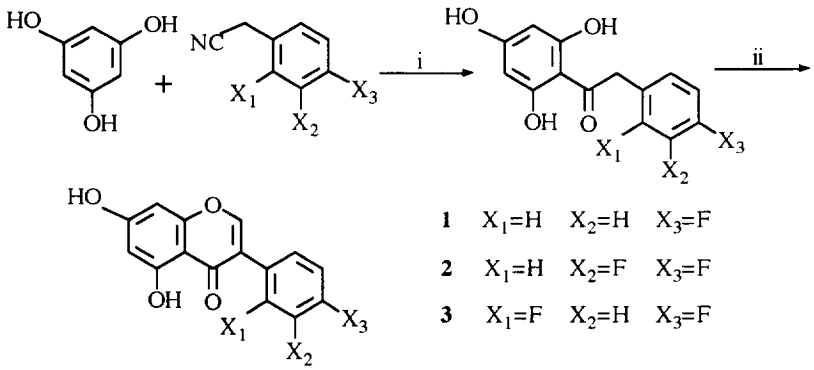
activity [8]. Once introduced, the high carbon-fluorine bond energy makes the substituents relatively resistant to metabolic transformations. The electronegativity of fluorine (4 vs 3.5 for oxygen) can have pronounced effects on the electron distribution in the molecule, affecting the basicity or acidity of neighboring groups, dipole moments within the molecule and the overall reactivity and stability of neighboring functional groups. As a consequence of the available electron density, fluorine can function as a hydrogen bond acceptor, when this observation is considered along with the fact that the carbon-fluorine bond length is 1.39 Å and the carbon-oxygen bond length is 1.43 Å, it is clear that replacement of hydroxyl by fluorine in an analog may be quite successful. Therefore substitution of fluorine can to help establish the effect of hydroxylation or other metabolic processes on the action of the molecule, as has been sucessfully applied in the synthesis of fluorinated vitamin D_3 analogs [9]. Herein, we describe the effect of substitution of 4'-hydroxyl group with fluorine on the biological activity. In particular, we describe the synthesis of 4'-fluoro-5,7-dihydroxyisoflavone **1**, 2',4'-difluoro-5,7-dihydroxyisoflavone **2** and and 3',4'-difluoro-5,7-dihydroxy-isoflavone **3**. Genistein, **1**, **2** and **3** were tested for their effects on guinea-pig airway smooth muscle.

Compounds listed in Scheme 1 were prepared starting from a substituted phenyl acetonitrile and phloroglucinol (1, 3, 5-trihydroxybenzene) [10].



Scheme 1. **i)** 1- HCl (gaz bubbling), $ZnCl_2$, Et_2O, 0°C. 2- HCl 2% aq., 3h (reflux).
ii) 1- BF_3-Et_2O, DMF, microwave (15 sec). 2- $MeSO_2Cl$, microwave (1 min).

Condensation of phloroglucinol and phenylacetonitrile catalyzed by zinc chloride gave the imine intermediate which was hydrolized *in situ* with aqueous HCl (2%) to provide the corresponding ketone. Microwave-mediated cyclization of the diaryl ketone by treatment with boron trifluoride-etherate and methanesulfonyl chloride in dimethylformamide (DMF) gave after chromatography the desired isoflavone as a white powder with an overall yield ranging from 50 to 60%. It is noteworthy that all steps can be performed without any purification of the intermediates [11].

All compounds were evaluated for their inhibitory properties on guinea-pig isolated trachea contractility. Guinea-pig trachea were cut into 4 rings. The rings were suspended in organ baths containing Krebs solution and tension developped by the tissue was measured isometrically as usually performed [12]. Concentration-relaxation curves to genistein and analogs were obtained in preparations precontracted with a concentration of acetylcholine (Ach), histamine or KCl giving 50% of the maximal tension. After a stable level of precontraction, the

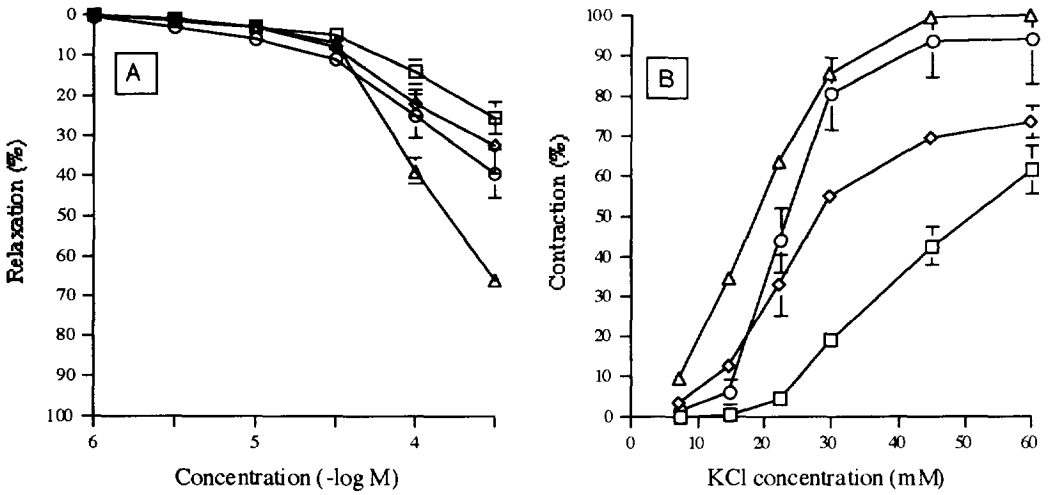


Figure 1. **A.** Relaxant effects of genistein (Δ), **1** (o), **2** (◊) and **3** (O) on guinea-pig trachea precontracted with acetylcholine. **B.** Effects of pretreatment with genistein on the contractile response to potassium chloride, KCl control (Δ), genistein 10^{-6} M (O), 10^{-5} M (◊) and 10^{-4} M (o). Each point represents the mean ± SEM for the number of experiments indicated in the text.

compounds were added to the bath in cumulative concentrations. Afterwards, aminophylline 3 mM was added to the bath to obtain the maximal relaxation. Only one concentration-response curve of each agent was obtained from each ring. The inhibitory effect of genistein and analog pretreatments (15 min incubation) has been also tested on the contraction induced by KCl, a depolarizing agent known to contract the airway smooth muscle by promoting the influx of extracellular calcium [12]. The relaxing effects of compounds are expressed as a percentage of the maximal relaxation produced by aminophylline 3 mM. For the studies on the inhibitory activity of genistein or analogs on KCl-induced contraction, results are expressed as percentages of the maximal contraction induced by Ach (3mM). Results are given as mean ± SEM. Statistical analysis was performed using analysis of variance. $P<0.05$ values were considered as significant. Genistein and analogs were all prepared in dimethyl sulfoxide (DMSO). At no time, the maximal concentrations of DMSO exceeded 1% by volume in the organ bath and produced by themselves any significant direct effect on basal tone of the trachea or on responses to reference compounds.

Genistein had the most potent relaxing activity on Ach-precontracted rings (Fig. 1-A). Its maximal effect measured at a 3 x 10^{-4}M concentration was 66 ± 2 % (n = 24) whereras the maximal effects of analogs **1**, **2** and **3** were respectively 26 ± 4 % (n = 9), 33 ± 7 % (n = 5) and 36 ± 6 % (n = 8), $P < 0.05$ vs genistein. Genistein and analogs **1**, **3** (analog **2** was not tested further due to its limited availability) were more potent on histamine- and KCl-precontracted trachea since their maximal effects were respectively 98 ± 21 % (n = 6), 69 ± 6 % (n = 5) and 73 ± 7 % (n = 6) on histamine contraction and 88 ± 5 % (n = 5), 71 % (n = 2) and 78 ± 6 % (n = 8) on KCl contraction. In all cases, the rank order of efficacy was genistein > analog **3** > (analog **2**) > analog **1**.

The -logEC_{50} value of genistein on Ach-precontracted rings, where EC_{50} is the concentration of the compound causing 50% relaxation, was 3.71 ± 0.04. As a relaxant on Ach-induced contraction, genistein was respectively 2,500-fold less potent than salbutamol (-logEC_{50} = 7.12) but 2.6-fold more potent than

aminophylline ($-\log EC_{50} = 3.28$) (data not shown). In addition, genistein potently and dose-dependently inhibited KCl-induced contraction which is known to be strickly dependent on extracellular Ca^{2+}-influx through VOC[12] (Fig. 1-B). Its maximal inhibitory effect on 30 mM KCl-induced contraction being 79 % at 10^{-4}M (n = 6) and 35 % at 10^{-5}M (n = 9), $P < 0.05$ vs control, whereas the inhibitory effects of analogs **1** and **3** at 10^{-4}M were respectively 36 % (n = 4) and 41 % (n = 5).

In summary, genistein exhibits a potent inhibitory effect on airway smooth muscle related at least in part to blockade of VOC. We have prepared three fluorinated analogs of genistein and evaluated them for smooth muscle inhibitory activity on guinea pig trachea. These results permit the following two conclusions. The 4'-hydroxyl group is necessary as well as 5 and 7 positions for genistein activity. The fact that substitution of 4'-OH by fluorine (a good hydrogen-bond acceptor) abolished the activity may suggest that 4'-hydroxyl group may participate in a hydrogen-donor phenomenon.

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