The Effect of Flavonol Glycosides on Opiate Withdrawal

Anna Capasso*

Dipartimento di Scienze Farmaceutiche, Università di Salerno, Via Ponte Don Melillo, 84084, Fisciano, Salerno Italia

Abstract: Our interest has been centered on flavonol glycosides from *Croton Menthodorus* (Euphorbiaceae) and *Aristeguietia discolor* (Asteraceae). In this respect, the effect of flavonol glycosides from *Croton Menthodorus* (Euphorbiaceae) and *Aristeguietia discolo*r (Asteraceae) was investigated on the naloxone-precipitated withdrawal contracture of the acute morphine-dependent guinea-pig ileum *in vitro*. Furthermore, the effect of these flavonol glycosides was also considered on DAGO (highly selective μ-agonist) and U50-488H (highly selective k-agonist) withdrawal to test whether the possible interaction of flavonol glycosides on opioid withdrawal involves μ- and/or k-opioid receptors. Flavonol glycosides from *Croton Menthodorus* ($1x10^{-5}$, $5x10^{-5}$ and $1x10^{-4}$ M) and from *Aristeguietia discolor* ($1x10^{-7}$-$1x10^{-6}$-$1x10^{-5}$ M) before or after the opioid agonists were able to both prevent and reverse the naloxone-induced contracture after exposure to μ (morphine and DAGO) or k (U50-488H) opiate agonists in a concentration-dependent fascion. Both acetylcholine response and electrical stimulation were reduced by flavonol glycosides treatment as well as the final opiate withdrawal was still reduced.

The results of the present study indicate that flavonol glycosides were able to produce significant influence on the opiate withdrawal *in vitro* and these compounds were able to exert their effects both at μ and k opioid agonists.

Key Words: Flavonol glycosides, croton menthodorus, aristeguietia discolor, opiate withdrawal, guinea pig ileum.

INTRODUCTION

Opiate withdrawal syndrome is a well known phenomenon and its cellular mechanisms have also been studied [1]. Although several methods may be used to induce opiate dependence both *in vivo* and *in vitro* [1], recently, the similarities of the enteric nervous system with the central nervous system make the first a most investigated tissue to study cellular biology of neurons [2]. Therefore, as the responses obtained from the isolated guinea-pig ileum share many features in common with those observed in the central nervous system, it has been thought that this tissue may provide a new and simple model for the study not only of the acute, but also of the long-term effects of opioids, such as tolerance and dependence [3]. Significant advances in understanding the dependence phenomenon have been obtained. For example, a strong naloxone-induced contraction could be obtained not only from the ilea of chronically opiate-treated animals but also from animals after a brief *in vitro* exposure to opioids [4]. This indicated that the cellular mechanisms of dependence may occur very rapidly following occupation of receptors and that these mechanisms are operating within the myenteric plexus. The characteristics of dependence development and its precipitation with naloxone in the guinea-pig ileum are very similar to those of acute dependence in experimental animals and men [5,6].

Croton species (Euphorbiaceae) widely distributed throughout tropical areas, are used in South America as folk medicine for the treatment of wounds, inflammation and cancer [7]. *Croton menthodorus* is rich in flavonol glycosides and they were identified as quercetin-3-*O*-(2-*O*-β-apiofuranosyl)-rutinoside (**Q**), rutin (**R**), Kaempferol-3-O-rutinoside (**K**) and kaempferol-3-O-(6-O-trans-p-coumaroyl)-b-D-glucopyranoside (**K1**) [8].

In the course of our systematic investigation of Peruvian medicinal Asteraceae, we examined *Aristeguietia discolor* DC. (vin-vino), a medicinal plant distributed in the Peruvian region and empirically used as an antispasmodic agent in local folk medicine. Four flavonol glycosides (**1-4**) were isolated from *Aristeguietia discolor* and identified their structures by spectral means as 3,5,6,7,8,4-hexahydroxyflavone-3-*O*-a-L-rhamnopyranosyl-(1→6)-b-D-galactopyranoside (**1**), 3,5,6,7,8,4-hexahydroxyflavone-3-*O*-a-L-rhamnopyranosyl-(1→6)-b-D-glucopyranoside (**2**), Kaempferol-3-O-rutinoside (**3**) and rutin (**4**) [9].

Flavonoids, natural compounds widely distributed in the plant kingdom, have been reported to display a number of biochemical and pharmacological activities [10]. Thus, several enzymes involved in intracellular signal transduction, such as lipoxygenase [11, 12] phospholipase A_2 [13], membrane ATPases [10] and a number of protein kinases including phosphatidylinositol 4-kinase [14] and protein kinase C [15] can be inhibited by flavonoids. Flavonoids can reduce LTB4 formation [10] as well as histamine release from mast cells and adhesion molecule expression [10). In addition, flavonoids have long been recognized as antioxidative and free radical scavenging agents [16-18]. Furthermore, flavonoids have been reported to inhibit guinea-pig ileal muscle contractions induced by PGE_2, LTD_4, acetylcholine, barium chloride, histamine, as well as electrical contractions [19,20]. Recently, we have demonstrated that flavonoids are also able to reduce *in vitro* morphine withdrawal [21].

Therefore, given the above evidence, we have decided to verify whether flavonol glycosides **Q**,**R**,**K** and **K1** from *Cro-*

*Address correspondence to this author at the Dipartimento di Scienze Farmaceutiche, Università di Salerno, *Via* Ponte Don Melillo, 84084, Fisciano, Salerno Italia; Tel/Fax: 089/964357; E-mail: annacap@unisa.it

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ton Menthodorus and Flavonol glycosides (**1-4**) from *Aristeguietia discolor* were also able to induce significant effects on morphine withdrawal.

Furthermore, the effect of flavonol glycosides were also determined on DAGO (highly selective μ-agonist) and U50-488H (highly selective k-agonist) withdrawal to test whether the possible interaction of these flavonol glycosides on opioid withdrawal involves μ- and/or k-opioid receptors.

MATERIALS AND METHODS

Animals

Male Charles River guinea-pigs (180-200 g) were used for all experiments. The animals were housed in colony cages (4 guinea-pigs to a cage) under standard light (light on from 7.00 a.m. to 7.00 p.m.), temperature (22±1° C) and room humidity (60%±10%) conditions for at least 1 week before the experimental sessions. Food and water were avalailable *ad libitum*.

Experimental Paradigm

As described previously [21], the ilea were allowed to equilibrate for 40-60 min without washing and the response to acetylcholine (Ach) was determined for three times (10^{-6} M) so that responses could be expressed as percentage of Ach maximum. A typical tracing of contraction responses of the ileum to repeated challenges with opiate and naloxone is shown in Fig. (**1**). After three similar Ach responses, the preparation was electrically stimulated for 10-20 min (0.5 msec pulse delivered transmurally, at a frequency of 10 sec at supramaximal voltage, 25 V). Before the addition of morphine to the bath, the electrical stimulation was switched off. Under these conditions, the first contact with the opioid agonist followed after a 4 min exposure by naloxone induced a strong contraction (about 60% of the Ach maximum). However, after washout, another Ach response was performed (to verify whether the ileum responsiveness was modified after withdrawal contraction) (Fig. (**1**); upper panel) and, after 30 min resting period under stimulation, a further 4 min exposure of the ileum (without electrical stimulation) to the opiate and naloxone elicited reproducible response. Following washout, Ach response (Fig. (**1**); middle panel) and another 30 min resting period under stimulation, the ileum responded again to the morphine and naloxone with the same intensiy (Fig. (**1**); lower panel). In our experiments, to avoid a possible tolerance to repeated morphine exposure, each prepara-

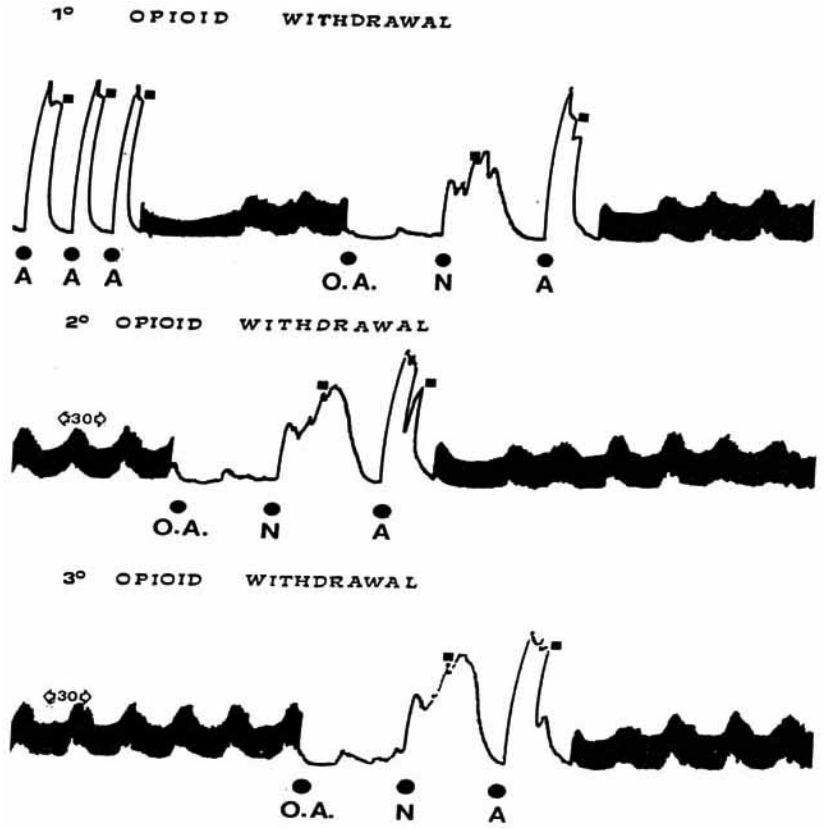

Fig. (1). Typical tracing of naloxone-precipitated withdrawal in isolated guinea-pig ileum exposed to opioid *in vitro*. Three similar acetylcholine response (upper panel), electrical stimulation, injection of the opioid agonist (O.A.) followed after 4 min of contact period by naloxone (N) which induces contraction (1st opioid withdrawal). After washout (□),another response was performed.

Middle panel: After 30 min resting period under electrical stimulation, a further 4 min exposure of the ileum to the O.A. and N elicited a reproducible response (2nd opioid withdrawal).

Lower panel: After another 30 min resting period under electrical stimulation, the ileum responded again to the O.A. and N with the same intensity (3nd opioid withdrawal).

tion was submitted only to three challenges with morphine and naloxone. Naloxone *per se* did not produce effects on "naive" preparations or those washed out after opiate contact.

Effects of Flavonol Glycosides on naloxone-precipitated withdrawal:

a) 3 Ach responses

b) electrical stimulation (10-20 min)

c) opiate injection in the absence of electrical stimulation (4 min) and addition of naloxone with a subsequent contraction (1st opioid withdrawal)

d) washout and Ach response

e) electrical stimulation (30 min)

f) flavonol glycosides **Q**,**R**,**K** and **K1** from *Croton Menthodorus* ($1x10^{-7}$-$1x10^{-6}$-$1x10^{-5}$ M) and flavonol glycosides (**1**-**4**) from *Aristeguietia discolor* ($1x10^{-5}$-$5x10^{-5}$-$1x10^{-4}$ M) without electrical stimulation injected 10 min before or after opioid followed by naloxone (2nd opioid withdrawal)

g) washout and Ach response

h) electrical stimulation (30 min)

i) final control opiate withdrawal (3nd opioid withdrawal)

Each experiment was performed at least on 6 to 9 isolated ilea from different animals.

During flavonol glycosides treatment, the duration of opioid exposure was 10 min when compared with the pre-drug period; accordingly, to avoid a possible influence of the contact period, preliminary experiments were performed to verify whether a contact period longer than 4 min might affect naloxone-induced contraction. No difference was observed when the contact period exposure of opioid agonist was 10 min.

Furthermore, in our experimental conditions to induce a strong contraction, each opioid agonist and naloxone were administered at the following concentrations:

Morphine (10^{-5} M) + naloxone (10^{-5} M)

DAGO (10^{-6} M) + naloxone (10^{-6} M)

U50-488H (10^{-8} M) + naloxone (10^{-5} M)

Drugs

Naloxone HCl, papaverine HCl and DAGO were purchased from Sigma (Milan, Italy); morphine HCl was from Carlo Erba (Milan, Italy) and U50-488H from Upjohn Co (Kalamazoo, MI).

Parameters Evaluation

Four parameters were evaluated:

1) Naloxone-induced contraction: The size of the contraction produced by the naloxone challenge was expressed as a fraction of the maximum contraction obtained with the subsequent addition of Ach in the same piece of tissue according to a modification of the method of Collier *et al*., 1981 [6]:

Tension ratio = (Response to naloxone / Maximum response to Ach) X100

2) Ach responses before and after treatment: Reduction or increase of the Ach responses in the post-drug state was expressed as a percentage of Ach response in the pre-drug state.

3) Electrically stimulation-induced contraction before and after treatment: Reduction or increase of the electrical stimulation contraction in the post-drug state was expressed as a percentage of the electrical stimulation contraction in the pre-drug state.

4) Naloxone-induced contraction before and after treatment: Reduction or increase of the naloxone contraction in the post-drug state was expressed as a percentage of the naloxone contraction in the pre-drug state.

Statistical Analysis

Results were tested for statistical significance using the Student's t-test for paired data when results before and after treatments on the same preparation were compared [22] and the ED50 were computed from the dose-response curve by the method of Litchfield and Wilcoxon [22].

RESULTS

In our experiments, flavonol glycosides **Q**,**R**,**K** and **K1** from *Croton Menthodorus* ($1x10^{-7}$-$1x10^{-6}$-$1x10^{-5}$ M) and flavonol glycosides (**1**-**4**) from *Aristeguietia discolor* ($1x10^{-5}$-$5x10^{-5}$-$1x10^{-4}$ M) were administered 10 min before the injection of opioid agonist. Therefore, as during the above treatments the contact period of the opioid agonist was 10 min when compared to the pre-drug period, to avoid a possible influence of the contact period, we performed a series of the preliminary experiments to verify whether a contact period longer than 4 min may affect naloxone contraction. No differences were observed when the contact period exposure of morphine was 4 or 10 min (data not shown).

Flavonol glycosides from *Croton Menthodorus* and *Aristeguietia discolor* although at different concentrations, were able to dose-dependently reduce the morphine, DAGO and U50-488H withdrawal. Tables **1** and **2** report the ED_{50} values of each flavonol glycosides.

Both acetylcholine response and electrical stimulation were also reduced by Flavonol glycosides treatment reduction *vs* control value and the final opiate withdrawal was still reduced (Table **3**).

DISCUSSION

While there are no data in literature on the effects exerted by flavonol glycosides **Q**,**R**,**K** and **K1** from *Croton Menthodorus* and flavonol glycosides (**1**-**4**) from *Aristeguietia discolor* on opioid withdrawal, the results of the present study indicate that flavonol glycosides were able to produce significant influence on the opiate withdrawal *in vitro* and to exert their effects on both μ and k opioid receptor agonists. This is the first paper indicating that flavonol glycosides are able to reduce opioid withdrawal and although the possible mechanisms by which flavonol glycosides from *Croton Men-*

Table 1. ED50 Values Flavonol Glycosides Q, R, K and K1 from *Croton Menthodorus* on Morphine, DAGO and U50-488H Withdrawal

Opioid Withdrawal	Q	R	K	K1
Morphine	$5.5x10^{-5}$ M ($1.1X10^{-5}$-$3.6x10^{-6}$)	$8.2x1^{-5}$ M ($1.7X10^{-5}$-$5x10^{-6}$)	$3.7x10^{-6}$ M ($2X10^{-5}$-$7x10^{-6}$)	$6.2x10^{-5}$ M ($8X10^{-5}$-$5x10^{-6}$)
DAGO	$4.5x10^{-6}$ M ($9X10^{-5}$-$2x10^{-6}$)	$5.3x10^{-5}$M ($2X10^{-5}$-$5x10^{-6}$)	$5.4x10^{-5}$ M ($4X10^{-5}$-$7x10^{-6}$)	$2.7x10^{-6}$ M ($3X10^{-5}$-$2x10^{-6}$)
U50-488H	$3.7x10^{-5}$ M ($2X10^{-5}$-$7x10^{-6}$)	$7.2x10^{-5}$ M ($3X10^{-5}$-$6x10^{-6}$)	$5.8x10^{-6}$ M ($1.13X10^{-5}$-$3.5x10^{-6}$)	$3.6x10^{-5}$ M ($2X10^{-5}$-$8x10^{-6}$)

Table 2. ED50 Values of Flavonol Glycosides 1-4 **from *Aristeguietia Discolor* on Morphine, DAGO and U50-488H Withdrawal**

Opioid Withdrawal	1	2	3	4
Morphine	$4.3x10^{-5}$ M ($6X10^{-5}$-$5x10^{-6}$)	$7.5x1^{-5}$ M ($1X10^{-5}$-$6x10^{-6}$)	$5.8x10^{-4}$ M ($3X10^{-5}$-$8x10^{-6}$)	$4.7x1^{-5}$ M ($7X10^{-5}$-$9x10^{-6}$)
DAGO	$6.7x10^{-5}$ M ($9X10^{-5}$-$3x10^{-6}$)	$4.9x10^{-5}$M ($5X10^{-5}$-$8x10^{-6}$)	$9.4x10^{-4}$ M ($2X10^{-5}$-$9x10^{-6}$)	$6.2x10^{-5}$M ($6X10^{-5}$-$4x10^{-6}$)
U50-488H	$7.2x10^{-5}$ M ($2X10^{-5}$-$7x10^{-6}$)	$3.6x10^{-5}$ M ($4X10^{-5}$-$7x10^{-6}$)	$3.3x10^{-5}$ M ($8X10^{-5}$-$2x10^{-6}$)	$8.2x10^{-5}$ M ($1X10^{-5}$-$7x10^{-6}$)

thodorus and *Aristeguietia discolor* control opiate withdrawal are still unclear, some possibilities should be considered.

Recently, it has been demonstrated that some flavonoids are able to reduce morphine withdrawal *in vitro* [21]. Flavonoids have been reported to possess good antiinflammatory activity by inhibiting the formation of arachidonic acid metabolites, [10-13]. Recently, it has been demonstrated that arachidonic acid metabolites are involved in the expression of opiate withdrawal since PLA2, cyclooxygenase and 5-lipoxygenase inhibitors are able to reduce opiate dependence [23]. Therefore, considering this relationship, we cannot exclude the possibility that reduction of opioid withdrawal by flavonol glycosides in our experiments may be related to their inhibition of arachidonic acid metabolites.

Considering the good activity of these flavonol glycosides, a concurrent mechanism could be considered in their opioid withdrawal control.

The ability of flavonol glycosides to reduce morphine withdrawal may be related to their anticholinergic properties. Our results strongly support the above hypothesis because the response to both acetylcholine and electrical stimulation were still reduced after washout of alkaloids, thus confirm-

Table 3. The Effect of Flavonol Glycosides Q, R, K **and** K1 **from *Croton Menthodorus* and Flavonol Glycosides** 1-4 **from *Aristeguietia Discolor* on Ach Response, Electrical Stimulation (E.S.) and Final Opioid Withdrawal (F.O.W.)**

Flavonol Glycosides	Ach Response	Electrical Stimulation	Final Opioid Withdrawal
Q	42.4±3.2**	47.8±2.4**	52.5±3.7**
R	43.7±2.9**	45.3±3.1**	58.4±2.1**
K	48.2±2.1**	43.8±2.7**	55.1±3.4**
K1	44.3±2.6**	46.5±2.9**	51.3±3.2**
1	52.4±2.2**	54.6±3.3**	48.3±2.7**
2	55.7±3.9**	51.0±3.8**	56.3±2.5**
3	60.1±4.5**	48.6±3.4**	57.2±3.6**
4	56.3±3.1**	57.5±4.1**	52.8±3.2**

Results are expressed as % of inhibition (mean±s.e.m.); *P<0.05; **P<0.01.

All data were compared to the pre-drug.

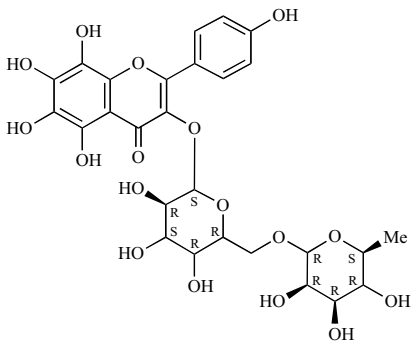

Fig. (2). An example of flavonol glycoside structure (flav4H-1-Benzopyran-4-one, 3-[[6-O-(6-deoxy-a-L-mannopyranosyl)-β-D- galactopyranosyl]oxy]-5,6,7,8-tetrahydroxy-2-(4-hydroxyphenyl)- (9CI).

ing a direct action both on post- and presynaptic acetylcholine receptors [11, 17].

It has been demonstrated that acetylcholine plays an important role in expression of opiate withdrawal because cholinergic agonist exacerbate opioid withdrawal; whereas, muscarinic and nicotinic blockers attenuate some aspects of the syndrome [24,25]. Furthermore, a large proportion of the contraction due to opioid withdrawal is caused by acetylcholine release since it can be blocked by atropine or hyoscine [26, 27].

The possible mechanism by which flavonol glycosides reduce opiate withdrawal is still unclear but some possibilities can be considered. It could be hypothesized that the effect observed may be related to the interaction of cholinergic system. A relationship between the cholinergic system and flavonoids has been reported [19,20] since the latter are able to inhibit contractions induced by Ach in isolated guinea-pig ileum as well as electrical stimulation [19,20]. Therefore, one may suggest that the effects observed may be related to the alteration of cholinergic neural activity and the ability of flavonoids to reduce opioid withdrawal may be related to their anticholinergic activity. Our results strongly support the above hypothesis since both Ach and electrical stimulation were still reduced after washout of flavonol glycosides, thus confirming a direct action of flavonoids both on post-and presynaptic acetylcholine receptors

Finally, whatever the mechanism may be, our data indicate that flavonol glycosides exert an important control on the opioid withdrawal phenomenon in the guinea pig ileum.

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ABBREVIATION

DAGO = (D-Ala2-methyl-Phe4-Gly5-ol)-enkephalin

U50-488H = (trans(±)-3,4,Dichloro-N-methyl-N[2-(1-pyrrolidinyl)ciclohexil]-benzene-acetamide methanesulphate salt)

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