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Synthesis and effects on cell viability of flavonols and 3-methyl ether derivatives on human leukemia cells

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ABSTRACT

Flavonoids are polyphenolic compounds which display an array of biological activities and are considered potential antitumor agents. Here we evaluated the antiproliferative activity of selected synthetic flavonoids against human leukemia cell lines. We found that 4′-bromoflavonol (flavonol **3**) was the most potent. This compound inhibited proliferation in a concentration-dependent manner, induced apoptosis and blocked cell cycle progression at the S phase. Cell death was found to be associated with the cleavage and activation of multiple caspases, the activation of the mitogen-activated protein kinase pathway and the up-regulation of two death receptors (death receptor 4 and death receptor 5) for tumor necrosis factor-related apoptosis-inducing ligand. Moreover, combined treatments using 4′-bromoflavonol and TRAIL led to an increased cytotoxicity compared to single treatments. These results provide a basis for further exploring the potential applications of this combination for the treatment of cancer.

1. Introduction

Flavonoids are naturally occurring polyphenolic compounds which may have beneficial effects for health and might be considered as potentially protective or therapeutic agents against cancer [1]. Among the different bioflavonoids, quercetin (3,3′,4′,5,7-pentahydroxyflavone) is one of the best studied and is widely distributed in nature. This natural product induces apoptosis in a variety of tumors [2–4], including leukemia [5]. Antitumor properties of flavonoids are mediated by different types of cell cycle arrest and induction of apoptosis, an active process of cell death which displays an essential role in the development and survival. It is also an important response to many chemotherapeutic agents. Cells that suffer this kind of cell death display features such as internucleosomal DNA fragmentation, the translocation of phosphatidylserine to the outside of the plasma membrane, apoptotic bodies formation and chromatin condensation. Caspases are fundamental executioners of apoptosis. They comprise a family of cysteine proteases which are synthesized as zymogens and are activated by proteolysis [6]. There are two main apoptotic pathways [7]. In the extrinsic pathway, apoptosis is mediated by death receptors (such as receptors for tumor necrosis factor-α, Fas and TRAIL) and involves caspase-8 activation. In the intrinsic pathway pro-apoptotic signals induce mitochondrial cytochrome *c* release to the cytosol, and promote the apoptosome assembly and caspase-9 activation. Both caspase-8 and caspase-9 activate caspase-3 which is responsible for specific cellular protein destruction during apoptosis [8]. The cellular response to this chemically-induced apoptosis is associated with the inactivation of the protein kinases involved in cell survival and with the activation of protein kinases that promote apoptosis. One of the most important aspects of apoptosis regulation is that it requires mitogen-activated protein kinases (MAPKs). MAPKs are a family of serine/threonine protein kinases directed to proline and function as intracellular mediators in response to diverse stimuli. They are activated by phosphorylation in threonine and tyrosine residues [9]. In mammals the MAPKs are mainly represented by the cascades

Abbreviations: ERK, extracelular signal-regulated kinase; IC_{50}, 50% inhibition of cell growth; JNK/SAPK, c-*jun* N-terminal kinases/stress-activated protein kinases; MAPK, mitogen-activated protein kinases; MEK, mitogen-activated extracellular kinases; MTT, 3-(4,5-dimethyl-2-thiazolyl-)-2,5-diphenyl-2*H*-tetrazolium bromide; $p38^{MAPK}$, p38 mitogen-activated protein kinases; TRAIL, tumor necrosis factor-related apoptosis-induced ligand.

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ERK (extracellular signal-regulated protein kinases) 1/2, the c-Jun NH_2-terminal kinases/stress-activated protein kinases (JNK/SAPK) and the p38 mitogen-activated protein kinases ($p38^{MAPK}$). The activation of ERK can trigger both anti-apoptotic and pro-apoptotic effects, depending on the stimuli and cell type [10]. In contrast, JNK/SAPK and $p38^{MAPK}$ are mainly activated by cytotoxic stimuli and are associated with pro-apoptotic actions in many cell types [11]. However, there are multiple exceptions to this rule.

Recent studies indicate that the combination of conventional cytotoxic compounds and new drugs directed at molecular targets can enhance the cell death induced by the tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) in tumor cells [12]. The identification of compounds that activate death receptors for TRAIL or that block anti-apoptotic effectors might constitute an important therapeutic advance. However, very little is known about the synergy between semi-synthetic compounds derived from natural products and apoptosis-inducing ligands such as tumor necrosis factor α (TNF-α), Fas and TRAIL. The search for compounds that can enhance TRAIL sensitivity in human leukemia cells is therefore of undoubted biomedical interest.

TRAIL induces selective apoptosis in several tumor cell lines and shows low toxicity against normal cells [13]. This distinctive feature has inspired the development of drugs to combat malignant diseases. Recombinant TRAIL and agonist antibodies that bind to death receptors have been used in clinical trials in phase I–II [14]. TRAIL can bind to the death receptors DR4 (death receptor 4) and DR5 (death receptor 5), which contain a cytoplasmic death domain, and to the decoy receptors DcR1 and DcR2, which lack a functional death domain. Decoy receptors compete with death receptors for the ligands and therefore block the apoptotic signals.

The results from several studies suggest that flavonoids with a hydroxy group at carbon 3 are potential anticancer agents [15] and also the presence of 3′-hydroxy-4′-methoxy groups on the B ring enhances cytotoxicity [16]. The justification for investigating methoxylated derivatives is provided by their chemopreventive properties, which are much greater than those seen in the more common unmethylated flavonoids, and the fact that they show increased metabolic stability [17]. In a previous study of naturally occurring and semi-synthetic flavonoids, we showed that methylation of hydroxyl groups of quercetin yields a compound with a higher antiproliferative activity against human cancer cell lines [18]. Moreover, it has recently been reported that molecules with halogen substituents in the B ring show improved anticancer activity relative to methoxylated, methylated or hydroxylated analogs [19].

Comparative studies that examine methylation of hydroxy group at C3 and the introduction of different substituents on the B ring and minor changes on the A ring, will greatly enhance knowledge of their impact on cytotoxicity. Here we synthesized a series of flavonols and methyl ether derivatives carrying varying substitutions at positions 5, 7, 2′, 3′, 4′, 5′. The substitutions included different electron-donating and electron-withdrawing groups, such as Cl, Br, CH_3O, CH_3, and OH, and allowed us to compare their anticancer activity with similar compounds tested against other tumor cell lines. We studied the effects of these synthetic flavonoids on viability of human leukemia cells and then examined the effects of the most potent, 4′-bromoflavonol, on apoptosis induction. This semi-synthetic flavonoid has been recently assessed for cytotoxicity against the human colorectal carcinoma cell line HCT116 [19] but, so far, its potential use in antileukemia therapy is largely unexplored. We have evaluated whether caspase activation and the MAPK cascade are involved in the mechanism of action. Finally, we have investigated whether 4′-bromoflavonol induces death receptors expression and the potential modulation of cell death in combination with TRAIL.

2. Chemistry

A selected combination of nine commercially available aldehydes (containing halogen, methoxy, methyl or aromatic groups) with two hydroxyacetophenones provided nine flavonols (**1**–**9**) and ten 3-methyl ether flavonols derivatives. The synthetic compounds differ in terms of their substituents on the B ring (2-phenyl group) and minor changes on the A ring (Fig. 1).

The synthesis takes advantage of the well-known two steps procedure for flavonols which combines a Claisen–Schmidt condensation of 2-hydroxyacetophenones and benzaldehydes with NaOH as base followed by a cyclization under Algar–Flynn–Oyamada reactions conditions (Scheme 1) [20]. Treatment of flavonols with 5 equiv. of iodomethane led to new methyl ethers **11**, **13**, **15**, **17** and **18** in high yield. The spectroscopic data obtained for known compounds were compared with those described in the literature in order to confirm the structures **1**–**5** [19], **7** [21], **9** [22], **10** [19], **12** [23], **14** [24], **16** [25], **19** [19], **20** [26]. The structure of new synthetic flavonols (**6**, **8**) and the methyl ether derivatives (**11**, **13**, **15**, **17** and **18**) was determined using 1D and 2D NMR experiments, high resolution mass spectroscopy and IR. Compound **19** was synthesized from quercetin by partial methylation reaction as previously described [21].

3. Biological results

3.1. Effect of the synthetic flavonoids on the growth and cell viability of human leukemia cell lines

In the present study, the potential cytotoxic properties of a series of 20 flavonoids, including nine flavonols and eleven 3-methyl ethers with different substituents on the B ring (2-phenyl group) and minor changes on the A ring were evaluated using HL-60 cells (Table 1). The results indicated that 4′-bromoflavonol (flavonol **3**) was the most cytotoxic compound with an IC_{50} value (the concentration that induces a 50% inhibition of cell growth) of 3.3 ± 0.7 μM. A 30-fold increase in antiproliferative activity was detected for 4′-substituted flavonols by increasing the size of the halogen from chlorine (flavonol **1**) to bromine (flavonol **3**). The properties of the bromine atom, including its higher volume and its polarizability together with the lower electron withdrawal at the benzene moiety compared with the chlorine atom, seem to play a key role in determining the potency of this compound on cell viability. The differences between flavonol **1** and flavonol **3** suggest that the higher potency of the latter might be due to steric and electronic properties conferred by the identity of the halogen on the B ring and are important in binding to the molecule target.

The introduction of a chlorine atom (flavonol **5**) or a methoxy group (flavonol **4**) on position 2′on the B ring improved the antiproliferative activity as compared with flavonol **1**, however, flavonol **5** was more potent than flavonol **4**. The position of chlorine on the B ring is important in conferring cytotoxicity, since 2′-chloroflavonol (flavonol **5**) exhibited a higher potency than 4′-chloroflavonol (flavonol **1**). The methylation of the hydroxyl group at position C3 of flavonol **1** to produce flavonol **12** improved the cytotoxicity. In contrast, methylation of the 3-hydroxy group of flavonol **3** to produce flavonol **13** did not enhance the potency against cell growth inhibition. The hydroxy group located at the C3 position seems to play a key role in determining the potency of flavonol **3** on cell viability since this is significantly more potent (7-fold) than the 3-methoxy derivative **13**. It thus seems unlikely that the C3 methoxy group is a major determinant of cytotoxicity. Although the exact target and binding site of this flavonoid has not