# Modulating Luminol-Dependent Chemiluminescence of Neutrophils by Flavones

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The effect of 14 flavones on luminol-dependent chemiluminescence of neutrophils was studied *in vitro*. Chemiluminescence was used in this study as an indicator for the production of a reactive oxygen species by neutrophils, stimulated by phorbol myristate acetate. While flavone-8-acetic acid, and most of the compounds tested, inhibited chemiluminescence, flavone and its 5-hydroxy-7-methoxy derivatives enhanced it by up to 150%. The most active inhibitors of photon emission were the glycosides. These results indicate that lipophilicity and some structural determinants modulate the chemiluminescent capacity of neutrophils.

Flavonoids are a group of naturally-occurring, low molecular-weight, benzo-gamma-pyrone derivatives abundant in plants [1]. They demonstrate a variety of pharmacological and biochemical effects in human and animal systems, such as anti-inflammatory, anti-allergic, anti-viral and anti-tumor properties [2–4]. Flavonoids are also known to inhibit a broad spectrum of enzymes and to scavenge free radicals [5–7]. They have a strong affinity for divalent metal ions, which is relevant to their cytotoxic mechanism [8–9]. As well, they activate immune-competent cells, through the induction of various cytokinins and interferons [10–11]. This divergence in their pharmacological and therapeutic applications stems from minor changes in their structures, indicating that a strong structure-activity relationship exists among this group [12].

The purpose of the present study was to evaluate, quantitatively, the activity of flavones on the metabolic activity of neutrophils, as they present themselves by generating oxygen radicals. As neutrophils are involved in a wide variety of immunological responses, this model was chosen because neutrophils have been known to be an integral part of the immune systems in all mammals. It is also known that many non-mitochondrial oxygen metabolites are involved in anti-bacterial and anti-tumoral activity, and, thus, it has been suggested that flavones are mediators in such processes [13–15]. The assay technique evaluates neutrophils' luminol-dependent chemiluminescence, using phorbol myristate acetate (PMA) as a stimulant for oxygen metabolism through the activation of protein kinase C [16–17].

## Materials and Methods

### *Chemicals*

Flavone-8-acetic acid (FAA; NSC-347512) was obtained from the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute, U.S.A., through the courtesy of Dr. K. Paull. All other flavones were purchased from Roth Chem. (Karlsruhe, Germany). Hanks' balanced salt solution (HBSS) without phenol red, phosphate-buffered saline (PBS), PMA, kit for myeloperoxidase assay, histopaque (densities of 1.119 and 1.077 g/l) and dimethylsulphoxide (DMSO) were purchased from Sigma Chem. Co. (St. Louis, M.O., U.S.A.). Luminol (5-amino-2,3-dihydro-1,4-phtalazinedione) was bought from LKB (Turku, Finland) and rabbit-liver glycogen was obtained from Fluka Chemie (Buchs, Switzerland). All compounds were of the highest available purity. The tested flavones were dissolved in DMSO, and the DMSO concentration in the final dilution did not exceed 1% v/v. The structural variations of the 14 test compounds are detailed in Table I.

### *Neutrophils*

Peritoneal exudate from male albino guinea pigs, 150–300 g BW, was collected by heparinized HBSS lavage, 18 h after intraperitoneal injection of 25 ml of 0.1% glycogen in PBS. The cells were

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purified by centrifugation on histopaque gradients and hypotonic lysis, and were suspended in HBSS. The final cell suspension contained a minimum of 98% neutrophils, as determined by Hanker's stain [18]. The method for cytochemical localization of myeloperoxidase involved the use of *p*-phenylenediamine and catechol. Viability was greater than 98%, as determined by trypan blue exclusion.

*Chemiluminescence* was measured as previously described [19], with a special low noise-count-rate photomultiplier (9514s, EMI, Middlesex, England). The bi-alkali photocathode of this photomultiplier has a spectral response of 300–660 nm. Washed neutrophils ($1 \times 10^6$) were suspended in HBSS and mixed with 10 µl of each of the test compounds' solution (each was 100 µM), or with 10 µl of DMSO as control. Luminol solution was then added, giving a final concentration of $5.6 \times 10^{-6}$ M, and the test-tubes were incubated at 37 °C. 5 min later, PMA solution was added, to yield a concentration of $1.6 \times 10^{-7}$ M, and chemiluminescence was measured for 15 min. The final volume of each sample was 1.0 ml.

## Results

The chemiluminescence kinetic profile of neutrophils pre-exposed to luminol and (5 min later) to $1.6 \times 10^{-7}$ M of PMA is presented in Fig. 1. The addition of luminol resulted in a slight, but significant, increase in the count rate, due to activation of the cells by glycogen before harvesting them. The increase in count rate, after the addition of PMA, was significantly larger, and peaked within 2 min. The total follow-up time of the chemiluminescence was 20 min, but its count rate was only registered from the time of addition of PMA, *i.e.* – for 15 min.

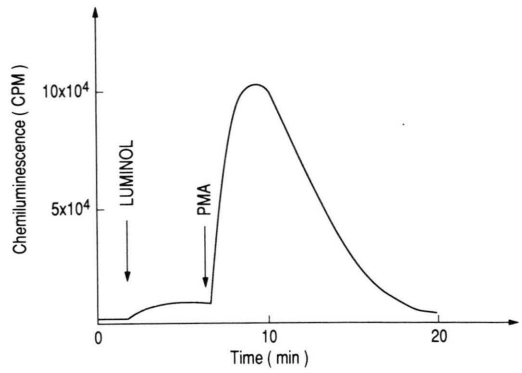
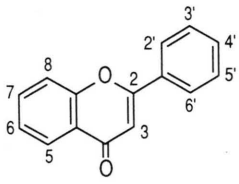



Fig. 1. Time course of chemiluminescence emitted from neutrophils exposed to $1.6 \times 10^{-7}$ M of PMA.

The level of inhibition or of stimulation (marked "–") of all 14 test compounds is presented in Table I, in descending order of their inhibitory activi-

Table I. Structure of the 14 flavones tested and their degree of inhibition of neutrophils' chemiluminescence ($n = 3-5$).




| | | | Inhibition (%, Mean ± SEM) |
|---|---|---|---|
| Stanin | 7-OH 8-OH | | 100.00 |
| Diosmin | 5-OH 7-ORu | 3'-OH 4'-$OCH_3$ | 86 ± 12 |
| Apigenin-7-glucoside | | 5-OH 7-OGl 4'-OH | 79 ± 08 |
| Apiin | 5-OH 7-OGl | 4'-OH | 76 ± 05 |
| Vitexin | 5-OH 7-OH 8-CGl | 4'-OH | 60 ± 04 |
| Gardenin | 5-OH 6,7,8,3',4',5'-hexamethoxy | | 56 ± 08 |
| Roifolin | 5-OH 7-ORGl | 4'-OH | 17 ± 02 |
| Flavone-8-acetic acid | | 8-$CH_2COOH$ | 14 ± 03 |
| Chrysin | 5-OH 7-OH | | 12 ± 03 |
| Acacetin | 5-OH 7-OH | 4'-$OCH_3$ | 10 ± 03 |
| Apigenin | 5-OH 7-OH | 4'-OH | 5 ± 03 |
| Mishkoin | 5-OH 7-$OCH_3$ | 4'-$OCH_3$ | –39 ± 06 |
| Genkwanin | 5-OH 7-$OCH_3$ | 4'-OH | –44 ± 04 |
| Flavone | | | –146 ± 22 |

OGl, CGl – glucosides; ORu – rutinoside; ORGl – rhamnoglucoside.

ty. Out of the 11 compounds which inhibited chemiluminescence, dihydroxyflavone, diosmin (two hydroxy and two esterified groups), apigenine-7-glucoside (two hydroxy and one esterified group) and apiin (two hydroxy and one esterified group) demonstrated the strongest inhibition. Vitexin (three hydroxy and one esterified group) and gardenin (one hydroxy and six esterified group) demonstrated a medium inhibition, while FAA (no hydroxyl groups), chrysin (two hydroxyls, no esterified groups), apigenin (three hydroxyls, no esterified groups), acacetin (two hydroxyls and one esterified group) and roifolin (two hydroxyls, one esterified groups) showed minor or practically no inhibition.

The flavone moiety exhibited the strongest enhancement of the neutrophils' chemiluminescence, while 5,4′-dihydroxy, 7-methoxy and 5-hydroxy, 7,4′-dimethoxy flavones exhibited a milder, but highly significant enhancement of the chemiluminescence. Hydrolyzing the glucose moiety at the 7 or 8 positions practically abolished the inhibitory activity of the compounds.

## Discussion

Chemiluminescence of neutrophils is a final result of luminol oxidation by strong oxidants like oxygen radicals and peroxides, two agents which emanate from enzymatic processes. On the other hand, flavonoids are well-known inhibitors of neutrophils' chemiluminescence (20), with flavonols (3-hydroxy-flavone derivatives) exerting the strongest inhibitory activity of them all. In a previous study from this laboratory we demonstrated that the trihydroxy-flavone galangin decreased neutrophils' chemiluminescence by 98%, while the dihydroxy-flavone chrysin decreased it by only 12%. A similar comparison was obtained between kaempferol (100% inhibition) *vs.* apigenin (5%), and kaempferide (99% inhibition) *vs.* acacetin (10%) [21]. The accepted mechanism(s) for decreased chemiluminescence is scavenging free radicals and peroxides by the flavonoids, inhibiting the enzymes involved in their generation [7, 8, 22] and inhibition of NADPH-oxidase and myeloperoxidase [23, 24].

In determining the possible structure-activity relationship of the investigated flavones, one should consider not only the number of the hydroxy group and their position on the benzopyrone ring, but also the hydrophobic/hydrophilic nature of each compound. This characteristic feature determines the rate of membrane transport and binding affinity [23]. Such properties may not only determine the mode of action (inhibition or stimulation) of each compound, but obviously its strength as well. Also we noticed that while the glycosidic derivatives, *e.g.* – diosmin (86%), apigenine-7-glucoside (79%), apiin (76%) and vitexin (60%) are much stronger inhibitors than their hydrolyzed counterparts, bare flavone is even a stronger inhibitor. It is also noteworthy that the concentration of the active compound is a major determinant of its activity: we have already shown that while low concentrations of quercetin stimulate activation of protein kinase C, high concentrations inhibits it [25]. This feature seems to be an important determinant in the immunotropic activity of flavonoids, where transmission processes of signals across cell membranes and other protein kinase-depending activities play an important role in their biological significance.

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