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SUPEROXIDE SCAVENGING ACTIVITY IN LEUKOCYTES AND ABSENCE OF CELLULAR TOXICITY OF A SERIES OF COUMARINS

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Abstract—Sixteen synthetic or plant-derived coumarins of dietary importance with different patterns of substitution were tested for their capacity to scavenge superoxide and for their cytotoxicity. Superoxide was generated by human polymorphonuclear leukocytes stimulated by phorbol myristate acetate and was measured using the reduction of ferricytochrome *c* or of nitroblue tetrazolium (NBT). Eleven of the coumarins, all lacking dihydroxy substitution, did not scavenge superoxide. Of the remaining five, the most potent scavenger was fraxetin (7,8-dihydroxy-6-methoxycoumarin) with an IC_{50} (concentration producing 50% inhibition) of 2.3 μM in the cytochrome assay and 5.8 μM using NBT. The other four coumarins (all containing *ortho*-dihydroxy catechol functions, and found previously to be pro-oxidant in cell-free systems by virtue of reduction of ferric to ferrous ions), themselves rapidly reduced cytochrome *c*. Therefore their effects on superoxide were measured using NBT, yielding IC_{50} values in the range 8.5 to 82.0 μM. Fraxetin and the other active and inactive coumarins were not directly cytotoxic at 100 μM to leukocytes or to erythrocytes, as shown by their failure to cause release of cytosolic lactate dehydrogenase or to cause haemolysis, respectively. However, all five dihydroxylated pro-oxidant coumarins were toxic to NS20Y neuroblastoma cells in 24 hr culture, whereas the other eleven coumarins were nontoxic. We conclude that 7,8-dihydroxylated coumarins such as fraxetin are agents which are not themselves directly cytotoxic and are capable of direct scavenging of superoxide anion radicals, an action which might be protective at sites of leukocyte activation during inflammation. However, in the presence of free ferric ions they may exert potentially damaging pro-oxidant actions, including cytotoxicity. This series of compounds provides a useful basis for structure–activity studies designed to achieve separation or combination of these properties.

Key words: coumarins; reactive oxygen species; radical scavengers; PMN leukocytes; anti-inflammatory drugs; cytotoxicity assays

In previous studies aimed at developing novel anti-inflammatory agents based on interactions with ROS§, we have investigated the abilities of a series of coumarins to scavenge superoxide anion radicals and peroxyl radicals [1, 2]. The prototype coumarin (1,2-benzopyrane) and its 7-hydroxy metabolite (umbelliferone) have been reported to have many biological activities (reviewed in Ref. 3), together with low mammalian toxicity [3, 4] but until recently the properties of the more complex plant-derived natural products have not been studied systematically. Such studies are of importance in view of the presence in the human diet of many coumarins and other plant polyphenolics, some of which are attracting interest on account of their anti-oxidant properties and possible roles in therapy and for food preservation [5–8].

There are some indications that coumarins might possess useful anti-inflammatory properties. For example, coumarin reduces tissue oedema and inflammation [9, 10], and the Japanese folk-medicine "Shinpi" which contains coumarins has anti-inflammatory properties (see Ref. 11 for comments). Furthermore, esculetin (6,7-dihydroxycoumarin) and various other related coumarin derivatives like fraxetin and daphnetin are recognised as inhibitors of the pro-inflammatory lipoxygenase and cyclo-oxygenase pathways of arachidonate metabolism [11–14]. In addition, esculetin has been reported to inhibit superoxide generation [15].

In view of this and our earlier studies on the inhibitory effects of coumarins on ROS generated in cell-free systems, we decided to evaluate their actions on the generation or scavenging of superoxide by human polymorphonuclear leukocytes and to establish if they demonstrate any cytotoxicity at effective concentrations in short term cell incubations (exposure to coumarins for 20 min), or after more prolonged exposure (24 hr) in a cell culture system.

MATERIALS AND METHODS

Reagents. PMA, NBT, MTT, histopaque 1083,

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§ Abbreviations: IC_{50}, concentration producing 50% inhibition; ROS, reactive oxygen species; PMA, phorbol myristate acetate; NADH, reduced form of nicotinamide adenine dinucleotide; PMN, polymorphonuclear neutrophil leukocyte; HBSS, Hank's balanced salt solution; LDH, lactate dehydrogenase; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; MTT, thiazolyl blue; NBT, nitroblue tetrazolium; OD, optical density.

NADH and pyruvic acid were obtained from Sigma Chemical Co. (Poole, U.K.). DMEM and FCS were from Gibco BRL, and dextran T-500 was from Pharmacia Fine Chemicals (Milton Keynes, U.K.). All other chemicals were from sources described previously [1].

Preparation of suspensions of human polymorphonuclear leukocytes. Blood from healthy non-medicated adult donors was drawn by venepuncture into one tenth volume 3.15% (w/v) trisodium citrate. Volunteers gave their consent, and local ethics committee guidelines were followed. The anti-coagulated blood was pooled in 50 mL polycarbonate tubes and centrifuged at 200 *g* for 15 min at room temperature. The upper platelet-rich layer was removed, and the residual blood was combined with an equal volume of dextran (prepared by dissolving 6 g of dextran T-500 plus 2.7 g of NaCl in 300 mL distilled water). After several inversions to ensure adequate mixing, the blood was left at room temperature for 45–60 min to permit the erythrocytes to sediment. The upper PMN-rich phase was then collected and concentrated by centrifugation at 200 *g* for 15 min at room temperature. Contaminating erythrocytes were removed by hypotonic lysis using ice-cold distilled water for 20 sec. The cell pellets were gently resuspended in 10 mL ice-cold modified HBSS free of Ca^{2+} and Mg^{2+}, and a solution of histopaque 1083 was carefully layered under the cell suspension to form a discontinuous gradient. This was achieved by dispensing 5 mL through a needle placed to the bottom of the centrifuge tubes, which were then spun for 40 min at 400 *g* at room temperature. The cell pellets were finally resuspended in HBSS containing 1.26 mM Ca^{2+} and 0.42 mM Mg^{2+}, so as to achieve a concentration of 2.5×10^6 cells/mL. The preparations contained more than 95% PMN, and viability of >95% was established by exclusion of Trypan Blue.

Incubation of human PMN for superoxide anion release: cytochrome c *method.* Aliquots of 1.0 mL human PMN leukocytes (2.5×10^6 cells/mL) were preincubated at 37° for 5 min with 10 μL of coumarin dissolved in ethanol (or an equivalent volume of ethanol for the controls). After this, the tubes were incubated for a further 5 min with 80 μM cytochrome *c* type III before the cells were stimulated with 1 μM PMA. After 10 min the reaction was terminated by centrifuging the tubes at 400 *g* for 10 min at 4°. The supernatants were poured into 1 mL cuvettes and the reduction of cytochrome *c* was measured as the change in absorbance at 550 nm. Compounds were screened initially at a concentration of 100 μM. Those showing at least 50% inhibition of cytochrome *c*-detectable superoxide generation were tested further at a range of five concentrations in order to determine the IC_{50} value. All incubations were performed in triplicate. Control experiments were performed using superoxide dismutase to verify that reduction of cytochrome *c* by PMA-treated leukocytes was due to the generation of superoxide, whereas the effects of the coumarins themselves on cytochrome *c* were tested by omitting the cells from incubations prepared as above.

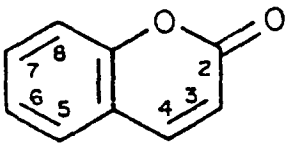
Incubation of human PMN for superoxide anion release: NBT method. Aliquots of 0.5 mL human PMN leukocytes (2.5×10^6 cells/mL) were pre-incubated at 37° for 5 min with 5 μL of coumarin dissolved in ethanol (or an equivalent volume of ethanol for the controls). After this, the tubes were incubated for a further 5 min with 5 μL of a 10 mM solution of NBT before the cells were stimulated with 1 μM PMA. After 10 min the reaction was terminated by centrifuging the tubes at 400 *g* for 10 min at 4°. The supernatants were poured off and 0.5 mL of a solution of DMSO/concentrated HCl (95:5 v/v) was added to the pellet. After solubilization with the aid of vigorous vortexing to lyse the cells, the reduced form of NBT (formazan) is released into the solvent. Aliquots of 0.25 mL were transferred to the wells of 96-well microtitre plates and the OD measured at 550 nm. Control experiments were performed using superoxide dismutase to verify that reduction of NBT in the PMA-treated leukocytes was due to the generation of superoxide. Appropriate blanks were run to rule out any direct interaction between coumarins and NBT.

Oxygen uptake by suspensions of human PMN. This was measured using a Clark-type oxygen electrode (37°, stirring, Hansatech Limited, Kings Lynn, U.K.). For these experiments, 1.5 mL of the leukocyte suspension (2.5×10^6 cells/mL) were placed into the sample compartment, and stimulated by adding 1 μM PMA. Consumption of oxygen was calculated as μmol O_2/min/mL. Where relevant, 100 μM test drug or equivalent volume of vehicle was added and preincubated with the cells for 5 min before adding PMA.

Cytotoxicity studies: human polymorphonuclear leukocytes. To measure LDH in the cell supernatants, we took 50 μL aliquots of cell supernatants prepared after pelleting the cells at the end of the PMA incubation period and added them to the wells of 96-well microtitre plates containing 200 μL 0.63 mM sodium pyruvate in 50 mM phosphate buffer pH 7.5 and 1.6 μL 40 mM NADH. The plates were then incubated at 37° with shaking every 20 sec, and the rate of decrease of absorption at 340 nm measured continuously in an Anthos HTIII microplate reader for 5 min. The amounts of LDH in the samples were obtained by comparing the slopes with those obtained for the "total content" in cells disrupted by treatment with 0.5% Triton X-100.

Cytotoxicity studies: haemolysis of washed human erythrocytes. Human blood was drawn into trisodium citrate and centrifuged as described above. The platelets and leukocytes were discarded. After this, the erythrocytes were resuspended in 30 mL PBS and centrifuged at room temperature for 10 min at 900 *g*. This was repeated three times, after which the red cells were resuspended in 20 mL PBS. Experiments were conducted using a 1:20 dilution of this stock which could be kept for up to 3 days at 4°. For the measurement of possible lytic agents, triplicated samples were added in 25 μL to tubes containing 0.9 mL 0.9% saline and 0.1 mL of the diluted erythrocytes (final concentration approximately 10^7 cells/mL). After 10 min incubation at 37°, the samples were centrifuged for 10 min at 3000 rpm and the OD measured at 405 nm in a microplate reader. Total cell haemoglobin was measured in cells

Table 1. Chemical structures of the coumarins used in this study



Number	Name	Trivial name	C-4	C-5	C-6	C-7	C-8
1	4-Hydroxycoumarin		OH	—	—	—	—
2	7-Hydroxycoumarin	Umbelliferone	—	—	—	OH	—
3	7-Methylcoumarin		—	—	—	CH_3	—
4	7-Methoxycoumarin	Herniarin	—	—	—	OCH_3	—
5	7-Hydroxy-4-methylcoumarin	4-Methylumbelliferone	CH_3	—	—	OH	—
6	7-Methoxy-4-methylcoumarin		CH_3	—	—	OCH_3	—
7	7,8-Dihydroxy-6-methoxycoumarin	Fraxetin	—	—	OCH_3	OH	OH
8	6,7-Dihydroxycoumarin	Esculetin	—	—	OH	OH	—
9	6,7-Dihydroxy-4-methylcoumarin	4-Methylesculetin	CH_3	—	OH	OH	—
10	7-Hydroxy-6-methoxycoumarin	Scopoletin	—	—	OCH_3	OH	—
11	7-Hydroxy-6-O-glucosylcoumarin	Esculin	—	—	OGlu	OH	—
12	7-Hydroxy-6-methoxy-8-O-glucosylcoumarin	Fraxin	—	—	OCH_3	OH	OGlu
13	5,7-Dihydroxy-4-methylcoumarin		CH_3	OH	—	OH	—
14	3,4-Dihydrocoumarin		—	—	—	—	—
15	7,8-Dihydroxycoumarin	Daphnetin	—	—	—	OH	OH
16	7,8-Dihydroxy-4-methylcoumarin	4-Methyldaphnetin	CH_3	—	—	OH	OH

lysed with 0.1% Triton X-100. Control incubations included appropriate volumes of the solvents.

Cytotoxicity studies: cultured NS20Y neuroblastoma cells. NS20Y mouse neuroblastoma cells were cultured in DMEM containing 10% FCS and 2 mM glutamine. Prior to confluence cells were transferred at about 10^4 cells/well to 96-well microtitre plates in DMEM containing 0.5% FCS and 2 mM glutamine. After allowing the cells to adhere overnight, test drugs dissolved in dimethylsulphoxide or vehicle (0.1%, v/v) were added to a final concentration of 100 μM and incubation continued for a further 24 hr. After this, 20 μL of a 5 mg/mL solution of MTT was added and left for 1 hr at 37°. The medium was then aspirated and 0.1 mL isopropanol added to solubilize the blue coloured tetrazolium. The plates were shaken for 5 min at room temperature and the absorption at 550 nm read in an Anthos HTIII plate reader. Control wells contained cells alone with no additions or cells plus DMSO.

RESULTS AND DISCUSSION

Sixteen coumarins with varying degrees of substitution (structures in Table 1) were tested for their ability to scavenge superoxide anion radicals generated by human polymorphonuclear leukocytes stimulated by PMA (Table 2). The generated superoxide radicals can be detected by their ability to reduce ferricytochrome *c* to ferrocytochrome *c* or by their ability to reduce NBT. It is assumed that compounds which apparently diminish the amount of cytochrome-reducible superoxide do so by scavenging (removing) the superoxide (as does superoxide dismutase, see Table 2), not by inhibiting the NADPH oxidase responsible for its generation (see below).

Eight of the coumarins (compounds **1**, **3**, **4**, **6**, **10**, **11**, **13** and **14**) did not significantly scavenge superoxide (Table 2) and can be regarded as inactive. Three compounds (**2**, **5** and **12**) showed intermediate scavenging ability which although significant was not of a magnitude to justify full dose response studies. Of the others, esculetin (**8**), 4-methylesculetin (**9**), daphnetin (**15**) and 4-methyldaphnetin (**16**) themselves rapidly reduced cytochrome *c* at the concentrations tested (10 and 100 μM). At lower concentrations these compounds neither reduced cytochrome *c* themselves nor scavenged the superoxide generated. Thus they could not be assessed for superoxide scavenging ability using this assay system, although the presumption is that they may well be effective scavengers of superoxide at the higher concentrations. This prediction was borne out by measuring their ability to quench the ROS-induced chemiluminscence in the xanthine/xanthine oxidase system: for example, compounds **15** and **16** inhibited luminescence by 98.9 and 99.6% at 100 μM (de las Heras and Hoult, unpublished studies), and by using the NBT assay, as described below.

Fraxetin (**7**) at 100 μM also reduced cytochrome *c*, but did not do so at 10 μM. At this lower concentration it proved to be a highly effective scavenger of superoxide radicals, inhibiting cytochrome *c* reduction by 99%, and was found to have an approximate IC_{50} value of 2.3 μM (Table 2).

Coumarins **8**, **9**, **15** and **16** (as well as fraxetin, **7**)

Table 2. Inhibitory effect of coumarins on superoxide generation by PMA-stimulated human PMN leukocytes

Number	Name	Trivial name	Percent inhibition at 100 μM cyt *c* method	IC_{50} μM cyt *c*	Percent inhibition at 100 μM NBT method	IC_{50} μM NBT
1	4-Hydroxycoumarin		1.1 ± 0.6	—	2.7 ± 0.7	—
2	7-Hydroxycoumarin	Umbelliferone	26.7 ± 3.3*	—	—	—
3	7-Methylcoumarin		8.3 ± 3.4	—	—	—
4	7-Methoxycoumarin	Herniarin	4.4 ± 0.6	—	—	—
5	7-Hydroxy-4-methylcoumarin	4-Methylumbelliferone	21.7 ± 2.2*	—	—	—
6	7-Methoxy-4-methylcoumarin		8.7 ± 2.7	—	—	—
7	7,8-Dihydroxy-6-methoxycoumarin	Fraxetin	99.1 ± 0.5*†	2.3	—	5.8
8	6,7-Dihydroxycoumarin	Esculetin	‡	—	77.4 ± 5.2*	33.9
9	6,7-Dihydroxy-4-methylcoumarin	4-Methylesculetin	‡	—	58.1 ± 2.9*	82.0
10	7-Hydroxy-6-methoxycoumarin	Scopoletin	9.0 ± 1.8	—	—	—
11	7-Hydroxy-6-O-glucosylcoumarin	Esculin	6.7 ± 0.9	—	—	—
12	7-Hydroxy-6-methoxy-8-O-glucosylcoumarin	Fraxin	44.2 ± 6.4*	—	—	—
13	5,7-Dihydroxy-4-methylcoumarin		2.0 ± 0.5	—	—	—
14	3,4-Dihydrocoumarin		0.6 ± 0.4	—	—	—
15	7,8-Dihydroxycoumarin	Daphnetin	‡	—	95.4 ± 1.9*	10.6
16	7,8-Dihydroxy-4-methylcoumarin		‡	—	97.0 ± 2.4*	8.5
Reference	Superoxide dismutase (70 U/ml)		99.3 ± 1.0*	3.1 U/ml	93.3 ± 2.3*	11.1 U/ml

Results show means ± SEM for six tests at each concentration; columns marked—not tested. For determination of IC_{50} value, five concentrations were used.

* Indicates statistically significant inhibition, $P < 0.01$ by Student's unpaired *t*-test.

† Fraxetin (**7**) reduces cytochrome *c* at 100 μM; the value shown was obtained using 10 μM.

‡ Not tested, because the coumarin itself reduces cytochrome *c* at 100 and 10 μM (see text).

Table 3. Effect of fraxetin on the oxygen uptake by PMA-activated human PMN leukocytes

Reaction conditions	O_2 consumed, nmol/min
Cells alone (2.5×10^6 cells/mL)	0.3 ± 0.4
Cells + PMA 1 μM	17.5 ± 0.7*
Cells + fraxetin 100 μM + PMA 1 μM	18.4 ± 0.7*
Cells + SOD 70 U/mL + PMA 1 μM	14.7 ± 0.2*†

Results show means ± SEM for six tests.
* Indicates significantly different from cells alone, $P < 0.01$ by Student's unpaired *t*-test.
† Indicates significantly different from PMA-activated cells, $P < 0.05$ by Student's unpaired *t*-test.

were therefore tested in the PMA-activated human neutrophils using NBT to detect scavenging of superoxide. As expected, all compounds were found to be active when tested at 100 μM (Table 2). Further experiments showed that fraxetin was the most potent scavenger (IC_{50} 5.8 μM, cf. 2.3 μM when using the cytochrome *c* assay), with the other 7,8-dihydroxy substituted coumarins also very active (IC_{50} values 8.5 and 10.6 μM for **16** and **15**, respectively). The two 6,7-dihydroxy coumarins (**8** and **9**) were somewhat less active.

In general, these results for the 16 coumarins as scavengers of phagocyte superoxide radicals agree with our earlier findings using superoxide generated by action of xanthine oxidase on hypoxanthine [1]. In that study, fraxetin was also the most active scavenger and compounds **8**, **9**, **15** and **16** similarly could not be assessed. However, there are some minor points of difference: compound **12** (fraxin) is active against neutrophil-derived superoxide but not in the xanthine oxidase system, whereas compound **13** (5,7-dihydroxy-4-methylcoumarin) shows the opposite profile. Whilst there are no obvious reasons for this discrepancy, it indicates that screening of potentially useful scavengers is best done on the precise target in question.

Insofar as the structure–activity relationships are concerned, it is notable that the active or potentially active compounds possess *ortho*-dihydroxyl functions characteristic of catechols, whereas substitution of a catecholic hydroxyl diminishes activity (**12** vs **7**). For example, *meta*-dihydroxy substitution (**13**) is highly detrimental, although single hydroxyl substitution at position 7 does allow some measure of activity (compounds **2** and **5**). Substitution of this hydroxyl abolishes activity (compare **3** and **4** vs **2**). The role of catecholic functions in determining a high potency has frequently been noted before in relation to the ability of flavonoids and other phenolics to inhibit arachidonate metabolism (e.g. Refs 16–21) and to scavenge superoxide and other ROS [22–27].

It has been proposed previously that the inhibition by coumarins of the reduction of ferricytochrome *c* by superoxide is due to their ability to scavenge the radicals, rather than by preventing their formation [1]. We therefore checked this by using an oxygen electrode to test the effects of fraxetin on the consumption of oxygen during the respiratory burst of human polymorphonuclear neutrophils activated by PMA (Table 3). The results show that fraxetin did not influence the enhanced oxygen consumption after stimulating the cells with PMA, demonstrating that it does not inhibit the NADPH oxidase. However, we observed a small reduction in the rate of oxygen consumption after addition of superoxide dismutase (Table 3); this is likely due to the more rapid dismutation of superoxide in the presence of this enzyme than in its absence, a process which effectively returns a proportion of molecular oxygen to the medium (see Ref. 28 for discussion). Incubation of cells with fraxetin alone did not alter oxygen consumption (data not shown), showing that any inhibitory effect it might have on superoxide in the presence of stimulant cannot be due to prior stimulation followed by desensitization.

Three sets of experiments were performed in order to determine whether the 16 coumarins exert any significant cellular toxicity at the highest concentrations used (100 μM). The intention was to determine short term "direct" cytotoxicity by incubating leukocytes and erythrocytes for 20 min with the coumarins, and to investigate whether prolonged contact (24 hr) might cause toxicity. For the latter experiments we chose a cultured mouse neuroblastoma cell line.

None of the 16 coumarins at 100 μM demonstrated marked toxicity to either human PMN leukocytes (Fig. 1) or erythrocytes (Fig. 2), although there was a small increase in LDH leakage from the leukocytes caused by **16** and small reductions caused by **1**, **2**, **4**, **7**, **9**, **11**, **13** and **15** (Fig. 1), some of which were significant. In the case of red cell haemolysis, **12**, **13** and **16** produced a slight increase in haemoglobin release, of a magnitude far less than that caused by a 100-fold smaller concentration of the bee venom peptide melittin (Fig. 2). Overall, it is clear that high concentrations of the coumarins do not compromise cell membrane integrity after short term contact.

In the case of their action on cultured neuroblastoma cells, 11 of the 16 coumarins again did not exhibit signs of cellular toxicity after 24 hr contact. However, coumarins **7**, **8**, **9**, **15** and **16** all caused a marked and significant reduction in MTT metabolism to its chromogen in the cells (Fig. 3). This indicates that mitochondrial dehydrogenase activity in the cells had been reduced (although not completely

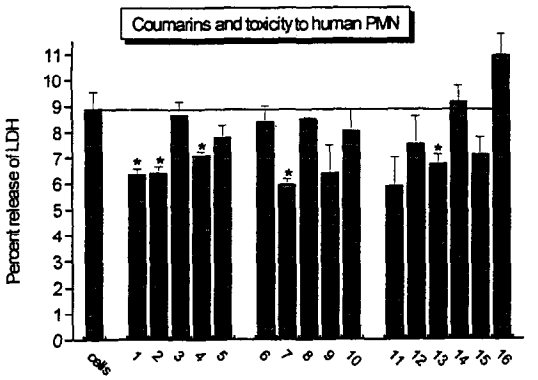


Fig. 1. Effects of coumarins on integrity of human PMN as measured in terms of release of cytosolic LDH. Coumarins were added at 100 μM in ethanol (an equivalent volume was added to cells alone, left hand column). Total lysis of the cells was achieved using 0.2% Triton X-100 for measurement of total cellular LDH activity. Results show mean ± SEM for three tests. *Indicates a statistically significant reduction in LDH leakage, $P < 0.05$ by Student's unpaired t-test.

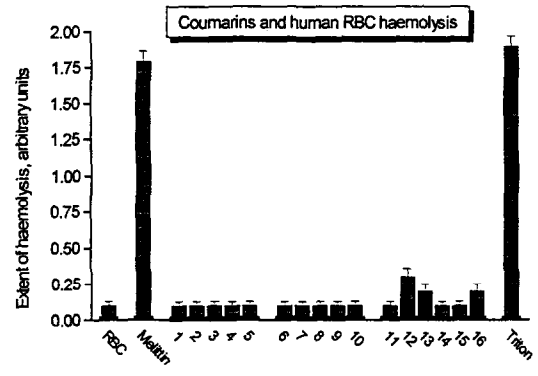


Fig. 2. Effects of coumarins on erythrocyte membrane integrity. Coumarins were added at 100 μM in ethanol (an equivalent volume was added to RBC alone, left hand column). Melittin was tested at 1 μM. Total lysis of the cells was achieved using 0.2% Triton X-100. Results show mean ± SEM for three tests.

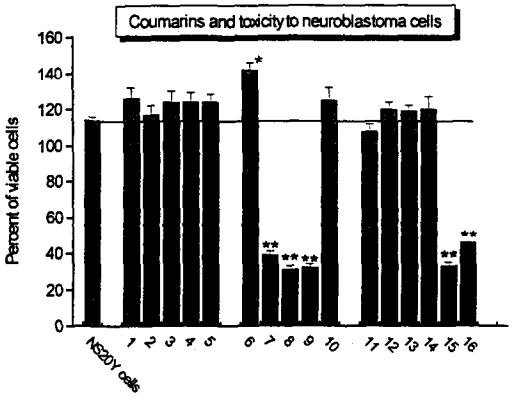


Fig. 3. Effects of coumarins on integrity of cultured NS20Y mouse neuroblastoma cells. Coumarins were added at 100 μM in DMSO (final concentration of solvent 0.1%) to microwells, incubated for 24 hr and assessed for cell viability using MTT as described in Materials and Methods. The column marked "NS 20Y cells" indicates results for wells to which DMSO was added on its own. The calculated values for cell viability are normalized against those obtained for parallel cell cultures which did not receive any addition at all (the DMSO solvent has a small stimulatory effect in terms of MTT metabolism over this time period). Results show mean ± SEM for nine wells (using cells obtained from three or more batches), except cells alone ($N = 60$), and *, ** indicates statistically significant difference with respect to cells alone, $P < 0.05$, 0.01 by Student's unpaired t-test.

abolished), a process which correlates directly with cell viability [29]. As shown above, these are the five coumarins showing high activity as scavengers of superoxide but which are also capable of exerting pro-oxidant effects [1]. This is thought to be due to their capacity to reduce ferric ions to the ferric form and may also underly their cytotoxic activity shown here.

Taken together, these results shown that various coumarins, especially those possessing *ortho*-dihydroxyl functions, are capable of scavenging superoxide anions generated by activated phagocytic neutrophils. These radicals are implicated in various aspects of the inflammatory process [30–32], either in their own right or as precursors of more damaging species such as hydroxyl radicals [33, 34]. Scavenging of such radicals by non-toxic compounds might therefore have beneficial protective effects. For this reason, substances such as fraxetin which are already present in the human diet and which have low toxicity in the absence of free transition metals (the normal state in extracellular fluids) may be worth evaluating in models of tissue injury in which reactive oxygen species are implicated [35, 36]. Moreover, because the coumarins also inhibit eicosanoid generation [37], they provide an interesting series of compounds on which to base more detailed structure–activity studies aimed at discovering combinations or separations of anti-oxidant and anti-eicosanoid properties in non-toxic molecules.

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