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Evaluation of the anti-inflammatory and cytotoxic effects of anthraquinones and anthracenes derivatives in human leucocytes

Rong-Fu Chen, Yuh-Chiang Shen, Hsu-Shan Huang, Jyh-Fei Liao, Li-Kang Ho, Yueh-Ching Chou, Wen-Yen Wang and Chieh-Fu Chen

Department & Institute of Pharmacology, National Yang-Ming University, Taipei, Taiwan

Rong-Fu Chen, Jyh-Fei Liao, Li-Kang Ho, Chieh-Fu Chen

National Research Institute of Chinese Medicine, Taipei, Taiwan

Yuh-Chiang Shen, Chieh-Fu Chen

Institute of Biological Sciences, College of Life Science, National Chung-Hshing University, Taichung, Taiwan

Yuh-Chiang Shen

School of Pharmacy, National Defense Medical Center, Taipei, Taiwan

Hsu-Shan Huang

Department of Pharmacy, Veterans General Hospital, Taipei, Taiwan

Yueh-Ching Chou

School of Pharmacy, Taipei Medical University, Taipei, Taiwan

Yueh-Ching Chou

Department of Surgery, Tao-Yuan General Hospital, Department of Health, Taiwan

Wen-Yen Wang

Correspondence: C.-F. Chen, National Research Institute of Chinese Medicine, No 155-1 Li-Nung Street, Section 2, Shih-Pai, Taipei 112, Taiwan.
E-mail: auth.tep@msa.hinet.net

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Abstract

A variety of anthracene- and anthraquinone-related derivatives, modified from three types of lead structures, including 9-acyloxy 1,5-dichloroanthracene (type I), 1,5-bisacyloxy-anthraquinones with O-linked substituents (type II) and 1,5-bisacyloxy-anthraquinones with S-linked substituents (type III), were synthesized and evaluated by an in-vitro bioassay for their anti-inflammatory and cytotoxic effects in human leucocytes. Among these derivatives, type I compounds displayed potent anti-inflammatory activity against phorbol-12-myristate-13-acetate (PMA)-induced superoxide anion production, a bio-marker of inflammatory mediator production by neutrophils, with 50% inhibition (IC50) concentrations (μM) for compounds **1f**, **1g**, **1h** and **1m** being 13.8 ± 3.0, 6.3 ± 4.1, 33.2 ± 1.3 and 33.9 ± 5.7, respectively. Type II and type III derivatives (i.e., 1,5-bisacyloxy anthraquinone-related compounds) and the reference compound, emodin, exhibited relatively minor (20–40%) inhibitory effect against superoxide production by neutrophils. Furthermore, none of these compounds showed a significant cytotoxic effect in human neutrophils. In conclusion, these results suggest that compounds modified from 9-acyloxy 1,5-dichloroanthracence (type I) are more powerful than the other two types as anti-inflammatory drugs. This is the first demonstration that derivatives modified from anthracenes or anthraquinones possess anti-inflammatory activity with no significant cytotoxicity in human neutrophils.

Introduction

Anthraquinone-related chemicals are widely used as anti-tumour drugs. However, their clinical effectiveness is limited by their side effects, especially dose-dependent cardiotoxicity. Numerous reports have demonstrated that the cardiac side effects of these drugs are the result of electron-transferring activity catalysed by NADPH dehydrogenase, NADPH cytochrome P450 reductase or xanthine oxidase, leading to the formation of reactive oxygen species (ROS) (Pawlowska et al 2003). However, in addition to pro-oxidative activity, anthraquinones have also been reported to exhibit anti-oxidative and radical-scavenging effects in rat hepatocytes (Malterud et al 1993).

Oxidative stress resulting in cell and tissue damage, such as lipid peroxidation and DNA damage, are widely regarded as the main pathological processes implicated in numerous diseases (Sies 1991). Many phenolic compounds, such as to those occurring naturally, including flavonoids, are known for their anti-oxidative potential, which has been shown to be beneficial in the prevention of oxidative stress-related disorders (Middleton et al 2000). Whether anthraquinones, with structure similar to those of the flavonoids, possess anti-oxidative or anti-inflammatory effects remains unclear. We have previously reported that modification of the structure of anthralin reduces its undesirable pro-inflammatory effects (Muller et al 1997). Recently, we have been engaged in the modification of anthracene and anthraquinone structures to develop novel analogues of anthracene or anthraquinone. We found that many of these modified compounds showed potent antioxidant activity (Huang et al 2001).

To further understand whether these anthracene- and anthraquinone-related derivatives could act as anti-inflammatory drugs, we synthesized different series of

anthracene- and anthraquinone-related derivatives, modified from three types of lead structures, including 9-acyloxy 1,5-dichloroanthracene (type I), 1,5-bisacyloxy-anthraquinones with O-linked substituents (type II) and 1,5-bisacyloxy-anthraquinones with S-linked substituents (type III). Herein, we compared the anti-inflammatory and cytotoxic effects of these three types of anthraquinones in human leucocytes as evaluated by an in-vitro bioassay system.

Materials and Methods

Anthraquinones and anthracenes derivatives

Anthracene and anthraquinone derivatives were synthesized in our laboratory as described in previous studies (Huang et al 2001). For the evaluation of the activity of these compounds, except where indicated, 10 μL of the solution of the test compound was added to 1.0 mL neutrophil suspension and incubated at 37°C for 10 min before the addition of 1 μM of phorbol-12-myristate-13-acetate (Sigma), a neutrophil activator.

Human neutrophil isolation

All experimental protocols were approved by our Institutional Review Board in accordance with international guidelines and informed consents were obtained from the subjects who participated in this study. Preparation of human neutrophils was obtained by vein-puncture from adult healthy subjects and collected into syringes containing heparin (20 U/mL blood) according to our previous report (Shen et al 2003). Neutrophils were isolated by the Ficoll gradient centrifugation method, followed by lysis of contaminating erythrocytes. Briefly, blood samples were mixed with an equal volume of 3% dextran solution in a 50-mL centrifuge tube and incubated in an upright position for 30–40 min at room temperature to allow sedimentation of erythrocytes. The upper, leucocyte-rich layer was then collected and subjected to centrifugation at 250 *g* for 15 min at 4°C. After centrifugation, the pellet was suspended immediately in a volume of phosphate-buffered saline (PBS) equal to the starting volume of the blood. The cell suspension was then apportioned, 6 mL per tube, into 15-mL centrifuge tubes, followed by laying 8 mL of 1.077 g mL^{-1} Ficoll solution (Histopaque 1077; Sigma Chemicals Co., St Louis, MO) beneath the cell suspension, using a pipette. After centrifugation at 400 *g* for 40 min at 20°C without braking, the upper (PBS) and lower (Ficoll) layers were carefully removed, leaving the granulocyte/erythrocyte pellet. To remove residual erythrocytes, the pellet was suspended in 10 mL cold lysis buffer containing (in mM) 155 NH_4Cl, 10 $KHCO_3$ and 0.1 ethylenediaminetetraacetate (EDTA), pH 7.4. The remaining neutrophils were then pelleted, washed twice with ice-cold PBS and suspended in an adequate volume of ice-cold Hanks' buffered saline solution (HBSS) until further manipulation. The preparation contained more than 95% neutrophils, as estimated by counting 200 cells under a microscope after Giemsa (Sigma) staining. In all cases, except where indicated, neutrophils were pre-treated with test compounds at concentrations in the range 1–100 μM in HBSS for 10 min at 37°C.

Measurement of extracellular generation of superoxide anion ($O_2^{\cdot -}$)

Extracellular $O_2^{\cdot -}$ generation was evaluated according to our previous report (Shen et al 2003). PMA (2.5 μg mL^{-1})-induced production of $O_2^{\cdot -}$ by leucocytes was determined in the presence or absence of 1–100 μM of test compounds by superoxide dismutase-inhibitable cytochrome *c* (80 μM) reduction by measuring the changes of absorbance at 550 nm (ΔOD_{550}) in the presence of superoxide dismutase (133 U mL^{-1}) at 37°C for 1 h. Staurosporine, a protein kinase C (PKC) inhibitor, was included as a positive control for the inhibition of extracellular $O_2^{\cdot -}$ production. Data are expressed as $O_2^{\cdot -}$ nmol/2×10^5 cells/h, using the molar extinction coefficient of 2.1×10^4 L mol^{-1} for cytochrome *c*, with a path length of 8 mm. The concentration dependency of each drug was tested and the 50% inhibitory concentration (IC50) for each drug was calculated using semi log-plot transformation of the data.

Estimation of cell viability

Cell viability was determined according to our previous report (Liou et al 2003) after incubation of cells (2×10^6 mL^{-1}) with test drugs for 4 h in 5-mL polystyrene round-bottomed tube (FALCON, Becton Dickinson). This method can be adapted for flow cytometry by adding 10 μg mL^{-1} of propidium iodide (PI), which is excluded by viable cells but which, when taken up by dead or dying cells, binds to nucleic acids and fluoresces red. The viable cells can be further identified by the addition of 100 ng mL^{-1} of fluorescein diacetate (FDA), which is not fluorescent but is taken up by the cells and converted to fluorescein by an intracellular esterase. The cell retains the fluorescein if the plasma membrane is intact. After incubation with the test compounds, cell suspensions were further incubated with PI and FDA at room temperature for 10 min and analysed immediately on a flow cytometer (FACSCalibur, Becton Dickinson) by recording forward and light scatter, red (>630 nm) and green (520 nm) fluorescence. After gating for light scatter to include single cells and to exclude clumps and debris, cell populations were differentiated by green (viable) versus red (dead) fluorescence. Cell viability (dead cell %) was calculated with the help of the CellQuest software (Becton Dickinson) on a Power Macintosh 7300/200 computer. Cell viability was further compared by using a cytotoxicity detection kit (Roche, Germany). This kit measures cytotoxicity and cell lysis by detecting lactate dehydrogenase (LDH) activity released from damaged cells.

Statistical analysis

All values in the text and figures are given as means ± s.e.m. Data were analysed by one-way analysis of variance followed by post-hoc Dunnett's *t*-test for multiple

comparisons. The concentration dependency of each drug was analysed by simple linear regression analysis of response levels against concentrations of drug and testing the slope of the regression line against 0 by Student's *t*-test. Values of $P < 0.05$ were considered significant.

Results and Discussion

We evaluated the structure–activity relationships (SARs) of three types of anthracene or anthraquinone lead structures for their anti-inflammatory effect and cytotoxicity in peripheral human neutrophils.

Among the three type of anthracene or anthraquinone derivatives being examined, 9-acyloxy 1,5-dichloroanthracene analogues (type I) displayed potent anti-inflammatory activity against superoxide anion production by human neutrophils. As shown in Table 1, compounds **1f**, **1g**, **1h** and **1m** were more effective than the other compounds in this assay with IC50s (μM) ranging from 6.3 to 33.9. Compounds **1f** and **1g** were almost 3–5 times more potent than **1h** and **1m**, indicating that a proper length (n = 3) of alkyl group in the substitution (R) of type I was optimal for anti-oxidative activity in human neutrophils. Furthermore, compounds **1c**, **1d**, **1j**, **1k** and **1l** also concentration-dependently inhibited the superoxide anion production with an inhibitory percentage of more than 40% at 100 μM. We reported previously that compound **1g** and **1c** exhibited an inhibitory effect on lipid peroxidation in rat brain (Huang et al 2001). Our results provide partial evidence for the explanation of the anti-oxidative effect of these anthracene-related derivatives (type I) as being possibly through scavenging or limiting the generation of free radicals by neutrophils. More detailed mechanism(s) for the inhibitory effect against superoxide anion production in neutrophils by type I-related compounds awaits further study.

An anthraquinone-related compound, emodin, has been reported to be a free radical generator with immunosuppressive effect (Huang et al 1992) but others have also reported that emodin can act as an antioxidant (Choi et al 2000) and protects tissues against lipid peroxidation in the rat brain (Scheibler 1997) or rat myocardium (Huang et al 1995; Sato et al 2000). To further understand whether anthraquinone-related compounds can display anti-oxidative/anti-inflammatory activity in human neutrophils, we synthesized some anthraquinone-related compounds (type II and type III) for the elucidation of SAR in this study. As compared to type I compounds' anti-oxidative activity, type II- and III-related compounds (anthraquinone-type derivatives) were relatively less effective in the inhibition of superoxide production by human neutrophils, with the inhibitory percentage (at 100 μM) being in the range 11–41.7% or 12.0–36.9%, respectively (Tables 2 and 3), although concentration dependency could also be observed. Emodin (Figure 1), an anthraquinone-related compound included as a reference drug, also had an inhibitory effect on superoxide production in human

Table 1 Summary of the effects of 9-acyloxy 1,5-dichloroanthracene-derived compounds (type I) on superoxide production in human neutrophils

Compound	R	IC50 (μM)	Inhibition (%) at 100 μM
1a	$-CH_3$	—	8.3 ± 4.1
1b	$-CH_2Cl$	—	28.3 ± 7.2
1c	$-CH_2CH_3$	—	48.5 ± 4.6
1d	$-CH(CH_3)_2$	—	43.4 ± 6.3
1e	$-CH(CH_3)Cl$	—	14.4 ± 2.5
1f	$-(CH_2)_2CH_3$	13.8 ± 3.0	—
1g	$-(CH_2)_3Br$	6.3 ± 4.1	—
1h	$-(CH_2)_4CH_3$	33.2 ± 1.3	—
1i	C_6H_5-	—	20.1 ± 8.8
1j	3-$CH_3C_6H_4-$	—	40.9 ± 4.2
1k	4-Cl, 2-$OCH_3C_6H_3-$	—	42.9 ± 11.7
1l	2,4-$Cl_2C_6H_3-$	—	50.4 ± 5.3
1m	$-CH_2C_6H_5$	33.9 ± 5.7	—
1n	$-(CH_2)_2C_6H_5$	—	15.4 ± 2.3

PMA (2.5 μg mL^{-1})-induced extracellular $O_2^{\cdot -}$ generation was determined in the presence or absence of 1–100 μM of test compounds (type I) by superoxide dismutase-inhibitable cytochrome *c* (80 μM) reduction. Data are expressed as 50% inhibitory concentration (IC50) or % of inhibition at 100 μM, when IC50 was not detectable. Values represent the means ± s.e.m. of 3–6 experiments performed on different days using cells from different donors.

Table 2 Summary of the effects of 1,5-bisacyloxy-anthraquinones with O-linked substituent-derived compounds (type II) on superoxide production in human neutrophils

Compound	R	Inhibition (%) at 100 μM	Inhibition (%) at 20 μM
2a	$-CH_2CH_3$	27.8 ± 7.8	21.2 ± 6.5
2b	$-(CH_2)_2CH_3$	25.8 ± 1.2	15.0 ± 1.9
2c	$-(CH_2)_4CH_3$	32.6 ± 5.2	15.6 ± 11.3
2d	$-C_6H_5$	18.0 ± 5.1	14.3 ± 3.9
2e	2-$CH_3C_6H_4-$	29.8 ± 1.8	24.2 ± 2.6
2f	3-$CH_3C_6H_4-$	36.8 ± 9.6	20.0 ± 8.6
2g	4-$CH_3C_6H_4-$	37.9 ± 3.8	27.1 ± 3.6
2h	3-ClC_6H_4-	37.4 ± 2.6	17.7 ± 2.0
2i	4-ClC_6H_4-	41.7 ± 2.0	29.7 ± 4.4
2j	2,4-$Cl_2C_6H_3-$	37.3 ± 4.7	41.6 ± 1.4
2k	$-CH_2C_6H_5$	37.7 ± 4.5	27.3 ± 2.0
2l	$-(CH_2)_2C_6H_5$	39.4 ± 3.0	24.3 ± 3.3
2m	2-ClC_6H_4-	11.0 ± 0.2	2.0 ± 1.2
2n	$-C(CH_3)_3$	29.7 ± 3.6	8.7 ± 0.8
Emodin	—	30.8 ± 5.5	27.4 ± 2.3

PMA (2.5 μg mL^{-1})-induced extracellular $O_2^{\cdot -}$ generation was determined in the presence or absence of 1–100 μM of test compounds (type II) by superoxide dismutase-inhibitable cytochrome *c* (80 μM) reduction. Data are expressed as inhibition (%) at 100 or 20 μM. Values represent the means ± s.e.m. of 3–6 experiments performed on different days using cells from different donors.

Table 3 Summary of the effects of 1,5-bisacyloxy-anthraquinones with S-linked substituent-derived compounds (type III) on superoxide production in human neutrophils

Compounds	R	Inhibition (%) at 100 μM	Inhibition (%) at 20 μM
3a	$-CH_2CH_3$	14.4 ± 4.0	19.6 ± 3.7
3b	$-(CH_2)_2CH_3$	31.4 ± 5.5	22.0 ± 7.9
3c	4-$CH_3C_6H_4-$	23.2 ± 6.5	13.0 ± 7.8
3d	$-CH_2C_6H_5$	20.8 ± 2.8	13.8 ± 4.5
3e	$-(CH_2)_2C_6H_5$	31.8 ± 3.3	23.4 ± 4.9
3f	$-CH_2C_6H_4$(4-OCH_3)	35.1 ± 2.3	23.2 ± 2.1
3g	$-(CH_2)_2OH$	16.4 ± 5.3	13.4 ± 2.5
3h	$-CH_2CH(OH)CH_2OH$	32.7 ± 7.8	17.9 ± 4.1
3i	$-(CH_2)_6OH$	25.9 ± 2.2	20.7 ± 2.5
3j	2-$NH_2C_6H_4-$	12.0 ± 4.4	12.4 ± 0.9
3k	3-$NH_2C_6H_4-$	36.9 ± 1.1	23.4 ± 5.6
3l	4-$NH_2C_6H_4-$	27.4 ± 4.9	10.1 ± 2.0

PMA (2.5 μg mL^{-1})-induced extracellular $O_2^{\cdot -}$ generation was determined in the presence or absence of 1–100 μM of test compounds (type III) by superoxide dismutase-inhibitable cytochrome *c* (80 μM) reduction. Data are expressed as inhibition (%) at 100 or 20 μM. Values represent the means ± s.e.m. of 3–6 experiments performed on different days using cells from different donors.

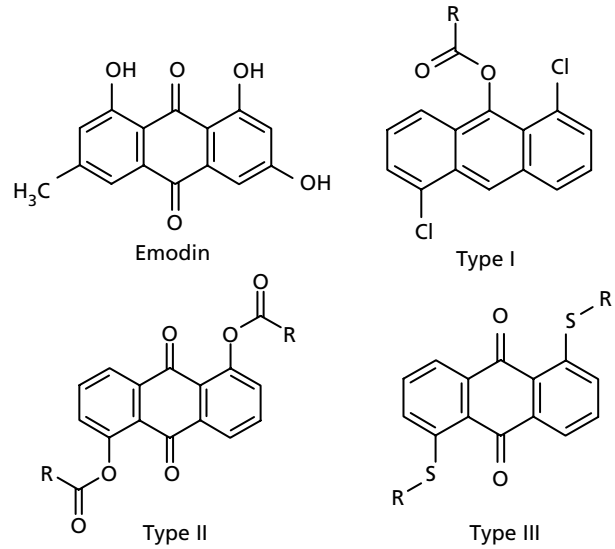

Figure 1 Structures of emodin (reference compound) and anthracene- and anthraquinone-related derivatives, modified from three types of lead structures, including 9-acyloxy 1,5-dichloroanthracene (type I), 1,5-bisacyloxy-anthraquinones with O-linked substituents (type II) and 1,5-bisacyloxy-anthraquinones with S-linked substituents (type III).

neutrophils at around 30% (Table 2). In the anthraquinone-related compounds examined, compounds **2f**, **2g**, **2h**, **2i**, **2j**, **2k**, **2l** and **3k** were as potent as emodin (one-way analysis of variance, $P > 0.05$) in this assay, indicating that substitution of both phenyl group and hydroxyl group at position (R) of type II or type III were effective for anti-oxidative activity.

Anthracene- or anthraquinone-derived compounds are regarded as cytotoxic chemicals due to their DNA-breaking effect (Fisher et al 1990). Therefore, we examined the cytotoxic effect of these compounds in peripheral human neutrophils. As shown in Table 4, no significant cytotoxic effect of these compounds could be observed at the concentration range of 1–100 μM (one-way analysis of variance, $P > 0.05$).

Table 4 Summary of the cytotoxic effects of type I, II or III derivatives in human neutrophils

Type I	Dead cells (%) at 100 μM	Type II	Dead cells (%) at 100 μM	Type III	Dead cells (%) at 100 μM
1a	5.2 ± 1.9	**2a**	1.1 ± 0.2	**3a**	0.8 ± 0.1
1b	3.5 ± 0.6	**2b**	1.2 ± 0.1	**3b**	1.2 ± 0.2
1c	1.9 ± 0.9	**2c**	1.6 ± 0.5	**3c**	2.3 ± 1.4
1d	1.5 ± 0.8	**2d**	1.2 ± 0.3	**3d**	1.5 ± 0.4
1e	1.0 ± 0.4	**2e**	1.3 ± 0.3	**3e**	1.4 ± 0.2
1f	1.8 ± 0.7	**2f**	1.2 ± 0.4	**3f**	3.3 ± 0.6
1g	1.1 ± 0.3	**2g**	1.4 ± 0.4	**3g**	6.5 ± 0.8
1h	1.4 ± 0.5	**2h**	1.3 ± 0.4	**3h**	2.0 ± 0.3
1i	1.7 ± 0.4	**2i**	3.5 ± 2.4	**3i**	3.2 ± 0.8
1j	1.2 ± 0.1	**2j**	2.0 ± 0.1	**3j**	2.4 ± 1.3
1k	1.7 ± 0.6	**2k**	1.6 ± 0.3	**3k**	8.1 ± 1.3
1l	1.3 ± 0.0	**2l**	1.2 ± 0.1	**3l**	3.7 ± 0.7
1m	1.3 ± 0.1	**2m**	1.5 ± 0.5	—	—
1n	1.2 ± 0.3	**2n**	1.5 ± 0.8	—	—

Cytotoxic effect was measured via flow cytometry as described in Material and Methods. After incubation with test compounds, vehicle control (0.25% DMSO) or triton (positive control) for 4 h, cell suspensions were further incubated with PI and FDA at room temperature for 10 min and analysed immediately on a flow cytometer. Cell viability was reflected as percentage of dead cells (%). Values represent the means ± s.e.m. of 3–5 experiments performed on different days using cells from different donors.

The percentage of dead cells (%) in the vehicle control (0.25% DMSO) and emodin groups (100 μM) were 5.0 ± 0.5 and 1.0 ± 1.2, respectively. In the positive controls where triton (0.1% and 0.01%) was used to disrupt the cell membrane to induce cytotoxicity, cell death increased significantly (98% and 27%, respectively). In summary, these results indicate that 9-acyloxy 1,5-dichloroanthracence derivatives are more potent than anthraquinone-related compounds as anti-inflammatory drugs, with no significant cytotoxicity in human neutrophils.

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