Novel Esters of Glaucarubolone as Inducers of Terminal Differentiation of Promyelocytic HL-60 Cells and Inhibitors of 7,12-Dimethylbenz[*a*]anthracene-Induced Preneoplastic Lesion Formation in Mouse Mammary Organ Culture

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In an effort to discover new chemotherapeutic/chemopreventive agents from natural sources, brusatol (**1**) was found to induce HL-60 cellular differentiation, accompanied by strong antiproliferative and cytotoxic effects. A series of natural and semisynthetic quassinoids (**1**−**48**) was designed to effect both antiproliferative and differentiation-inducing properties. Compounds were assessed in vitro using the HL-60 promyelocytic cell model. Changes in activity due to structural modification of the core structure glaucarubolone (**24**) were consistent with activities reported in other cell systems. However, the following were novel SAR findings: (1) semisynthetic analogues with a hydroxylated ring at the β-position of the ester side chain at C-15 were able to induce cellular differentiation at concentrations lower than those inducing cell growth arrest, and (2) quassinoids inhibiting DNA synthesis with greater efficacy than reducing cellular viability possessed alkyl substitutions at the α-position of the C-15 ester side chain. Analogues from this latter group and brusatol (**1**) and bruceantin (**2**) inhibited dimethylbenz(*a*)anthracene-induced preneoplastic lesion formation in a mouse mammary organ culture. The novel finding of **1** and glaucarubolone analogues as potent inducers of differentiation leads to potential novel applications in the field of cancer.

The concept that aberrant cell differentiation is a consistent and important characteristic of malignant cells has been exploited to develop novel chemotherapeutic and/or chemopreventive agents. Evidence that induction of differentiation is sufficient to control malignancy was obtained from studies using somatic cell hybridization. It has been demonstrated that malignant cells fused with normal diploid cells of the same species result in hybrid cells that retain their transformed phenotype in culture. However, when inoculated into immune-deficient animals, these cells fail to form tumors due to induction of differentiation in the host animal.[1,2] In a similar manner, nonphysiological agents are known to induce differentiation in malignant cells that have lost their normal response to the physiological inducers of maturation.[3] The HL-60 cell system has been utilized as a tool to study the molecular and cellular events that lead to maturation. Various chemical entities have shown remarkable activities as inducers of HL-60 cell differentiation. These compounds act through gene expression modulation of important signals that regulate differentiation, proliferation, and cell death processes. For instance, all-*trans*-retinoic acid was discovered as a differentiating agent using this system[4] and, together with its natural and synthetic analogues, constitutes one of the most important categories of chemopreventive and chemotherapeutic agents.[5,6]

In our search for novel anticancer agents, we have utilized the HL-60 system as a screening tool of natural sources,[7,8] and this led to the isolation of brusatol (**1**) from the seed extract of *Brucea javanica* (Simaroubaceae) as a potent natural inducer of cellular differentiation.[9] Brusatol belongs to the chemical type of nortriterpenoids termed quassinoids (simaroubolides), which are biogenetically derived by degradation of C_{30}-precursors. These compounds are known to mediate several biological activities including antileukemic and cytotoxic responses.[10] The major mechanism responsible for antineoplastic activity at the molecular level by the quassinoids has been attributed to inhibition of site-specific protein synthesis.[11] Such inhibition has been shown to occur via interference at the peptidyltransferase site, thus preventing peptide bond formation. However, quassinoids are not universal protein synthesis inhibitors; they mediate cytotoxic effects with normal and transformed lymphocytic and hepatic cell lines, while they can enhance proliferation of normal and transformed kidney and lung cells.[12] Further, we have recently demonstrated more complex mechanisms involving down-regulation of nm23 and *c-myc* (Mata-Greenwood, E.; Sher, D.; Gustin, D.; Stock, W.; Pezzuto, J. M. *Leukemia*, submitted).

In the current investigation, we have evaluated the potential of 48 quassinoids to induce HL-60 cell differentiation and determined structure−activity relationships (SAR).

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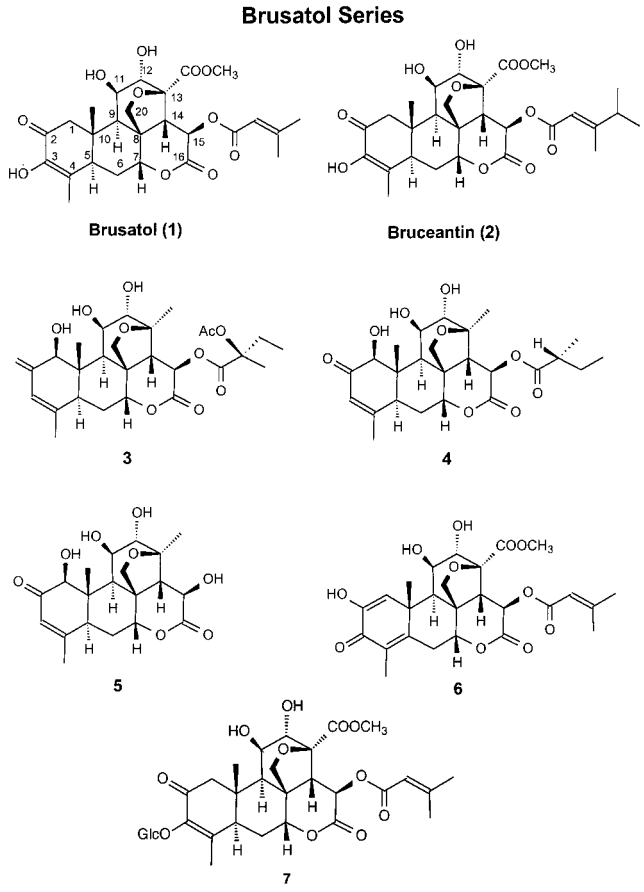
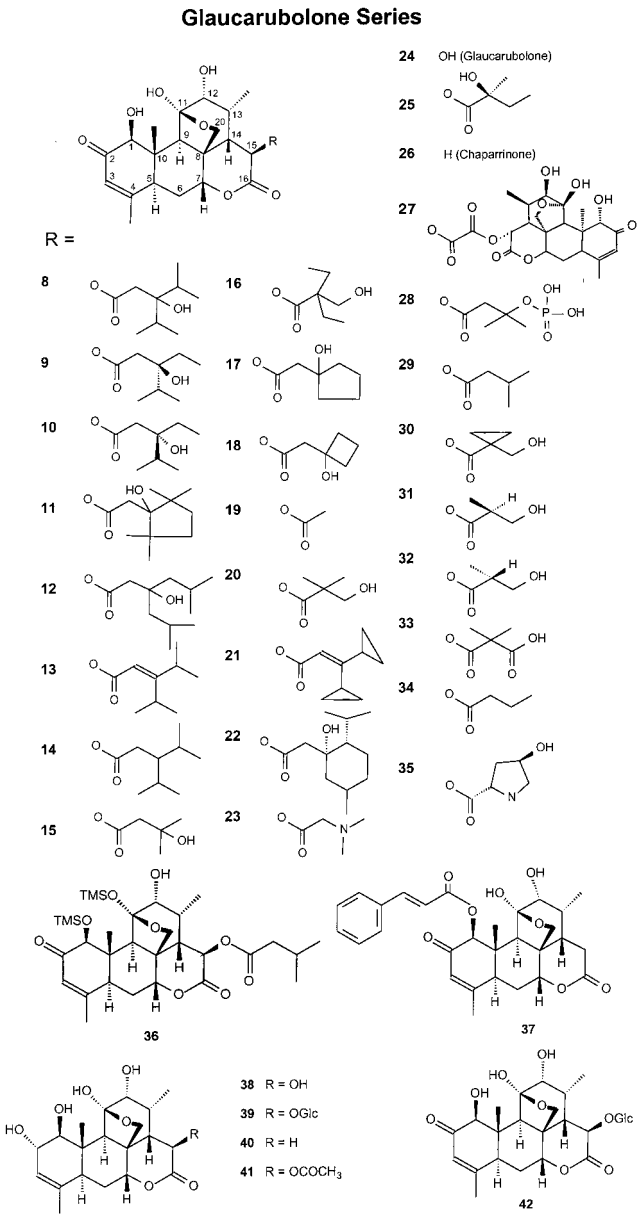
As an initial evaluation of the relevance of these effects, we tested a group of selected quassinoids for their potential to inhibit dimethylbenz(*a*)anthracene (DMBA)-induced preneoplastic lesions in a mouse mammary organ culture.



Results and Discussion

A set of 48 natural and semisynthetic quassinoid analogues (**1**−**48**) was studied using the HL-60 system to determine SAR. Induction of differentiation was determined by the ability of treated cells to produce superoxide anions [nitroblue tetrazolium (NBT)-reduction], a functional marker of mature macrophages or granulocytes. Proliferation capacity is equivalent to cell growth and was measured by incorporation of [^{3}H]thymidine into DNA over a period of 18 h, and cytotoxic activity was evaluated by the loss of membrane integrity as shown by trypan blue exclusion. Thirty-three quassinoids showed activity as either cytotoxic, antiproliferative, and/or inducers of cellular differentiation (Table 1). Inactive quassinoids (IC_{50} >5 μM) lacked either the epoxymethano bridge in ring D (i.e., quassin series **43**−**47**), or a free hydroxyl group at positions 1, 3, 11, and 12 (i.e., due to glycosylation, compounds **7**, **39**, **42**), or a freely conjugated ketone in ring A (i.e., **6**, and due to reduction, compounds **38**−**41**). Although members of the brusatol and glaucarubolone series were active, when comparing members of both series that varied only in the positioning of the epoxymethano bridge, great differences were noted, as shown with yadianzolide C (**5**) and glaucarubolone (**24**) and analogues **4** (brusatol series) and **32** (glaucarubolone series).

The effect of an ester side chain at position C-15 on cytotoxicity and cellular differentiation was studied in greater detail with analogues of glaucarubolone (compounds **8**−**35**). The absence of a side chain at C-15 is associated with a 100−500-fold decrease of potency [as



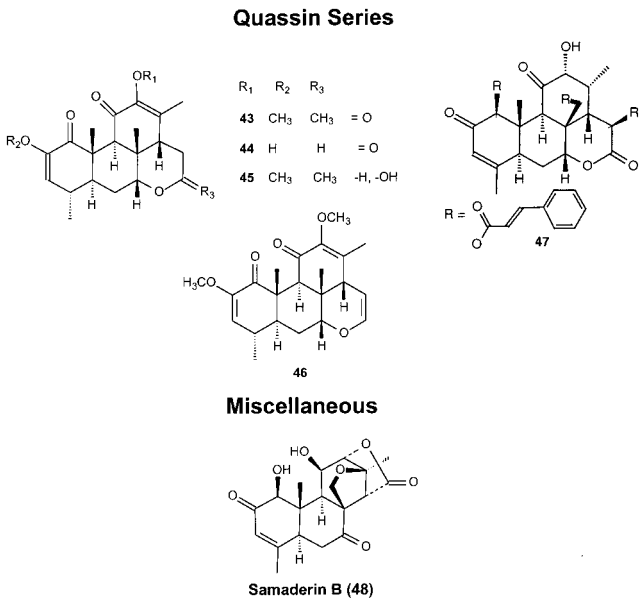
compared to brusatol (**1**)] and an increased selective inhibition of DNA synthesis as compared to cytotoxicity (i.e., compounds **5**, **24**, and **26**). The nature of the side chain is also of importance. There were no correlations between the lipophilicity of the ester side chain and HL-60 cell differentiation induction. Analysis of two pairs of enantiomers (**9**,**10** and **31**,**32**) revealed that the stereochemistry at the α- or *β*-positions does not affect biological activity. On the contrary, the addition of alkyl groups resulting in a branched side chain correlated with increase in potency as shown between side chains **11** and **17**, **14** and **29**, and **16** and **20**. The presence of hydroxyl substituents at the *β*-position of the side chain correlated with an 8−10-fold increase in potency as shown by comparing the following pairs: **8** with **14**, and **15** with **29**. The presence of alkyl substituents in the α-position correlated with increased selectivity (2−6-fold) for antiproliferative activity versus cytotoxicity, but less potency as inducers of differentiation (analogues **16**, **20**, **25**, **30**−**33**). An increase in selectivity (1.5−2-fold) between induction of differentiation and antiproliferative/cytotoxic activity was observed only in those analogues possessing side chains with cyclic rings in the *β*-position (**11**, **17**, **18**, and **22**). Others were cytotoxic but

Table 1. Induction of HL-60 Cell Differentiation and Growth Arrest by Quassinoids (**1**−**48**)[a]

compound	inhibition of proliferation (IC_{50}, μM)	induction of differentiation (EC_{50}, μM)	cytotoxicity (IC_{50}, μM)	selectivity index[b]
1	0.07 ± 0.007	0.07 ± 0.001	0.17 ± 0.001	2.5 ± 0.3
2	0.04 ± 0.003	0.02 ± 0.002	0.04 ± 0.003	1.0 ± 0.2
3	0.06 ± 0.005	0.09 ± 0.001	0.13 ± 0.008	2.2 ± 0.5
4	>0.2	>0.2	0.2 ± 0.04	
5	1.5 ± 0.08	10 ± 1.5	>10	>6.7
8	0.009 ±0.0007	0.009 ± 0.0002	0.009 ± 0.0007	1.0 ± 0.2
9	0.011 ± 0.0004	0.017 ± 0.0004	0.013 ± 0.001	1.2 ± 0.2
10	0.009 ± 0.001	0.019 ± 0.0004	0.013 ± 0.001	1.5 ± 0.4
11	0.04 ± 0.008	0.025 ± 0.002	0.05 ± 0.004	1.3 ± 0.5
12	0.04 ± 0.003	>0.05	0.04 ± 0.002	1.0 ± 0.2
13	0.07 ± 0.003	0.09 ± 0.003	0.13 ± 0.01	1.9 ± 0.3
14	0.075 ± 0.009	0.055 ± 0.01	0.15 ± 0.02	2.0 ± 0.7
15	0.1 ± 0.02	~0.2 ± 0.004	0.4 ± 0.02	4.2 ± 1.5
16	0.15 ± 0.02	0.5 ± 0.05	0.95 ± 0.02	6.5 ± 1.4
17	0.19 ± 0.04	0.13 ± 0.01	0.38 ± 0.04	2.1 ± 0.9
18	0.26 ± 0.009	0.14 ± 0.003	0.28 ± 0.01	1.1 ± 0.1
19	0.2 ± 0.01	0.2 ± 0.001	0.5 ± 0.07	2.5 ± 0.7
20	0.2 ± 0.02	0.5 ± 0.01	0.6 ± 0.08	3.1 ± 1
21	0.2 ± 0.03	0.6 ± 0.07	0.75 ± 0.02	3.9 ± 1
22	0.4 ± 0.03	0.3 ± 0.008	0.9 ± 0.1	2.3 ± 0.6
23	0.5 ± 0.03	0.5 ± 0.04	0.8 ± 0.1	1.6 ± 0.4
24	0.4 ± 0.03	~1.3 ± 0.03	1.3 ± 0.2	3.3 ± 1
25	0.5 ± 0.09	1.8 ± 0.09	1.0 ± 0.1	2.1 ± 0.8
26	1.2 ± 0.2	~2.4 ± 0.1	5.0 ± 0.3	4.3 ± 1.4
27	0.5 ± 0.04	>1.5	1.8 ± 0.1	3.6 ± 0.7
28	0.7 ± 0.09	~3.0 ± 0.03	4.4 ± 0.3	6.5 ± 1.7
29	1.0 ± 0.1	>2.0	1.5 ± 0.1	1.5 ± 0.4
30	1.0 ± 0.15	~2.0 ± 0.03	2.8 ± 0.2	2.9 ± 0.9
31	0.6 ± 0.1	1.9 ± 0.04	2.5 ± 0.05	4.3 ± 1.1
32	1.5 ± 0.2	>6.0	6.2 ± 0.06	4.2 ± 0.9
33	3.2 ± 0.35	>7.0	>7.0	>2.2
34	>3.0	>3.0	3.0 ± 0.5	
48	0.4 ± 0.05	1.0 ± 0.02	>2	>6

[a] [^{3}H]Thydimidine incorporation was used as a proliferation marker, NBT reduction as a differentiation marker, and trypan blue exclusion as a viability marker. Inhibitory (IC_{50}) and effective (EC_{50}) concentrations required to induce a 50% response were determined using dose−response studies with at least five different data points. Compounds **6**, **7**, and **35**−**47** were tested and found to be inactive (IC_{50} > 5 μM). [b] Selectivity index was calculated as the ratio of cytotoxic IC_{50} over antiproliferative IC_{50}.

not antiproliferative nor inducers of cellular differentiation (**4**, **34**). In sum, novel esters of glaucarubolone (**24**) were shown to be either more potent or more selective than the parent compound and than brusatol (**1**).



A smaller set of quassinoids (i.e., **1**, **2**, **10**, **14**, **16**, **18**, **26**, **34**, and **48**) was tested for potential to inhibit DMBA-induced preneoplastic lesion formation in the mouse mammary organ culture (MMOC) model. This model correlates with in vivo chemopreventive activity in models such as the DMBA-induced rat mammary adenocarcinoma and the DMBA/12-*O*-tetradecanoylphorbol 13-acetate (TPA) two-stage mouse skin papilloma models.[13] All nine quassinoids were tested at the same concentration (2 μM); however, only four were active (Table 2). Interestingly, potency in the HL-60 assay did not correlate with activity in the MMOC assay, considering that quassinoids **10** and **14** were among the most potent inducers of HL-60 differentiation (EC_{50} <0.05 μM) but were found inactive in the MMOC model at concentrations as high as 2 μM. Activity in the MMOC assay seemed to favor those analogues that pos-

Table 2. Quassinoid Inhibition of DMBA-Induced Preneoplastic Lesion Formation Using the Mouse Mammary Organ Culture Model

quassinoid (2 μM)	% inhibition[a]
brusatol (**1**)	70
bruceantin (**2**)	70
10	44
14	40
16	70
18	33
chaparrinone (**26**)	25
34	82
samaderin B (**48**)	0

[a] Percent inhibition was calculated in comparison with a DMBA (carcinogen) control. Based on historical controls,[26] samples are classified as active if preneoplastic lesions are reduced to >60%. No visible signs of toxicity (as indicated by dilation of mammary ducts or disintegration of mammary structure yielding amorphous material) was manifested by any of the tested quassinoids.

sessed alkyl substituents at the α-position of the C-15 ester side chain (Table 2), contrary to what was observed in HL-60, where potency correlated with the presence of β-branched ester side chains (Table 1). For instance, compound **34** was only moderately antiproliferative in the HL-60 cell line ($EC_{50} = 3.2\ \mu M$), but greatly inhibited preneoplastic lesion formation (82% inhibition).

It has been reported that quassinoids regulate DNA and RNA synthesis by blocking several metabolic sites necessary for nucleic acid synthesis,[14] while protein synthesis is regulated by binding to the ribosome.[11] Inhibition of protein synthesis has been linked to cytotoxicity and antineoplastic activity of quassinoids,[12] since resistant tumors and cell lines are still sensitive to quassinoid inhibition of DNA and RNA synthesis while resistant to protein synthesis inhibition. In the current study, utilizing HL-60 cells in culture, quassinoids were antiproliferative agents and potent inducers of cellular differentiation. As illustrated through analysis of 48 quassinoids using the HL-60 cell system, inhibition of DNA synthesis/cellular growth and potential to induce differentiation are greatly influenced by structural alterations. As demonstrated by previous literature reports,[15−20] some correlations can be drawn with antineoplastic activity, but exceptions are obvious. For example, brusatol dimers are more potent as antineoplastic agents than brusatol (**1**) itself,[20] whereas brusatol is more potent as an antiinflammatory[15] or differentiating agent. Analogues that lacked the ester side chain inhibited DNA synthesis at lower concentrations than those required to inhibit cellular growth (and protein synthesis), while other analogues were cytotoxic without inhibiting DNA synthesis. These data suggest that selectivity for a particular cellular target can be achieved by structural modification of the parent quassinoid.

Extensive studies on agents that induce metabolic arrest have shown a correlation between DNA synthesis inhibitors and induction of differentiation. For instance, the inhibition of DNA synthesis has been shown to be an initial event necessary to induce cell differentiation by antineoplastic agents such as ara-C and actinomycin D.[21] It has been proposed that inhibition of DNA synthesis allows the slow production of some proteins necessary for fulfillment of the differentiation program. However, SAR studies demonstrated that some quassinoids with potent antiproliferative activity did not induce differentiation, and analogues **4** and **33** were cytotoxic but neither antiproliferative nor differentiation inducers. In addition, known inhibitors of DNA synthesis, i.e., aphidicolin, were incapable of inducing maturation of HL-60 cells (data not shown). These observations make it unlikely that inhibition of DNA synthesis is the mechanism of induction of differentiation.

Certain protein synthesis inhibitors have also been reported to induce HL-60 cell differentiation. Although inhibition of protein synthesis and gene expression activation seem to be mutually exclusive events, some reports have shown that selective gene expression and translation can occur with as little as 10% of control protein synthesis levels.[22] Several theories have been proposed for the observed results. One is that inhibitors that act by blocking the elongation step of protein synthesis, like the quassinoids, increase the stability of weak mRNAs and decrease the degradation of certain proteins necessary for the induction of differentiation. In support of this idea, quassinoids and *Cephalotaxus* alkaloids (i.e., homoharringtonine) are efficient differentiating agents[9,23] that bind to similar sites in the ribosome.[24] Quassinoids and *Cephalotaxus* alkaloids induce disaggregation of polyribosomes, while other protein synthesis inhibitors (cycloheximide and anisomycin) function by other modes of action[24] and are not capable of inducing cellular differentiation. Studies on differentiation of cell lines with mutated ribosomal sites would clarify this issue.

The differentiation-inducing and antiproliferative effects of retinoic acid were identified first with the HL-60 cell line[25] and then confirmed with other cell systems. Subsequently, studies with in vitro and in vivo chemically induced models of carcinogenesis established a correlation between induction of differentiation and chemopreventive activity; for example, inducers of cell differentiation inhibit preneoplastic lesion formation in MMOC[26] and adenocarcinomas in the Sprague−Dawley rat mammary model.[27] Moreover, retinoic acid and novel retinoids have shown chemopreventive activity against primary and secondary tumor formation in human clinical trials of lung and head and neck cancers.[28,29] In the present study, initial assessment of the chemopreventive potential of brusatol (**1**) and glaucarubolone esters was performed using the MMOC model. It was found that analogues bearing α-dialkylated C-15 ester side chains were more selective in the cell differentiation tests, as well as being active in the MMOC model. Since active agents in the MMOC test system are often active as in vivo chemopreventive agents,[13] it would be of interest to further evaluate selective antiproliferative quassinoids of this series.

Experimental Section

Preparation of Quassinoids. Brusatol (**1**), yadanziolide C (**5**), dehydrobrusatol (**6**), and bruceoside A (**7**) were isolated from *Brucea javanica*,[9] and bruceantin (**2**) was obtained from the NCI. Quassinoids belonging to the glaucarubolone series (**37**−**42**, **47**)[30,31] and quassin series (**43**−**46**)[31−33] were obtained by J. D. and J. D. M. Peninsularinone (**10**) was isolated from *Castela peninsularis*.[34] Glaucarubolone (**24**),[35] glaucarubinone (**25**),[35] chaparrinone (**26**),[35] samaderin B (**48**),[36] quassimarin (**3**),[37] and simalikalactone D (**4**)[38] were prepared via total synthesis. Semisynthetic analogues **8**, **9**, **11**−**23**, and **27**−**36** were prepared via a four-step protocol[39] starting with glaucarubolone (**24**), which was isolated from *Castela polyandra*.[40]

Differentiation/Proliferation and Cytotoxicity Assays Using HL-60 Cells. HL-60 (human promyelocytic) cells were tested using a 4-day incubation protocol.[8] In brief, cells in log phase (approximately 10^6 cells/mL) were diluted to 10^5 cells/mL and preincubated overnight (18 h) in 24-well plates to allow cell-growth recovery. Then, samples dissolved in DMSO were added, keeping the final DMSO concentration at 0.1% (v/v). Control cultures were treated with the same concentration of DMSO. After 4 days of incubation, the cells were analyzed to determine the percentage of cells undergoing maturation as determined by NBT reduction. Concomitantly, the effect on viability and proliferation of HL-60 cells was determined. In each experiment, 1α,25-dihydroxyvitamin D_3 ($EC_{50} = 0.01\ \mu M$) and brusatol ($EC_{50} = 0.07\ \mu M$) were used as reference controls. EC_{50} and IC_{50} values were calculated using 5−7 test concentrations (in duplicate), and consistent results were obtained, indicating the data reported for the related quassinoids are reliable.

(1) Nitroblue Tetrazolium (NBT) Reduction. Evaluation of NBT reduction was used to assess the ability of sample-treated HL-60 cells to produce superoxide when challenged with 12-*O*-tetradecanoylphorbol 13-acetate (TPA). A 1:1 (v/v) mixture of a cell suspension (10^6 cells) and TPA/NBT solution (2 mg/mL NBT and 1 μg/mL TPA in phosphate buffered solution) was incubated for 1 h at 37 °C. Positive cells reduce NBT, yielding intracellular black-blue formazan deposits, which were quantified by microscopic examination of >200 cells. Results are expressed as a percentage of positive cells.

(2) Cytotoxicity. Since loss of membrane integrity is an early feature of necrotic cells and a late feature of apoptotic

cells, trypan blue, a cationic blue dye, was used to stain cells with compromised plasma membranes, while leaving intact cells unstained. Cells (100 μL) were stained with 400 μL of trypan blue (0.2% w/v in PBS), incubated for at least 5 min at room temperature, and counted using a hematocytometer. Viability percentages were calculated with duplicate samples.

(3) Cell Proliferation Assay. Inhibition of [^{3}H]thymidine incorporation into DNA was determined to assess the level of HL-60 cell proliferation. Cells were treated with the test samples for 4 days and then placed into 96-well plates (100 μL) and treated with [^{3}H]thymidine (0.5 μCi/mL, 65 Ci/mmol) for 18 h at 37 °C in a 5% CO_2 incubator. Cells were then collected on glass fiber filters (90 × 120 mm; Wallac, Turku, Finland) using a TOMTEC Harvester 96. The filters were counted using a Microbeta liquid scintillation counter (Wallac, Turku, Finland) with scintillation fluid. Finally, the percentage of [^{3}H]thymidine incorporation per 10^6 cells was calculated by dividing the dpm of sample-treated cells by the dpm of DMSO-treated cells.

Inhibition of DMBA-induced Preneoplastic Lesion Formation in Mouse Mammary Organ Culture (MMOC). The identification of potential inhibitors of DMBA-induced preneoplastic lesion formation in mammary organ culture has been described previously.[26] Briefly, four-week old BALB/c female mice (Charles River) were pretreated for 9 days with 1 μg of estradiol and 1 mg of progesterone. The thoracic pair of mammary glands was dissected on silk and incubated with growth-promoting hormones in the presence of test compounds (2 μM) for 10 days. DMBA (2 μg/mL) was included in the medium (containing 5 μg/mL insulin, 5 μg/mL prolactin, 1 μg/mL aldosterone, and 1 μg/mL hydrocortisone) for 24 h on the third day of culture to induce preneoplastic mammary lesions. Following 10 days of growth promoting phase, all hormones except insulin were withdrawn and the glands were allowed to regress to lobuloalveolar structures during a 14-day incubation period. Glands were then fixed in 10% buffered formalin and stained with alum carmine. Incidence of lesion formation (percentage of glands per group with mammary lesions) was recorded, and percent inhibition was calculated by comparison with the DMBA control group that was not treated with test sample. Active samples induce 60% inhibition, based on historical controls.[26]

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