Journal of Medicinal Chemistry

Article

pubs.acs.org/jmc

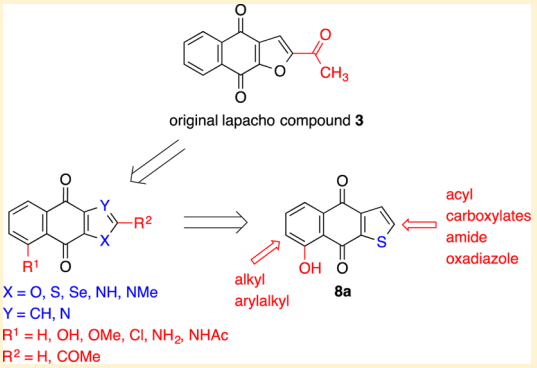
Synthesis and Structure–Activity Relationships of Lapacho Analogues. 2. Modification of the Basic Naphtho[2,3-*b*]furan-4,9-dione, Redox Activation, and Suppression of Human Keratinocyte Hyperproliferation by 8-Hydroxynaphtho[2,3-*b*]thiophene-4,9-diones

Sven Bannwitz, Dirk Krane, Silke Vortherms, Tobias Kalin, Cathrin Lindenschmidt, Nader Zahedi Golpayegani, Jan Tentrop, Helge Prinz, and Klaus Müller*

Institute of Pharmaceutical and Medicinal Chemistry, PharmaCampus, Westphalian Wilhelms University, Corrensstrasse 48, D-48149 Münster, Germany

Supporting Information

ABSTRACT: The basic structure of linearly anellated lapacho quinones, naphtho[2,3-*b*]furan-4,9-dione (**7**), was modified in the search for novel agents against keratinocyte hyperproliferation. The synthesis and structure–activity relationships of several heterocycle-fused naphthoquinones as well as a full range of 2- and 7-substituted derivatives of one of these, 8-hydroxynaphtho[2,3-*b*]thiophene-4,9-dione (**8a**), are described. Out of a total of 71 analogues, particularly 2-thenoyl-substituted **26l**, 2-nicotinoyl-substituted **26m**, and 2-oxadiazole-substituted **35a** compared favorably with the antipsoriatic agent anthralin. Their potency for suppression of keratinocyte hyperproliferation, which was evaluated using HaCaT cells as a model, was combined with comparably low membrane-damaging effects toward keratinocytes, as established by the release of lactate dehydrogenase activity from the cytoplasm of the cells. With respect to the mechanism of action, redox activation of lapacho quinones by one- and two-electron reduction in isolated enzymatic assays was studied, and their potential to generate superoxide was confirmed in the keratinocyte-based hyperproliferation assay.

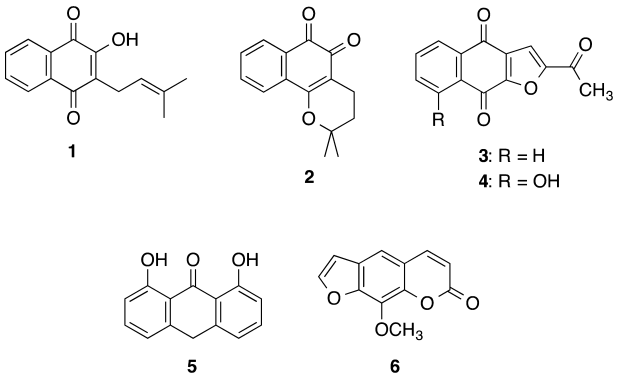


INTRODUCTION

Derivatives of the naturally occurring substance lapachol (**1**), such as the angularly anellated *β*-lapachone (**2**) or the linearly anellated naphtho[2,3-*b*]furan-4,9-diones **3** and **4** (Chart 1), are among the principal constituents of the inner bark and heartwood of the lapacho (Tabebuia) tree from the Bignoniaceae family.[1−3] Commonly known as "pau-d'arco", lapacho is widely used as a folk medicine for the treatment of a variety of diseases by the local people in South America.[4,5] Its remarkable pharmacological properties, including antibacterial, antifungal, antiinflammatory, and antitumor activities, are cited in the literature.[6−11] As a consequence of its biological significance, quinone **3** has become an attractive synthetic target, and several studies have been carried out toward the development of antitumor agents.[12−17]

Moreover, lapacho is today used against disorders of the immune system and skin diseases such as psoriasis,[4] which is supported by an earlier study in which we assessed potent inhibitory action of a number of lapacho compounds against the growth of human keratinocytes at sufficiently low concentration, and in particular, quinones **2**–**4** displayed activity comparable to that of the antipsoriatic drug anthralin (**5**).[18] In our continuing search for potent antiproliferative agents based on a three-ring structure such as anthracenones,[19]

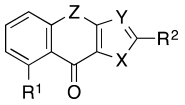
Chart 1. Lapacho Quinones 1–4 and Antipsoriatic Drugs 5 and 6



Received: May 15, 2014

   dx.doi.org/10.1021/jm500754d | J. Med. Chem. XXXX, XXX, XXX–XXX

Table 1. Suppression of Human Keratinocyte Hyperproliferation by Simple Analogues of Basic Naphtho[2,3-*b*]furan-4,9-dione



compd	X	Y	Z	R^1	R^2	AA,[a] IC_{50} (μmol/L)
3[b]	O	CH	C=O	H	COMe	0.50
5, anthralin						0.70
7[b]	O	CH	C=O	H	H	3.85
8	S	CH	C=O	H	H	4.80
8a	S	CH	C=O	OH	H	2.75
8b	S	CH	C=O	OMe	H	13.50
8c	S	CH	C=O	Cl	H	3.77
8d	S	CH	C=O	NH_2	H	>30
8e	S	CH	C=O	NHCOMe	H	2.76
9	S	CH	CH_2	OH	H	2.50
10	Se	CH	C=O	H	H	6.12
11	NH	CH	C=O	H	H	>30
12	NH	N	C=O	H	H	>30
12a	NMe	N	C=O	H	H	>30
13	O	N	C=O	H	H	0.90
14	S	N	C=O	H	H	1.20
18	S	CH	C=O	H	COMe	0.44
18a	S	CH	C=O	OH	COMe	0.40
18b	S	CH	C=O	OMe	COMe	3.50
18c	S	CH	C=O	Cl	COMe	1.28
21	NH	N	C=O	H	COMe	>30
21a	NMe	N	C=O	H	COMe	>30

[a]Antihyperproliferative activity against keratinocytes. IC_{50} = concentration of test compound required for 50% inhibition of cell growth (HaCaT). Inhibition of cell growth was significantly different with respect to that of the control, $N = 3$, $P < 0.05$. [b]Reference 23.

naphtho[2,3-*b*]thiophen-4(9*H*)-ones,[20] acridones,[21] and phenoxazines and phenothiazines,[22] these findings encouraged the design and synthesis of a novel series of linearly anellated lapacho analogues and their evaluation for suppression of keratinocyte hyperproliferation, which we have reported recently.[23] Anthralin (**5**) and 8-methoxypsoralen (**6**) exemplify established drugs with a tricyclic structure (Chart 1) in the clinical treatment of psoriasis.[24,25]

In this paper we describe the initial modifications of the basic naphtho[2,3-*b*]furan-4,9-dione (**7**; Table 1) of lapacho ingredient **3** in which several closely related ring systems were compared and also a full range of analogues of one of these, 8-hydroxynaphtho[2,3-*b*]thiophene-4,9-dione (**8a**), which is bioisosteric to the basic structure of lapacho constituent **4** (Chart 1) and is found to be among the most active structural types. Thus, the first goal of this investigation was the synthesis and biological evaluation of heterocyclic-ring-fused naphthoquinones and derivatives thereof. These thiophene (**8**, **8a**–**8e**), selenophene (**10**), pyrrole (**11**), imidazole (**12**, **12a**), oxazole (**13**), and thiazole (**14**) analogues are chromophore-modified naphtho[2,3-*b*]furan-4,9-diones and should maintain the planarity and spatial and electronic characteristics of the basic lapacho quinone **7** that may be necessary for molecular recognition at the cellular level. The second goal was to further delineate the structure–activity relationships (SARs) for lapacho quinone analogues. To do this, many congeners of **8a** with side chains occupying the 2- or 7-position on the thiophene-anellated skeleton were synthesized and evaluated for their ability to suppress keratinocyte hyperproliferation. This may be critical for the management of psoriasis, a common, immune-mediated inflammatory and scaling skin disorder, mainly characterized by excessive growth of keratinocytes.[26]

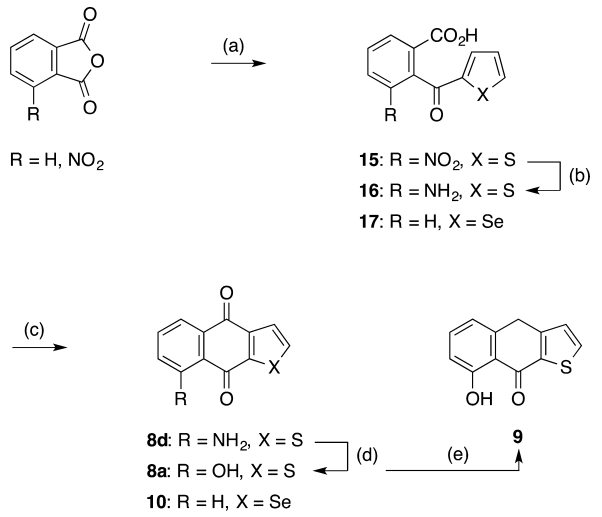
With respect to redox activation of the novel analogues, testing procedures for the generation of superoxide by the compounds are identical to those described in our recent paper.[23]

CHEMISTRY

The target molecule for the synthesis of the desired analogues was 8-hydroxynaphtho[2,3-*b*]thiophene-4,9-dione (**8a**). The methodology for its preparation is outlined in Scheme 1. In following the procedure of Weinmayr,[27,28] thenoylbenzoic acid **15** was prepared from 3-nitrophthalic anhydride by Grignard reaction and separated from its isomer. The nitro group of **15** was reduced with ferrous sulfate in aqueous ammonia to the amino intermediate **16**, and direct ring closure upon heating with sulfuric/boric acid yielded aminonaphtho[2,3-*b*]-thiophene-4,9-dione (**8d**). While the conditions used do not ensure control of regioselectivity and could result in rearrangements to produce the 5-amino isomer, this process gave only isomerically pure **8d**. The latter was converted to the target molecule **8a** by diazotation and subsequent hydrolysis of the diazonium salt. The X-ray crystal structure of **8a** (see the Supporting Information for details) clearly confirmed the 8-position of the substituent, whereas the corresponding 5-hydroxy isomer[29] could not be detected. Furthermore, reduction of **8a** with stannous chloride in acetic acid occurred at the keto group remote from the hydroxyl group, as in the case of anthraquinone reduction to anthrones,[30] and provided naphthothiophenone **9**. Even though this analogue lacks the quinone moiety, it is closely related to anthralin (**5**) of the

dx.doi.org/10.1021/jm500754d | J. Med. Chem. XXXX, XXX, XXX–XXX

Scheme 1[a]

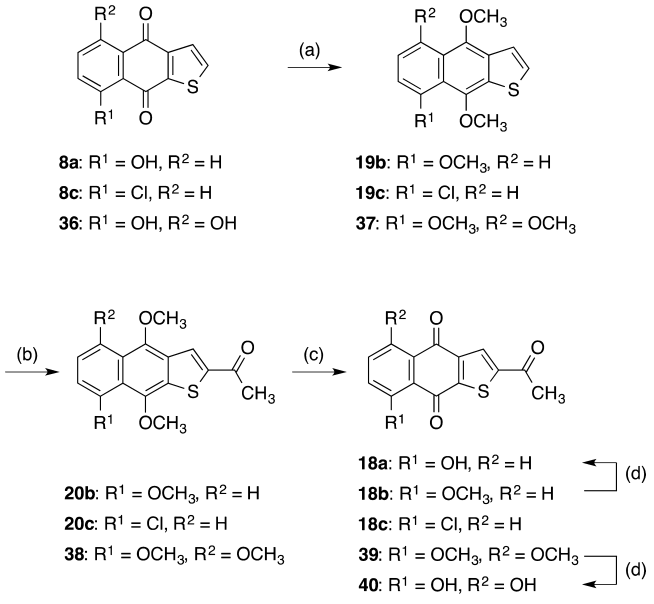


[a]Reagents and conditions: (a) 2-bromothiophene, Mg, Et_2O, benzene, 45 °C, or selenophene, $AlCl_3$, CH_2Cl_2; (b) $FeSO_4$, NH_3, reflux; (c) H_2SO_4, 120 °C; (d) (1) HOAc, H_2SO_4, 70 °C; (2) $NaNO_2$, H_2SO_4, 0 °C; (3) H_2O, H_2SO_4, reflux; (e) $SnCl_2$, HCl, HOAc, reflux.

anthrone class of antipsoriatic agents. For the synthesis of seleno analogue **10**, Friedel–Crafts conditions proved entirely satisfactory for the condensation of phthalic anhydride with selenophene to the acid **17**.

The 2-acetyl analogues **18a–18c** were prepared from the corresponding basic structures **8a** and **8c** (Scheme 2), which were subjected to reductive methylation. The phase-transfer method of Kraus[31] with sodium dithionite and dimethyl sulfate in the presence of tetrabutylammonium bromide afforded the electron-rich nucleophilic 8-methoxy- and 8-chloro-substituted 4,9-dimethoxynaphtho[2,3-*b*]thiophenes **19b** and **19c**, respectively. These were treated with *n*-butyllithium and acetylated with *N*,*N*-dimethylacetamide to afford the 2-acetylated naphthohydroquinone dimethyl ethers **20b** and **20c**. Final oxidation of the naphthohydroquinone ethers with diammonium cerium(IV) nitrate proceeded with concomitant ether cleavage to give the desired 2-acetylated target compounds **18b** and **18c**, and ether cleavage of the methoxy derivative **18b** with aluminum chloride provided the 8-hydroxy derivative **18a**. Likewise, 5,8-dihydroxy-substituted 2-acetyl analogue **40** was prepared starting from **36**. 8-Unsubstituted 2-acetyl analogue **18** (not depicted; see Table 1) was obtained in an alternative approach from naphtho[2,3-*b*]thiophene[32] by 2-acetylation as described above, and the resulting 2-acetylnaphthothiophene (**20**) was then oxidized with chromium(VI) oxide.

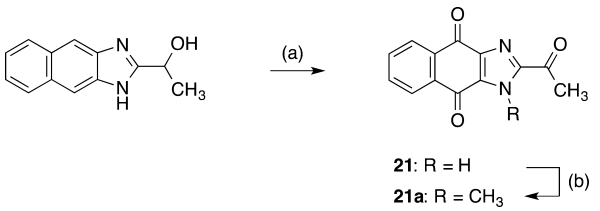
Scheme 2[a]



[a]Reagents and conditions: (a) $Na_2S_2O_4$, Bu_4NBr, THF, KOH, Me_2SO_4, N_2; (b) *n*-BuLi, THF, −15 °C, *N*,*N*-dimethylacetamide, N_2; (c) $(NH_4)_2[Ce(NO_3)_6]$, MeCN, H_2O, 0 °C; (d) $AlCl_3$, CH_2Cl_2, rt.

The 2-acetyl analogues **21** and **21a** of the naphtho[2,3-*d*]imidazole-4,9-diones **12**[33] and **12a**[34] (Table 1), respectively, were obtained by oxidation of 2-(1-hydroxyethyl)-1*H*-naphtho[2,3-*d*]imidazole[35] with an excess of potassium dichromate to afford **21**, which upon treatment with methyl iodide gave the *N*-methyl analogue **21a** (Scheme 3).

Scheme 3[a]



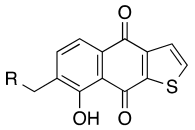
[a]Reagents and conditions: (a) $K_2Cr_2O_7$, H_2SO_4, H_2O, reflux; (b) CH_3I, K_2CO_3, DMF, −60 °C.

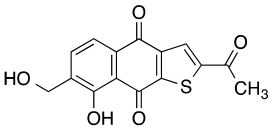
The preparation of 7-substituted derivatives (Table 2) of the selected lapacho skeleton **8a** is outlined in Scheme 4. As in our earlier studies on anthrone chemistry,[36] we have used the Marschalk reaction,[37,38] which enables the direct attachment of alkyl side chains to hydroxyanthraquinones. We have now successfully adopted this method to provide access to ortho-substituted 8-hydroxynaphtho[2,3-*b*]thiophene-4,9-diones. While **8a** itself is an electron-deficient quinone system, reduction with sodium dithionite results in an electron-rich, highly nucleophilic hydroxynaphthothiophene intermediate that reacts with aldehydes and after thermal elimination of the hydroxy substituent directly yields the alkylated products. The direct alkylation of **8a** proceeded cleanly with aliphatic aldehydes, benzaldehyde, and benzaldehydes with substituents deactivating the phenyl ring to provide the 7-substituted compounds **22a–22d** and **22i–22p**. Attempts to alkylate **8a** with benzaldehydes bearing electron-donating groups failed. However, hydroxymethylation of **8a** and reoxidation of the reaction mixture afforded the alcohol **22u**, which was converted into the chloride **22v** by thionyl chloride. Friedel–Crafts alkylation of methoxybenzenes with **22v** gave analogues **22s** and **22t**. Carboxylate esters **22e–22h** and **22q,r** were obtained by esterification of the carboxylic acids **22e** and **22o,p**, respectively.

The 2-substituted 8-hydroxynaphtho[2,3-*b*]thiophene-4,9-diones of Table 3 were prepared by the general method of Scheme 5. For functionalization of **8a** by a substitution reaction, protection of the phenolic system was necessary and a more activated tricyclic structure was required. Therefore, **8a** was exhaustively methylated as described above (Scheme 2) to

dx.doi.org/10.1021/jm500754d | J. Med. Chem. XXXX, XXX, XXX–XXX

Table 2. Suppression of Human Keratinocyte Hyperproliferation by 7-Substituted 8-Hydroxynaphtho[2,3-*b*]thiophene-4,9-diones





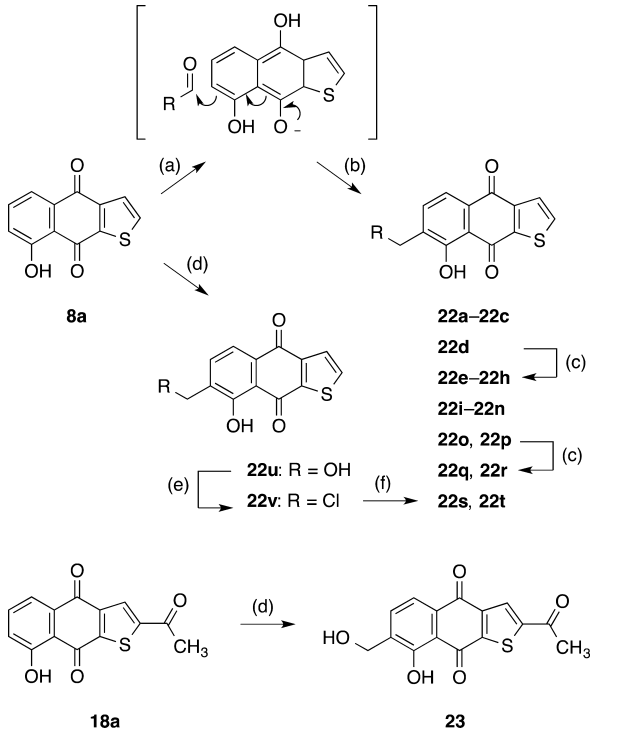
23

compd	R	AA,[a] IC_{50} (μmol/L)	LDH[b] (mU/mL)
22a	H	5.3	*d*
22b	Me	11.4	*d*, *c*
22c	Pr	>30	*d*
22d	CO_2H	21.5	*d*
22e	CO_2Me	2.8	*d*
22f	CO_2Et	2.1	*d*
22g	CO_2Pr	4.5	*d*
22h	CO_2Bn	>30	*d*
22i	furan	8.5	127
22k	Ph	10.1	107
22l	Ph-4-Me	13.8	*d*
22m	Ph-4-CF_3	16.7	*d*
22n	Ph-4-CN	14.2	*d*
22o	Ph-2-CO_2H	25.7	*d*
22p	Ph-4-CO_2H	>30	*d*
22q	Ph-2-CO_2Me	19.1	*d*
22r	Ph-4-CO_2Me	>30	*d*
22s	Ph-4-OMe	23.2	*d*
22t	Ph-3,4-$(OMe)_2$	>30	*d*
22u	OH	1.2	214[c]
23		0.70	284[c]
anthralin		0.70	239[c]
β-lapachone		0.90	268[c]
menadione		15.8	*d*

[a]Antihyperproliferative activity against keratinocytes. IC_{50} = concentration of test compound required for 50% inhibition of cell growth (HaCaT). Inhibition of cell growth was significantly different with respect to that of the control, $N = 3$, $P < 0.05$. [b]Activity of LDH (mU) release in HaCaT cells after treatment with 2 μmol/L test compound ($N = 3$, SD < 10%). [c]Values are significantly different with respect to those of the vehicle control (0.2% DMSO in the culture medium, 110 mU/mL), $P < 0.05$. Brij 35 (polyoxyethylene glycol dodecyl ether)/ultrasound was the positive control (409 mU/mL). [d]Not determined.

provide the electron-rich 4,8,9-trimethoxynaphtho[2,3-*b*]-thiophene (**19b**), and the desired 2-acyl precursors **24a–24m** as well as carboxylic acid **27a** were prepared by metalation of **19b** with *n*-butyllithium and quenching the resulting anion with the appropriate *N*,*N*-dimethylcarboxamide or dry ice, respectively, analogously to the procedure of Tanaka.[39] In a similar manner, carboxamide **30** was obtained with *N*,*N*-diethyl-2,2,2-trifluoroacetamide, as in this case the trifluoromethyl group was eliminated from the organolithium intermediate rather than diethylamine. 2-Carboxylic ester analogues **27b–27e** were obtained from **27a** and appropriate alcohols by Steglich esterification.[40] Furthermore, 1,2,4-oxadiazole analogues **33a** and **33b** were also prepared from carboxylic acid **27a** and *N*-hydroxypropionamidine or *N*-hydroxy-3-phenylpropionamidine, respectively, in a one-pot reaction.[41] As also outlined in Scheme 5, these 2-substituted 4,8,9-trimethoxynaphtho[2,3-*b*]thiophenes **24a–24m**, **27a–27e**, and **30**, as well as **33a** and **33b**, were then oxidized with diammonium cerium(IV) nitrate to the quinone stage (**25a–25m**, **28a–28e**, **31**, **34a**, **34b**)

Scheme 4[a]



[a]R is defined in Table 2. Reagents and conditions: (a) $Na_2S_2O_4$, NaOH, N_2, 60 °C, 20 min; (b) RCHO, $Na_2S_2O_4$, N_2, 90 °C, 12 h, O_2, HCl; (c) appropriate alcohol, H_2SO_4, reflux, 3 h; (d) (1) $Na_2S_2O_4$, MeOH, NaOH, HCHO, N_2, 0–5 °C, 3 h; (2) H_2O_2, HCl; (e) CH_2Cl_2, $SOCl_2$; (f) anisole or veratrole, $AlCl_3$, CH_2Cl_2, reflux, 4 h.

followed by deprotection of the 8-methoxy group with aluminum chloride to give the desired 8-hydroxy-substituted target compounds, i.e., 2-acyl analogues **26a–26m**, 2-carboxylic acid **29a**, esters **29b–29e**, amide **32**, and oxadiazoles **35a** and **35b**.

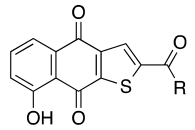
BIOLOGICAL EVALUATION AND DISCUSSION

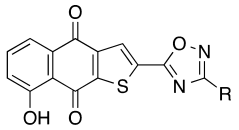
Initial Evaluation of Simple Analogues as Inhibitors of Keratinocyte Hyperproliferation and SARs for the Heterocyclic Ring System. As shown in Table 1 we initially examined the antihyperproliferative activity against keratinocytes of some basic heterocycle-fused naphthoquinones, i.e., simple analogues of the basic structure **7** of lapacho-derived 2-acetylnaphtho[2,3-*b*]furan-4,9-dione (**3**) in which the furan oxygen was replaced by sulfur (**8**), selenium (**10**), or nitrogen (**11**). Furthermore, an additional heteroatom was introduced (**12–14**).

The ability of these compounds to suppress keratinocyte hyperproliferation is potentially useful in the treatment of psoriasis because a rebalanced homeostatic control of keratinocyte growth and differentiation is crucial for recovery from psoriatic to normal epidermis.[42] As a good model for the evaluation of our compounds, we used HaCaT cells, a rapidly dividing immortalized human keratinocyte line.[43] These cells mimic the hyperproliferative epidermis, show a keratinization pattern similar to that of psoriatic skin, and have been widely used for the preclinical evaluation of novel antipsoriatic agents.[44] The antipsoriatic agent anthralin and the naturally occurring β-lapachone were used as standard compounds (Table 2).

dx.doi.org/10.1021/jm500754d | J. Med. Chem. XXXX, XXX, XXX–XXX

Table 3. Suppression of Human Keratinocyte Hyperproliferation by 2-Substituted 8-Hydroxynaphtho[2,3-*b*]thiophene-4,9-diones





35a, 35b

compd	R	AA,[a] IC_{50} (μmol/L)	LDH[b] (mU/mL)
26a	H	1.00	*d*
26b	Et	0.38	244[c]
26c	$CHMe_2$	0.45	*d*
26d	CH_2Ph	4.42	*d*
26e	$(CH_2)_2Ph$	>30	*d*
26f	CH_2OPh	11.15	*d*
26g	Ph	0.80	419[c]
26h	Ph-4-Cl	5.64	*d*
26i	Ph-4-OMe	4.72	*d*
26k	Ph-2-OH-4-OMe	7.07	*d*
26l	2-thienyl	0.29	158[c]
26m	3-pyridine	0.30	154[c]
29a	OH	>30	*d*
29b	OEt	0.76	233[c]
29c	$OCHMe_2$	0.92	*d*
29d	OCH_2Ph-4-OMe	11.90	*d*
29e	$O(CH_2)_2Ph$	>30	*d*
32	NEt_2	0.36	330[c]
35a	Et	0.19	150[c]
35b	$(CH_2)_2Ph$	0.48	*d*

[a]Antihyperproliferative activity against keratinocytes. IC_{50} = concentration of test compound required for 50% inhibition of cell growth (HaCaT). Inhibition of cell growth was significantly different with respect to that of the control, $N = 3$, $P < 0.05$. [b]Activity of LDH (mU) release in HaCaT cells after treatment with 2 μmol/L test compound ($N = 3$, SD < 10%). [c]Values are significantly different with respect to those of the vehicle control (0.2% DMSO in the culture medium, 110 mU/mL), $P < 0.05$. Brij 35 (polyoxyethylene glycol dodecyl ether)/ultrasound was the positive control (409 mU/mL). [d]Not determined.

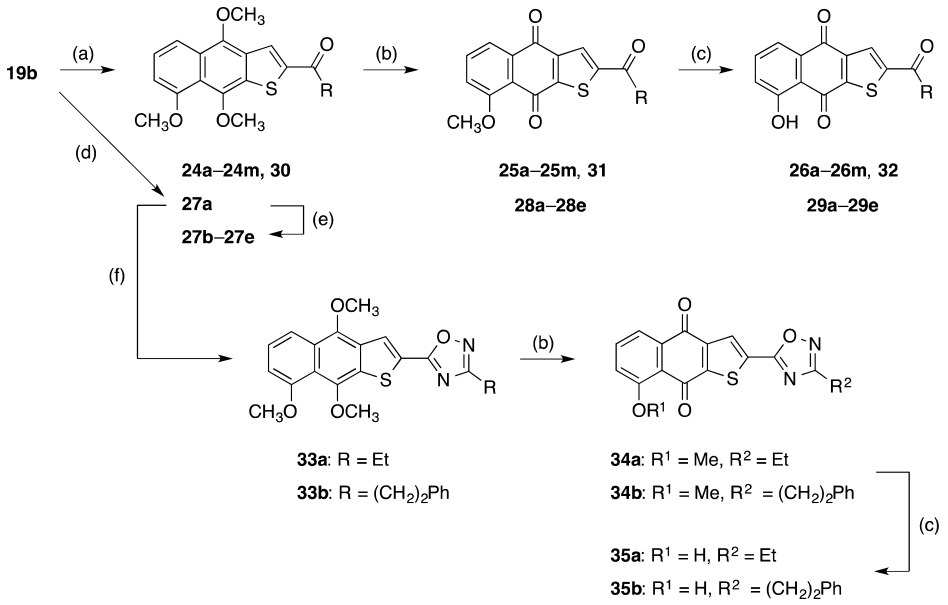
The preliminary evaluation of SARs for the basic naphthoquinone skeletons in this model indicated that most of them indeed possessed valuable, but not outstanding, activity. Attempts at changing the nature of the heterocyclic ring system met with varied success. Bioisosteric replacement of the furan oxygen of the basic lapacho structure **7** with nitrogen or introduction of a second nitrogen atom was detrimental to activity, as the pertinent pyrrole-fused (**11**) and imidazole-fused (**12** and **12a**) agents were totally inactive. This was also observed for their 2-acetylated derivatives **21** and **21a**, respectively, which are aza analogues of the original lapacho quinones **3** and **4**. On the other hand, oxazole (**13**) and thiazole (**14**) analogues revealed interesting activity; however, attempts to introduce a 2-acetyl group as in the original lapacho quinones **3** and **4** failed. Furthermore, replacement of oxygen for sulfur (**8**) almost retained activity. This led us to evaluate some 8-substituted naphthoquinone skeletons (**8a**–**8e**) of the thiophene analogue **8**. These structures were then acetylated at C-2 as in **3**. From inspection it became clear that introduction of an 8-chlorine atom, **8c**, did not substantially alter the inhibitory potency in the keratinocyte hyperproliferation assay relative to that of **7** and **8**, whereas an 8-methoxy group, **8b**, led to a clearly less potent structure, and an 8-amino group, **8d**, even abolished activity, unless it was converted into its *N*-acetylated form **8e**. However, interesting inhibitory activity was obtained by adding an 8-hydroxy group (**8a**), and an even more potent compound was found when the 2-position of the fused heterocycle was acetylated, as in **18a**. Finally, quinone **8a** was also reduced to the naphthothiophenone **9** to explore an analogue that contains chemical features on which the minimum structure of antipsoriatic activity is based[45] and is most similar to the potent antipsoriatic agent anthralin (**5**). Unfortunately, this analogue was only slightly more potent than **8a** and substantially less potent than anthralin.

Evaluation of 7-Substituted 8-Hydroxynaphtho[2,3-*b*]thiophene-4,9-diones. With a rudimentary understanding of the tricyclic skeleton SARs, we turned our attention to side chain modifications on naphthoquinone skeleton **8a**. Among our first thoughts was to introduce carbon side chains into position 7, as this is favored by the presence of the 8-hydroxy group. The ability of the resulting compounds to suppress keratinocyte hyperproliferation is shown in Table 2. Compounds **22a**–**22c** and **22i**–**22t** explore the consequences of adding an aliphatic or arylmethyl side chain to the 7-position of the naphthoquinone skeleton. Increasing the lipophilic character of the compounds appears to be disadvantageous for their potency, which is demonstrated by the loss of potency for the set of 7-alkyl homologues; the order was methyl > ethyl > butyl. Furthermore, 7-benzyl-substituted analogues were all less active than the parent **8a**. Compounds **22l**–**22t** were prepared to study the effects of further substitution on the benzyl group, but these agents were less potent, which was also observed for those bearing hydrophilic groups (**22o**–**22r**). This indicated that both electronic and solubility effects might not be at play in the SARs of 7-substituted analogues. Analogues **22d**–**22h** explore introduction of an acetic acid side chain and some corresponding esters. This was done primarily to improve the hydrophilic character of the molecules. Methyl ester **22e** was equipotent to parent **8a**, whereas ethyl ester **22f** was somewhat more potent. Finally, 7-hydroxymethylated **22u** was the most potent compound in this series. Potency was further improved by an additional 2-acetyl group, with an IC_{50} of 0.70 μM for analogue **23**, but not as potent as 2-acetylated **8a**; cf. lapacho compound **18a**, IC_{50} = 0.40 μM (Table 1). The overall trend of lower potency seems to indicate that introduction of this structural modification in position 7 of the naphtho[2,3-*b*]thiophene-4,9-dione is generally unfavorable, however.

Effects of Varying the 2-Acetyl Side Chain. The third part of this investigation examined the effects of varying the 2-acetyl group (**18a**) on the selected 8-hydroxynaphtho[2,3-*b*]thiophene-4,9-dione (**8a**) to define SARs for this position (Table 3). The choice of the 2-acyl and 2-carboxylic ester groups was guided by our previous study on the naphthofuran series of analogues in which a carbonyl group at the 2-position was found optimal for substitution.[23] Comparing the homologues series of small and branched alkyl groups (**18a** of Table 1, **26b**, **26c**), these compounds were active in the submicromolar range, whereas a terminal phenyl ring in **26d** and **26f** lowered potency or gave an inactive compound (**26e**). Furthermore, a phenyl (**26g**) ring directly attached to the carbonyl group was tolerated well, with an IC_{50} of 0.80 μM, which is of the same order of magnitude as those of anthralin and β-lapachone. However, aromatic substitution was not successful, independently of more hydrophobic and electron-withdrawing (**26h**) or electron-donating (**26i**, **26k**) effects, leading to a decrease in potency. Replacing the phenyl ring in

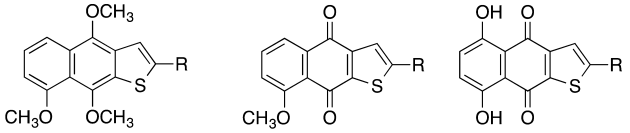
dx.doi.org/10.1021/jm500754d | J. Med. Chem. XXXX, XXX, XXX–XXX

Scheme 5[a]



[a]R is defined in Table 3. Reagents and conditions: (a) *n*-BuLi, THF, −15 °C, appropriate *N*,*N*-dimethylcarboxamide, N_2; (b) $(NH_4)_2[Ce(NO_3)_6]$, MeCN, H_2O, 0 °C; (c) $AlCl_3$, CH_2Cl_2, rt; (d) *n*-BuLi, THF, −15 °C, CO_2, THF, −70 °C, N_2; (e) DCC, DMAP, ROH, 0 °C, rt; (f) DCC, CH_2Cl_2, 0 °C, appropriate *N*-hydroxyalkylamidine, pyridine, reflux.

Table 4. Suppression of Human Keratinocyte Hyperproliferation by 4,8,9-Trimethoxynaphtho[2,3-*b*]thiophenes, 8-Methoxynaphtho[2,3-*b*]thiophene-4,9-diones, and 5,8-Dihydroxynaphtho[2,3-*b*]thiophene-4,9-diones



19b, 24b, 24m, 27b, 33a **8b, 25b, 25m, 28b, 34a** **36, 40**

compd	R	AA,[a] IC_{50} (μmol/L)	compd	R	AA,[a] IC_{50} (μmol/L)
8b	H	13.50	**27b**	CO_2Et	>30
19b	H	>30	**28b**	CO_2Et	2.87
24b	COEt	>30	**33a**	5-(1,2,4-oxadiazole)	>30
24m	nicotinoyl	>30	**34a**	5-(1,2,4-oxadiazole)	3.52
25b	COEt	3.50	**36**	H	2.70
25m	nicotinoyl	5.70	**40**	COMe	0.50

[a]Antihyperproliferative activity against keratinocytes. IC_{50} = concentration of test compound required for 50% inhibition of cell growth (HaCaT). Inhibition of cell growth was significantly different with respect to that of the control, $N = 3$, $P < 0.05$.

26g with thiophene (**26l**) or pyridine (**26m**) gave bioisosteric molecules whose potency was nearly 3-fold improved. An acid substituent at position 2 renders the compound inactive (**29a**). The deleterious effect of the presence of the free carboxylic acid group on potency could be overcome by esterification. Thus, ethyl ester **29b** demonstrated activity, and with an IC_{50} of 0.76 μM against keratinocyte hyperproliferation it was equipotent to anthralin, supporting the hypothesis that cellular inactivity of **29a** was due to insufficient cell penetration. Similar to the 2-acyl series, a terminal phenyl ring of the ester chain diminished potency (**29d**) or abolished activity (**29e**). Potency was further enhanced when the ester group of **29b** was replaced with a pertinent amide group, with analogue **32** (IC_{50} = 0.36 μM) being among the most potent compounds of this series. As the replacement of the ester with an amide was compatible with good activity, it was obviously of interest to see what would happen when the same replacement was made with a bioisosteric group that would mimic the carbonyl-containing ester or amide groups of **29b** and **32**, such as the 1,2,4-oxadiazole moiety.[46,47] Accordingly, we found that the oxadiazoles **35a** and **35b** were highly potent, IC_{50} = 0.19 and 0.48 μM, confirming a similar observation made in our previous study.[23]

Other Structural Features. Table 4 shows, for a limited set of analogues, the effect on activity against keratinocyte hyperproliferation of other structural features. There are four pairs of compounds where the effect of *O*-methylation of the 8-hydroxy group can be explored by comparing the corresponding 8-methoxy and 8-hydroxy analogues of Table 3. In all cases the 8-hydroxy analogues are the more potent inhibitors, which is in good agreement with the observation made in the initial study with the pairs of basic structures **8a**/**8b** and **18a**/**18b** (Table 1). The differences vary from 4- to 9-fold (**25b**/**26b**, **28b**/**29b**) and up to 19-fold for the nicotinoyl-substituted **25m**/**26m** and oxadiazoles **34a**/**35a**. In addition, there are five pairs of compounds where the basic structures (**8b**/**19b**) and

dx.doi.org/10.1021/jm500754d | J. Med. Chem. XXXX, XXX, XXX−XXX

Table 5. Rates of Superoxide Generation by 8-Hydroxynaphtho[2,3-*b*]thiophene-4,9-diones through One-Electron Reduction by Human Recombinant CPR and Two-Electron Reduction by Human Recombinant NQO-1

	rate of $O_2^{\bullet-}$ generation			rate of $O_2^{\bullet-}$ generation	
compd	CPR[a]	NQO-1[b]	compd	CPR[a]	NQO-1[b]
8a	1.8 ± 0.4[c]	0.3 ± 0.2	menadione	2.1 ± 0.4[c]	1.0 ± 0.4[c]
18a	1.1 ± 0.1[c]	0.6 ± 0.2[c]	control[d]	0.2 ± 0.1	0.2 ± 0.1
35a	1.0 ± 0.1[c]	0.7 ± 0.1[c]			

[a]The rate of superoxide generation in the presence of CPR is expressed as the rate of SOD-inhibitable reduction of succinoylated cytochrome *c* (μmol/L/min/2 mU of enzyme) by the test compound (100 μmol/mL). Each value represents the mean ± SD, $N \geq 3$ [b]The rate of superoxide generation in the presence of NQO-1 is expressed as the rate of SOD-inhibitable reduction of cytochrome *c* (μmol/L/min/U of enzyme) by the test compound (25 μmol/mL). Each value represents the mean ± SD, $N \geq 3$. [c]Values are significantly different from those of the vehicle control, $P < 0.0001$. [d]Rate of reduction with no test compound present (DMSO).

those bearing propionyl (**24b**/**25b**), nicotinoyl (**24m**/**25m**), ethyl carboxylate (**27b**/**28b**), and oxadiazole (**33a**/**34a**) side chains can be directly compared. The results for these sets of compounds suggest the requirement for a quinone moiety. When the quinone form is masked by reductive methylation to afford the 4,8,9-trimethoxynaphtho[2,3-*b*]thiophenes, in all five of these cases activity against keratinocyte hyperproliferation is completely lost. Finally, compounds **36** and **40** explore the consequences of an additional 5-hydroxy group, which would allow the presence of a fully internally hydrogen-bonded quinone function. There appears to be no requirement for hydrogen bond donor capability at position 5, as the 2-acetyl derivative **40** and the monohydrogen-bonded analogue **18a** as well as the pair of basic structures **36** and **8a** differ little in their potency

Lactate Dehydrogenase Release. A major concern in the testing of potential inhibitors of keratinocyte hyperproliferation is to confirm that the compound does not interfere with the functioning of the cell membrane by causing leakage of cytoplasm through it. Therefore, the action on the cell cultures by the lapacho analogous compounds was assessed by the activity of lactate dehydrogenase (LDH) activity released into the culture supernatant. The release of LDH is commonly used as an indicator of plasma membrane damage.[48,49] Tables 2 and 3 show the membrane-damaging properties of some selected potent inhibitors of keratinocyte hyperproliferation. The most potent 7-substituted inhibitor of Table 2, hydroxymethyl-substituted **22n**, also significantly released LDH, while LDH released by weaker inhibitors such as furan **22i** and compound **22k** was not significant. Whereas all tested compounds of Table 3 released significantly large amounts of LDH, there was clear distinction in potency of inducing cell lysis within the 2-substituted series. Thus, comparing the strongest inducers of membrane damage with the weakest, i.e., compound **26g** with **26l**/**26m**, and **32** with **35a**, respectively, less than half the amount of LDH was released when the phenyl ring or the amide group was replaced with a thiophene/pyridine or an oxadiazole ring, respectively, suggesting that bioisosteric replacement is a useful measure for substantially reducing membrane-damaging effects. In this assay, the heterocycle-substituted analogues **26l**, **26m**, and **35a** also compared favorably with anthralin, the clinical efficacy of which is limited by staining and irritation of the nonaffected skin,[24] and *β*-lapachone, as LDH release by these agents significantly exceeded that of the vehicle control (Table 2) and was much more pronounced than that of the bioisosteric analogues.

Enzymatic Redox Activation. While a number of biological effects of lapacho-derived quinones have been described, their mechanism of action and their key cellular targets remain largely unknown. Due to their electrophilic and redox-active nature, the major mechanisms of quinones include alkylation of cellular nucleophiles such as thiols and redox cycling initiated through one-electron reduction by NADPH–cytochrome P450 oxidoreductase (CPR) or two-electron reduction by NAD(P)H:quinone oxidoreductase 1 (NQO-1).[50−52] Both the desired biological action and some other effects such as skin toxicity of quinones have been attributed to the generation of reactive oxygen species (ROS) through enzyme-mediated redox cycling.[50−56] The reduction of quinones typically occurs in two sequential reactions to generate hydroquinones via semiquinone radicals that can react back to the parent compound, thus perpetuating a redox cycle. Concomitantly, electron transfer to molecular oxygen generates superoxide and other species therefrom, such as H_2O_2 and hydroxyl radicals ($^{\bullet}OH$). Finally, the depletion of the antioxidant defense systems may cause oxidative stress,[57] which results in the failure of normal cellular functions and even cell death.[58] Our recent study revealed that lapacho quinones were activated by both CPR- and NQO-1-catalyzed one- and two-electron reduction, respectively, to generate superoxide, which we also confirmed in a keratinocyte-based assay.[23]

Superoxide Generation in Isolated Enzyme Assays. The rate of superoxide generation was measured using a spectrophotometric assay that employs ferricytochrome *c* as the terminal electron acceptor, which was specified by (1) succinoylation of cytochrome *c*, (2) addition of superoxide dismutase (SOD) to ensure that only the SOD-inhibitable, superoxide-dependent extent of cytochrome *c* reduction was measured,[59] (3) addition of catalase to scavenge H_2O_2, and (4) DTPA to prevent the generation of hydroxyl radicals.[60]

The ability of selected 8-hydroxynaphtho[2,3-*b*]thiophene-4,9-diones to stimulate superoxide generation was determined, and the results are presented in Table 5. For comparison, the known redox cycler menadione[61,62] (2-methylnaphthalene-1,4-dione) was used as a positive control. In the one-electron reduction catalyzed by human recombinant CPR and NADPH, the 2-unsubstituted structure **8a** generated the highest amount of superoxide, whereas 2-acetylation (**18a**) or an oxadiazole ring in position 2 (**35a**) substantially decreased activation through one-electron transfer, which was significant for all three lapacho analogues. Accordingly, the semiquinone radicals generated from these compounds in turn can efficiently react with molecular oxygen and generate superoxide. However, when the compounds were incubated with human recombinant NQO-1/NADPH to study the contribution of two-electron reduction, superoxide generation of the basic structure **8a** did not significantly exceed the control value. By contrast, both the 2-acetyl (**18a**) and oxadiazole (**35a**) analogues significantly

dx.doi.org/10.1021/jm500754d | J. Med. Chem. XXXX, XXX, XXX−XXX

Table 6. Superoxide Generation in Human Keratinocytes and Percentage of Apoptotic Cells Following Acute and Chronic Treatment with 8-Hydroxynaphtho[2,3-*b*]thiophene-4,9-diones

	$O_2^{\bullet-}$ generation (MFI)[a]			percentage of apoptotic cells	
compd	acute treatment[b]	chronic treatment[c]	dicoumarol pretreatment[c,d]	chronic treatment[c]	dicoumarol pretreatment[c,d]
8a	23.8 ± 3.7[e]	610.6 ± 258.7[e]	717.7 ± 91.1[e]	4.9 ± 1.4[e]	6.1 ± 3.1
18a	23.7 ± 3.8[e]	921.4 ± 149.2[e]	813.0 ± 85.0[e]	23.0 ± 13.2[e]	7.3 ± 4.7
35a	20.9 ± 16.6[e]	802.2 ± 400.1[e]	747.0 ± 36.3[e]	77.6 ± 23.0[e]	20.3 ± 12.1[e]
menadione	44.0 ± 11.6[e]	431.9 ± 83.5	457.2 ± 103.4	4.8 ± 2.3	3.1 ± 1.1
control[f]	7.1 ± 5.2	367.5 ± 80.1	407.6 ± 68.7	3.0 ± 1.0	4.3 ± 2.6

[a]Superoxide generation is expressed in terms of changes in the mean fluorescence intensity (MFI) of 2-OH-E^+. Each value represents the mean ± SD, $N = 3$. [b]HaCaT keratinocytes were incubated with test compound (50 μmol/L) for 30 min. [c]HaCaT keratinocytes were incubated with test compound (5 μmol/L) for 18 h. [d]HaCaT keratinocytes were preincubated with the NQO-1 inhibitor dicoumarol (5 μmol/L) for 25 min before treatment with test compounds. [e]Values are significantly different from those of the vehicle control, $P < 0.05$. [f]Mean fluorescence intensity with no test compound present (DMSO).

generated increased amounts of superoxide, suggesting that their primary hydroquinones produced by two-electron reduction were not stable and readily autoxidized. This may be related to an electron-withdrawing substituent in position 2, which enables redox cycling and superoxide generation.[23]

Superoxide Generation in Keratinocytes. To prove that redox cycling of lapacho quinones also results from the normal enzyme activities of keratinocytes, superoxide generation was directly evaluated in a whole-cell assay. For the detection and quantification of superoxide under the conditions of the hyperproliferation assay in HaCaT keratinocytes, we employed flow cytometry using dihydroethidium (DHE) as the fluorescent probe.[63] The fluorescence intensity arising from the product of the DHE/superoxide reaction, 2-hydroxyethidium (2-OH-E^+), is a measure of intracellular superoxide generation and a specific marker for superoxide.[64,65] Menadione served as a positive control, as intracellular superoxide generation by the use of DHE has been described for this naphthoquinone.[64,66]

Acute and Chronic Treatment of HaCaT Keratinocytes with Lapacho Analogues. Quantitative measurements of the mean fluorescence intensities from the samples demonstrated significantly increased superoxide generation following both acute and chronic treatment with the three lapacho compounds (Table 6). However, menadione significantly enhanced the levels of 2-OH-E^+ fluorescence only at relatively high concentrations (50 μmol/L) where superoxide generation in human spermatozoa has been described.[66]

Effects of Pretreatment with the NQO-1 Inhibitor Dicoumarol. To demonstrate the role of NQO-1 through a two-electron reduction of lapacho quinones, we determined intracellular superoxide generation in keratinocytes after preincubation with dicoumarol. This is a commonly used inhibitor of NQO-1,[61,67] which competes with NADH for binding and prevents reduction of various quinones. As shown in Table 6, chronic treatment of dicoumarol-pretreated keratinocytes with lapacho analogues significantly increased 2-OH-E^+ fluorescence in each case. In control experiments, alone or with dicoumarol, chronic treatment of keratinocytes with menadione did not significantly increase superoxide generation at a concentration of 5 μmol/L, which is in line with the observation that relatively high concentrations of menadione were required for suppression of HaCaT cell hyperproliferation (IC_{50} = 15.8 μmol/L, Table 2).

To further examine the action of lapacho analogues in HaCaT keratinocytes after chronic treatment, apoptosis was measured by 7-aminoactinomycin D (7-AAD) staining. Cells in the early phase of apoptosis stain positive for 7-AAD in flow cytometry, depending on the concentration of the fluorescent intercalator.[68] All lapacho analogues (5 μM/L) significantly increased early apoptosis compared to the control (Table 6), with oxadiazole **35a** having the greatest effect. In contrast, the basic structure **8a** only slightly triggered apoptosis, and induction of cell death by menadione was not significant. In contrast to its effect on intracellular superoxide generation, pretreatment of the keratinocytes with dicoumarol substantially reduced induction of apoptosis by about 3-fold.

The biological implications of oxidative stress[57] in human keratinocytes induced by lapacho analogues with respect to their potential therapeutic action for the treatment of psoriasis or other hyperproliferative skin disorders has been fully discussed in the first part of this study.[23] According to the results of this paper, the selected 8-hydroxynaphtho[2,3-*b*]thiophene-4,9-dione (**8a**) and its 2-substituted analogues **18a** and **35a** were potent generators of superoxide radicals in the chronic treatment experiments under conditions comparable to those of the hyperproliferation model. The observation that pretreatment of keratinocytes with the NQO-1 inhibitor dicoumarol did not substantially modulate increased superoxide generation may suggest that NQO-1 cannot protect keratinocytes against oxidative stress induced by these compounds. The large amounts of superoxide produced may indicate an increased availability of these quinones for one-electron reduction and redox cycling by CPR. Surprisingly, when we extended these studies to compare the ability of the three lapacho quinones to trigger cell death, either alone or in the presence of dicoumarol, keratinocytes pretreated with the NQO-1 inhibitor were protected against apoptosis induced by the 2-substituted analogues **18a** and **35a**. This clearly shows that NQO-1 is involved in the activation of these quinones. However, the nature of the activated species responsible for a possible reaction with DNA or other cellular macromolecules, such as lipids and proteins, to cause damage to the cell, remains unclear, as superoxide formation did not correlate with the induction of apoptosis. In this context, the possible contribution of the oxadiazole substituent at the quinone, as in the highly potent quinone **35a**, is currently under investigation in our laboratory.

CONCLUSIONS

In summary, we have described modifications to three structural features present in the basic naphtho[2,3-*b*]furan-4,9-dione (**7**) of lapacho compounds that could influence biological activity and may help to further delineate SARs for

dx.doi.org/10.1021/jm500754d | J. Med. Chem. XXXX, XXX, XXX−XXX

suppression of keratinocyte hyperproliferation. To this end, a total of 71 analogues of **7** were prepared and evaluated. Information obtained from our initial SAR studies that pyrrole- or imidazole-fused systems were inactive, coupled to the fact that 2-acylation of pertinent thiazole- or oxazole-fused systems was not efficient, led us to explore the effect of introducing a variety of substituents at the 2- and 7-positions of the selected thiophene-fused 8-hydroxy-substituted naphthoquinone **8a**. While 7-substitution was generally less beneficial for improving potency, potent inhibitors of keratinocyte hyperproliferation were obtained with electron-withdrawing functionalities such as acyl, carboxylic acids, and carboxamide or their bioisosteric replacement with a 1,2,4-oxadiazole ring at position 2 of the tricyclic system. In particular, analogues **26l**, **26m**, and **35a** with a thiophene, a pyridine, or an oxadiazole ring, respectively, compared favorably with the antipsoriatic agent anthralin, as their potency was combined with comparably low membrane-damaging effects toward keratinocytes. The biological activity of the lapacho analogues is thought to be due, in part, to their activation by enzymatic reductases such as CPR or NQO-1 to the semiquinones or hydroquinones, respectively. These intermediates react with oxygen to generate superoxide, which we have detected in the hyperproliferation assay. However, the amount of superoxide produced by lapacho quinones did not correlate with their ability to induce cell death.

EXPERIMENTAL SECTION

Melting points were determined with a Kofler melting point apparatus and are uncorrected. ^{1}H NMR spectra were recorded on a Varian Mercury 400 plus spectrometer (400 MHz) using tetramethylsilane as an internal standard. Fourier transform IR spectra were recorded on a Jasco FT/IR-4100 (attenuated total reflection, ATR) spectrometer by applying ATR correction. Mass spectra (EI, unless otherwise stated) were obtained on a Finnigan MAT GCQ instrument (70 eV). Thin-layer chromatography (TLC) was conducted on Merck 60 F_{254} precoated silica gel plates. Chromatography refers to column chromatography, which was performed on Acros Organics silica gel (0.060−0.200 mm, 6 nm) with CH_2Cl_2 as the eluant unless otherwise stated. Yields have not been optimized. Elemental analyses were performed by the Microanalysis Laboratory, University of Münster, using a Vario EL III elemental analyzer. Analytical data confirmed the purity of the test compounds was ≥95%.

Compounds **5**,[30] **7**,[23] **8**,[69] **8c**,[27] **8d**,[27,28] **11**,[70] **12**,[33] **12a**,[34] **13**,[71] **14**,[72] **15**,[28] **16**,[28] and **36**[73] were prepared as described.

8-Hydroxynaphtho[2,3-*b*]thiophene-4,9-dione (8a). To a solution of 8-aminonaphtho[2,3-*b*]thiophene-4,9-dione (**8d**;[27] 2.50 g, 10.91 mmol) in glacial acetic acid (36 mL) was added 96% H_2SO_4 (10 mL) at 70 °C. The mixture was stirred for 10 min, and a small amount of insoluble material was removed by filtration. The filtrate was cooled in an ice bath (0 °C), a solution of $NaNO_2$ (0.982 g, 14.23 mmol) in water (2 mL) was added dropwise over 30 min, and the reaction mixture was kept at 0 °C for 75 min. Urea (0.325 g, 5.41 mmol) in water (2 mL) was added to quench any unreacted nitrous acid, and the reaction mixture was stirred for an additional 30 min at 0 °C. The cold solution was added over 1 h to a refluxing solution of 96% H_2SO_4 (33 mL) in water (260 mL). The reaction was heated at reflux for 3 h until N_2 evolution ceased and then cooled in an ice bath. The brown-red crystals were filtered by suction, dried in vacuo, and purified by chromatography to afford orange crystals: 64% yield; mp 188 °C; FTIR 1665, 1626 (CO···HO) cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 12.18 (s, 1H), 7.77 (d, 1H), 7.77 (d, J = 5.08 Hz, 1H), 7.71 (d, J = 5.09 Hz, 1H), 7.63 (t, 1H), 7.27 (dd, 1H); MS m/z 230 (100, M$^+$). Anal. ($C_{12}H_6O_3S$) C, H. For the crystallographic data of **8a**, see the Supporting Information.

8-Methoxynaphtho[2,3-*b*]thiophene-4,9-dione (8b). To a solution of **8a** (0.50 g, 2.17 mmol) in dry acetone (20 mL) with stirring was added anhydrous K_2CO_3 (3.00 g, 21.72 mmol), and the reaction mixture was heated to reflux. Dimethyl sulfate (1.03 mL, 10.86 mmol) was added dropwise, and the mixture was refluxed for an additional 4 h until the reaction was completed (TLC control). Then it was cooled to rt, filtered by suction, and washed with acetone (20 mL). The solvent was evaporated and the residue purified by chromatography to afford light yellow, felted needles: 72% yield; mp 164 °C (lit.[69] mp 164−166 °C); FTIR 1670 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 7.91 (dd, ^3J = 7.62 Hz, ^4J = 1.18 Hz, 1H), 7.70 (t, 1H), 7.63 (d, 2H), 7.33 (d, 1H), 4.04 (s, 3H); MS m/z 244 (100, M$^+$). Anal. ($C_{13}H_8O_3S$) C, H.

***N*-(4,9-Dioxo-4,9-dihydronaphtho[2,3-*b*]thiophene-8-yl)-acetamide (8e).** A mixture of **8d** (0.25 g, 1.09 mmol) in acetic anhydride (5 mL) and H_2SO_4 (96%, 0.10 mL) was heated to reflux for 20 min. Then it was poured into ice−water (100 mL) and extracted with CH_2Cl_2 (3 × 50 mL). The combined organic phase was washed with water (3 × 50 mL), dried over Na_2SO_4, concentrated, and purified by chromatography to afford orange needles: 54% yield; mp 198−200 °C; FTIR 1702 (CONH), 1662 (CO), 1627 (CO···HN) cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 12.04 (s, 1H), 9.02 (dd, ^3J = 8.6 Hz, ^4J = 1.17 Hz, 1H), 7.92 (dd, 1H), 7.67 (d, J = 5.08 Hz, 1H), 7.65 (t, J = 8.21 Hz, 1H), 7.61 (d, J = 5.08 Hz, 1H), 2.25 (s, 3H); MS m/z 271 (23, M$^{\bullet+}$), 229 (100). Anal. ($C_{14}H_9NO_3S$) C, H.

8-Hydroxynaphtho[2,3-*b*]thiophen-9(4*H*)-one (9). To a solution of **8a** (0.30 g, 1.30 mmol) in glacial acetic acid (40 mL) heated to reflux was added, dropwise over 3 h, a solution of $SnCl_2$ (5.55 g, 24.60 mmol) in 37% HCl (9.7 mL). The solution was then cooled to rt, filtered, diluted with water (40 mL), and extracted with CH_2Cl_2 (2 × 40 mL). The combined organic phase was washed with water (3 × 40 mL), dried over Na_2SO_4, concentrated, and purified by chromatography to afford yellow crystals: 3% yield; mp 165−168 °C; FTIR 1627 (CO···HO) cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 12.60 (s, 1H), 7.73 (d, J = 5.08 Hz, 1H), 7.39 (t, 1H), 7.10 (d, J = 5.08 Hz, 1H), 6.88 (dd, ^3J = 7.62 Hz, ^4J = 0.98 Hz, 1H), 6.86 (dd, J = 8.21 Hz, 1H), 4.25 (s, 2H); MS m/z 216 (100, M$^{\bullet+}$). Anal. ($C_{12}H_8O_2S$) C, H.

Naphtho[2,3-*b*]selenophene-4,9-dione (10). A solution of **17** (1.00 g, 3.58 mmol) in 96% H_2SO_4 (20 mL) was heated at 100 °C for 1 h. Then the mixture was poured onto ice−water (100 mL) and extracted with CH_2Cl_2 (2 × 50 mL). The combined organic phase was dried over Na_2SO_4, concentrated, and purified by chromatography to afford yellow-orange crystals: 41% yield; mp 239 °C; FTIR 1674 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 8.42 (d, 1H), 8.26−8.20 (m, 2H), 8.01 (d, 1H), 7.79−7.71 (m, 2H); MS m/z 262 (100, M$^+$). Anal. ($C_{12}H_6O_2Se$) C, H.

2-(Selenophene-2-ylcarbonyl)benzoic Acid (17). Phthalic anhydride (14.80 g, 100 mmol) in CH_2Cl_2 (100 mL) was slowly added to a suspension of anhydrous $AlCl_3$ (29.43 g, 220 mmol) in CH_2Cl_2 (75 mL), and the mixture was stirred at rt for 30 min. Then selenophene (13.10 g, 100 mmol) was added dropwise within 30 min under stirring. The mixture was stirred for an additional 2 h, treated with HCl (0.5 mol/L, 125 mL), and stirred for a further 1 h. Then the aqueous phase was extracted with CH_2Cl_2 (3 × 50 mL) and the combined organic phase extracted with NaOH (2 mol/L, 2 × 100 mL). The product was precipitated with 37% HCl, filtered by suction, and recrystallized from MeOH/water (1/1) to afford white crystals: 42% yield; mp 147 °C; FTIR 1701, 1652 cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 8.03−8.02 (d, 1H), 7.95−7.93 (d, 1H), 7.87−7.86 (d, 1H), 7.72−7.62 (m, 2H), 7.27−7.21 (m, 2H); MS m/z 280 (23, M$^+$), 187 (100).

2-Acetylnaphtho[2,3-*b*]thiophene-4,9-dione (18). To a solution of 1-(naphtho[2,3-*b*]thiophene-2-yl)ethan-1-one (**20**; 0.20 g, 0.88 mmol) in glacial acetic acid (10 mL) was added with stirring at room temperature, dropwise over 1 h, a solution of CrO_3 (0.33 g, 3.29 mmol) in glacial acetic acid (5 mL) and water (5 mL). The solution was stirred for an additional 60 min at rt, poured into water (100 mL), and extracted with CH_2Cl_2 (3 × 50 mL). The combined organic phase was washed with a saturated solution of NaCl (2 × 50 mL), dried over Na_2SO_4, concentrated, and purified by chromatography to afford yellow crystals: 64% yield; mp 253−254 °C; FTIR 1668 (CO), 1650 (CO) cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 8.26−8.14 (m, 2H), 8.10 (s, 1H), 7.80−7.65 (m, 2H), 2.60 (s, 3H); MS m/z 256 (100). Anal. ($C_{14}H_8O_3S$) C, H.

dx.doi.org/10.1021/jm500754d | J. Med. Chem. XXXX, XXX, XXX−XXX

General Procedure for the Cleavage of Methyl Ethers. *2-Acetyl-8-hydroxynaphtho[2,3-b]thiophene-4,9-dione (**18a**).* Anhydrous $AlCl_3$ (0.54 g, 4.05 mmol) was added in portions to a solution of **18b** (0.100 g, 0.349 mmol) in dry CH_2Cl_2 (17 mL), and the mixture was stirred at rt for 30 min. Then it was treated with water (150 mL), acidified with HCl (2 mol/L), stirred for an additional 1 h, and extracted with CH_2Cl_2 (3 × 50 mL). The combined organic phase was washed with water (3 × 50 mL), dried over Na_2SO_4, concentrated, and purified by chromatography to afford orange crystals: 64% yield; mp 253−254 °C; FTIR 1664 cm^{-1}; ^1H NMR ($CDCl_3$) δ 12.05 (s, 1H, OH), 8.15 (s, 1H), 7.80 (dd, ^3J = 7.43 Hz, ^4J = 0.97 Hz, 1H), 7.68 (t, 1H), 7.31 (d, 1H), 2.68 (s, 3H); MS *m/z* 272 (79, M^+), 257 (100). Anal. ($C_{14}H_8O_4S$) C, H.

General Procedure for the Oxidative Demethylation to Naphtho[2,3-*b*]thiophene-4,9-diones. *2-Acetyl-8-methoxynaphtho[2,3-b]thiophene-4,9-dione (**18b**).* To a suspension of **20b** (0.30 g, 0.95 mmol) in MeCN (8 mL) and water (1.5 mL) at 0 °C was added dropwise within 20 min $(NH_4)_2[Ce(NO_3)_6]$ (1.39 g, 2.54 mmol) in MeCN (2.5 mL) and water (2.5 mL) under vigorous stirring. The mixture was allowed to react for 20 min under the same conditions. Then it was poured onto ice−water (50 mL) and extracted with CH_2Cl_2 (3 × 50 mL). The combined organic phase was washed with a saturated solution of NaCl (2 × 50 mL), dried over Na_2SO_4, concentrated, and purified by chromatography to afford light yellow, felted needles: 64% yield; mp 260−262 °C; FTIR 1668 cm^{-1}; ^1H NMR ($CDCl_3$) δ 8.10 (s, 1H), 7.92 (dd, ^3J = 7.62 Hz, ^4J = 1.17 Hz, 1H), 7.74 (t, 1H), 7.35 (d, *J* = 8.40 Hz, 1H), 4.05 (s, 3H), 2.65 (s, 3H); MS *m/z* 286 (100, M^+). Anal. ($C_{15}H_{10}O_4S$) C, H.

Analogously, compounds **18c** and **39** were prepared from **20c** and **38**, respectively. See the Supporting Information for details.

*4,8,9-Trimethoxynaphtho[2,3-b]thiophene (**19b**).* To a solution of **8a** (5.00 g, 21.72 mmol) and tetrabutylammonium bromide (0.70 g, 0.22 mmol) in dry THF (220 mL) under N_2 was added dropwise sodium dithionite (22.72 g, 130.43 mmol) in water (110 mL). The mixture was vigorously stirred for 30 min and then treated with sodium hydroxide (28.04 g, 500 mmol) in water (110 mL). After 30 min it was cooled to 0 °C in an ice bath, dimethyl sulfate (43.48 mL, 458.48 mmol) was added, and the mixture was then stirred for 2 h at 0 °C. Then it was allowed to warm to rt, stirred for an additional 2 h, and extracted with CH_2Cl_2 (3 × 200 mL). The combined organic phase was washed with a saturated solution of NaCl (200 mL), dried over Na_2SO_4, and evaporated, and the residue was purified by chromatography to afford yellow crystals: 77% yield; mp 82 °C; FTIR 3077, 2836 cm^{-1}; ^1H NMR ($CDCl_3$) δ 7.88 (dd, 1H), 7.53 (d, *J* = 5.47 Hz, 1H), 7.45 (d, *J* = 5.67 Hz, 1H), 7.37 (t, *J* = 7.42 Hz, 1H), 6.85 (d, *J* = 7.23 Hz, 1H), 4.09 (s, 3H), 4.05 (s, 3H), 4.04 (s, 3H); MS *m/z* 274 (72, $M^{\bullet+}$), 259 (100). Anal. ($C_{15}H_{14}O_3S$) C, H.

Analogously, compounds **19c** and **37** were prepared from **8c** and **36**,[73] respectively. See the Supporting Information for details.

General Procedure for 2-Acylation of 19b. *1-(4,8,9-Trimethoxynaphtho[2,3-b]thiophene-2-yl)ethan-1-one (**20b**).* To a mixture of *n*-butyllithium (1.6 M, 5.40 mL, 8.64 mmol) in dry THF (8.5 mL) at −15 °C under N_2 was added dropwise a solution of **19b** (1.20 g, 4.37 mmol) in dry THF (43 mL). The mixture was stirred for 4 h at −15 °C, and then *N,N*-dimethylacetamide (0.86 mL, 9.17 mmol) in THF (7 mL) was added. The mixture was stirred at rt for 3 h, poured onto ice−water (85 mL), acidified with HCl (2 mol/L), and extracted with ether (3 × 50 mL). The combined organic phase was washed with a saturated solution of NaCl (3 × 50 mL), dried over Na_2SO_4, concentrated, and purified by chromatography to afford light yellow crystals: 23% yield; mp 190−192 °C; FTIR 1665 cm^{-1}; ^1H NMR ($CDCl_3$) δ 8.14 (s, 1H), 7.86 (dd, 1H), 7.39 (t, 1H), 6.90 (d, *J* = 7.62 Hz, 1H), 4.14 (s, 3H), 4.05 (s, 3H), 4.03 (s, 3H), 2.72 (s, 3H); MS *m/z* 316 (93, $M^{\bullet+}$), 301 (100).

Analogously, 2-acetyl derivatives **20**, **20c**, and **38** were prepared from naphtho[2,3-*b*]thiophene,[32] **19c**, and **37**, respectively. See the Supporting Information for details.

*2-Acetyl-1H-naphtho[2,3-d]imidazole-4,9-dione (**21**).* A solution of (±)-2-(1-hydroxyethyl)-1*H*-naphtho[2,3-*d*]imidazole[35] (2.00 g, 9.43 mmol) in 96% H_2SO_4 (8.3 mL) and water (150 mL) was heated at reflux and treated with $K_2Cr_2O_7$ (8.83 g, 30 mmol). After 1 h the mixture was cooled and filtered. The product was dried in vacuo over P_2O_5 and purified by chromatography to afford yellow crystals: 53% yield; mp > 350 °C; FTIR 1686, 1662 cm^{-1}; ^1H NMR (DMSO-d_6) δ 14.96 (br s, 1H), 8.10−8.08 (m, 2H), 7.88−7.84 (m, 2H), 2.65 (s, 3H); MS *m/z* 240 (62, M^+), 212 (100). Anal. ($C_{13}H_8N_2O_3$) C, H, N.

*2-Acetyl-1-methyl-1H-naphtho[2,3-d]imidazole-4,9-dione (**21a**).* A mixture of **21** (0.50 g, 2.08 mmol) and anhydrous K_2CO_3 (2.00 g, 15 mmol) in dry DMF (50 mL) was cooled to −60 °C in a dry ice/acetone bath under stirring while iodomethane (0.31 mL, 5 mmol) was added dropwise. After 30 min the mixture was allowed to return to rt and stirred for an additional 18 h. Then it was poured into water (150 mL) and filtered by suction. The product was dried in vacuo over P_2O_5 and purified by chromatography to afford a pale yellow powder: 79% yield; mp 204 °C; FTIR 1679 cm^{-1}; ^1H NMR ($CDCl_3$) δ 8.28−8.15 (m, 2H), 7.78−7.75 (m, 2H), 4.43 (s, 3H), 2.82 (s, 3H); MS *m/z* 254 (100, M^+). Anal. ($C_{14}H_{10}N_2O_3$) C, H, N.

*Methyl 2-(8-Hydroxy-4,9-dioxo-4,9-dihydronaphtho[2,3-b]-thiophene-7-yl)acetate (**22e**).* To the carboxylic acid **22d** (0.15 g, 0.52 mmol) in MeOH (15 mL) was added 96% H_2SO_4 (0.1 mL), and the mixture was heated to reflux for 3 h. Then it was allowed to cool to rt, diluted with ice−water (50 mL), and extracted with CH_2Cl_2 (3 × 30 mL). The combined organic phase was washed with water, dried over Na_2SO_4, concentrated, and purified by chromatography to provide an orange product: 51% yield; mp 173 °C; FTIR 1738 (ester), 1658 (CO), 1622 (CO···HO) cm^{-1}; ^1H NMR ($CDCl_3$) δ 12.55 (s, 1H), 7.77 (d, ^3J = 5.08 Hz, 1H), 7.73 (d, ^3J = 7.83 Hz, 1H), 7.70 (d, ^3J = 5.08 Hz, 1H), 7.57 (d, ^3J = 7.44 Hz, 1H), 3.78 (s, 2H), 3.74 (s, 3H); MS *m/z* 302 (100, $M^{\bullet+}$). Anal. ($C_{15}H_{10}O_5S$) C, H.

Analogously, compounds **22f−22h**, **22q**, and **22r** were prepared from the appropriate alcohols and **22d**, **22o**, and **22p**, respectively. See the Supporting Information for details.

General Procedure for the Marschalk Reaction of 8-Hydroxynaphtho[2,3-*b*]thiophene-4,9-dione (8a) with Various Benzaldehydes. *7-Benzyl-8-hydroxynaphtho[2,3-b]-thiophene-4,9-dione (**22k**).* To a solution of **8a** (0.80 g, 3.47 mmol) in NaOH (1 mol/L, 100 mL) was added $Na_2S_2O_4$ (0.91 g, 5.21 mmol) under N_2. The solution was heated to 60 °C for 20 min. Benzaldehyde (1.84 g, 17.35 mmol) was added, and the temperature was raised to 90 °C. After 12 h the reaction mixture was cooled, aerated for 30 min, and allowed to cool to rt. Then it was poured onto water (200 mL), acidified with HCl (2 mol/L), and extracted with CH_2Cl_2 (3 × 100 mL). The combined organic phase was washed with water, dried over Na_2SO_4, concentrated, and purified by chromatography (CH_2Cl_2/hexane, 8/2) to provide red crystals: 7% yield; mp 145 °C; FTIR 1658 (CO), 1621 (CO···HO) cm^{-1}; ^1H NMR ($CDCl_3$) δ 12.61 (s, 1H), 7.75 (d, ^3J = 5.08 Hz, 1H), 7.70 (d, ^3J = 7.82 Hz, 1H), 7.69 (d, ^3J = 4.70 Hz, 1H), 7.41 (dd, ^3J = 7.82 Hz, ^4J = 0.78 Hz, 1H), 7.31−7.25 (m, 5H), 4.09 (s, 2H); MS *m/z* 320 (100, $M^{\bullet+}$). Anal. ($C_{19}H_{12}O_3S$) C, H.

Analogously, compounds **22a−22d**, **22i**, and **22l−22p** were prepared from the appropriate aldehydes. See the Supporting Information for details.

*8-Hydroxy-7-(4-methoxybenzyl)naphtho[2,3-b]thiophene-4,9-dione (**22s**).* To a suspension of **22v** (0.25 g, 0.90 mmol) and anhydrous $AlCl_3$ (0.54 g, 4.04 mmol) in CH_2Cl_2 (30 mL) was added anisole (0.15 g, 1.35 mmol). The reaction mixture was refluxed for 4 h and then poured onto ice−HCl (100 mL) with stirring. The product was extracted with CH_2Cl_2 (3 × 50 mL), the organic phase dried over Na_2SO_4, and the residue purified by chromatography (CH_2Cl_2/hexane, 6/4) to give an orange powder: 19% yield; mp 140 °C; FTIR 1654 (CO), 1621 (CO···HO) cm^{-1}; ^1H NMR ($CDCl_3$) δ 12.61 (s, 1H), 7.75 (d, ^3J = 5.09 Hz, 1H), 7.68 (d, ^3J = 5.09 Hz, 1H), 7.67 (d, ^3J = 7.82 Hz, 1H), 7.39 (d, ^3J = 8.21 Hz, 1H), 7.18 (d, ^3J = 8.61 Hz, 2H), 6.85 (d, ^3J = 9.00 Hz, 2H), 4.02 (s, 2H), 3.78 (s, 3H); MS *m/z* 350 (100, $M^{\bullet+}$). Anal. ($C_{20}H_{14}O_4S$) C, H.

Analogously, compound **22t** was prepared from veratrole. See the Supporting Information for details.

*8-Hydroxy-7-(hydroxymethyl)naphtho[2,3-b]thiophene-4,9-dione (**22u**).* A suspension of **8a** (1.00 g, 4.34 mmol) in MeOH (100

dx.doi.org/10.1021/jm500754d | J. Med. Chem. XXXX, XXX, XXX−XXX

mL) was cooled to 0 °C, and NaOH (2 mol/L, 15 mL) was added under N_2. The mixture was stirred for 15 min, treated with $Na_2S_2O_4$ (3.00 g, 17.21 mmol) in water (15 mL), and stirred for an additional 20 min. A solution of formaldehyde (37%, 9 mL) was added dropwise, and then the mixture was stirred for 3 h at 0−5 °C under N_2 (TLC control). The reaction mixture was then treated with H_2O_2 (2 mL), poured onto ice−water (200 mL), and acidified with HCl (6 mol/L) until the color changed to orange. The aqueous phase was extracted with CH_2Cl_2 (3 × 100 mL). The combined organic phase was washed with water, dried over Na_2SO_4, and concentrated. Petroleum ether was added to induce precipitation of the product at 0 °C. The precipitate was filtered by suction, washed with petroleum ether/CH_2Cl_2 (1/1, 20 mL), dried in vacuo, and purified by chromatography (CH_2Cl_2/ethyl acetate, 1/1) to provide orange crystals: 37% yield; mp 185 °C; FTIR 3311 (OH), 1660 (CO), 1612 (CO···HO) cm^{-1}; ^1H NMR (DMSO-d_6) δ 12.32 (s, 1H), 8.24 (d, ^3J = 4.92 Hz, 1H), 7.83 (d, ^3J = 7.82 Hz, 1H), 7.68 (d, ^3J = 5.08 Hz, 1H) 7.67 (d, 1H), 5.45 (t, ^3J = 5.48 Hz, 1H), 4.61 (d, ^3J = 5.09 Hz, 2H); MS m/z 260 (95, $M^{\bullet+}$), 231 (100). Anal. ($C_{13}H_8O_4S$) C, H.

Analogously, compound **23** was prepared from **18a**. See the Supporting Information for details.

*7-Chloromethyl-8-hydroxynaphtho[2,3-b]thiophene-4,9-dione (**22v**).* A solution of **22u** (1.65 g, 6.34 mmol), thionyl chloride (2.26 g, 19.02 mmol), and DMF (1 mL) was stirred in dry CH_2Cl_2 (30 mL) for 2 h at rt. Then the solvent and excess thionyl chloride were removed in vacuo. The resulting residue was treated with a mixture of ether (20 mL) and petroleum ether (10 mL) and filtered by suction to afford an orange product: 83% yield; mp 130 °C; FTIR 1657 (CO), 1619 (CO···HO) cm^{-1}; ^1H NMR ($CDCl_3$) δ 12.50 (s, 1H), 8.27 (d, ^3J = 4.69 Hz, 1H), 7.90 (d, ^3J = 7.82 Hz, 1H), 7.69 (d, ^3J = 5.09 Hz, 1H), 7.65 (d, ^3J = 7.82 Hz, 1H), 4.82 (s, 2H); MS m/z 280 (20, $M^{\bullet+}$) (^{37}Cl), 278 (50, $M^{\bullet+}$) (^{35}Cl), 243 (100).

According to the general procedure for 2-acylation as described for **20b**, compounds **24a−24m** were prepared from 4,8,9-trimethoxynaphtho[2,3-*b*]thiophene (**19b**) and the appropriate *N,N*-dimethylcarboxamides and **30** was prepared from **19b** and *N,N*-diethyl-2,2,2-trifluoroacetamide. See the Supporting Information for details.

According to the general procedure for the oxidative demethylation as described for **18b**, compounds **25a−25m** were prepared from **24a−24m**, compounds **28a−28e** from **27a−27e**, and compounds **31**, **34a**, and **34b** from **30**, **33a**, and **33b**, respectively. See the Supporting Information for details.

According to the general procedure for the cleavage of methyl ethers as described for **18a**, compounds **26a−26m** were prepared from **25a−25m**, compounds **29a−29e** were prepared from **28a−28e**, compounds **32**, **35a**, and **35b** were prepared from **31**, **34a**, and **34b**, and compound **40** was prepared from **39**, respectively. See the Supporting Information for details.

*4,8,9-Trimethoxynaphtho[2,3-b]thiophene-2-carboxylic Acid (**27a**).* To a mixture of *n*-butyllithium (1.6 M, 5.40 mL, 8.60 mmol) in dry THF (8.5 mL) at −15 °C under N_2 was added dropwise a solution of **19b** (1.50 g, 5.47 mmol) in dry THF (55 mL). The mixture was stirred and cooled to −78 °C, and then dry ice (27.00 g, 613.62 mmol) was added in portions under N_2. The mixture was stirred at −78 °C for 10 min, allowed to warm to −20 °C within 30 min, and then poured into HCl (2 mol/L, 45 mL). The organic phase was separated and the aqueous phase extracted with ether (2 × 50 mL). The combined organic phase was extracted with NaOH (1 mol/L, 3 × 100 mL) and the aqueous phase acidified with HCl (2 mol/L) to pH 2. The carboxylic acid was extracted with CH_2Cl_2 (3 × 200 mL), the organic phase dried over Na_2SO_4, and then the solvent removed in vacuo to afford a light yellow solid: 82% yield; mp 273−275 °C; FTIR 1677 (CO_2H) cm^{-1}; ^1H NMR (DMSO-d_6) δ 8.15 (s, 1H), 7.78 (d, J = 8.40 Hz, 1H), 7.43 (t, 1H), 7.03 (d, J = 7.63 Hz, 1H), 4.06 (s, 3H), 3.97 (s, 3H), 3.90 (s, 3H); MS m/z 318 (84, $M^{\bullet+}$), 303 (100).

*Ethyl 4,8,9-Trimethoxynaphtho[2,3-b]thiophene-2-carboxylate (**27b**).* To the carboxylic acid **27a** (1.50 g, 4.71 mmol) in absolute ethanol (0.82 mL, 14.13 mmol) was added DMAP (0.05 g, 0.41 mmol) under stirring. Then DCC (1.07 g, 5.18 mmol) was added at 0 °C, and the mixture was stirred for 10 min and then for an additional 3 h at rt. The mixture was filtered and the filtrate evaporated and dissolved in CH_2Cl_2 (100 mL). The solution was washed with HCl (0.5 mol/L, 2 × 50 mL) and then with a saturated solution of $NaHCO_3$ (2 × 50 mL), and the organic phase was dried over Na_2SO_4. Then the solvent was evaporated and the product purified by chromatography to give yellow needles: 53% yield; mp 134−135 °C; FTIR 1702 (CO) cm^{-1}; ^1H NMR ($CDCl_3$) δ 8.27 (s, 1H), 7.87 (d, J = 8.6 Hz, 1H), 7.38 (t, 1H), 6.88 (dd, J = 7.62 Hz, 1H), 4.43 (q, 2H), 4.13 (s, 3H), 4.05 (s, 3H), 4.03 (s, 3H), 1.45 (t, 3H); MS m/z 346 (83, $M^{\bullet+}$), 331 (100). Anal. ($C_{18}H_{18}O_5S$) C, H.

Analogously, compounds **27c−27e** were prepared from **27a**. See the Supporting Information for details.

*3-Ethyl-5-(4,8,9-trimethoxynaphtho[2,3-b]thiophene-2-yl)-1,2,4-oxadiazole (**33a**).* To a solution of **27a** (1.20 g, 3.77 mmol) in dry CH_2Cl_2 (20 mL) was added DCC (0.39 g, 1.89 mmol) under N_2, and the mixture was stirred at 0 °C for 1 h. Then it was filtered and the solvent evaporated. The residue was treated with pyridine (20 mL), and a solution of *N*-hydroxypropionamidine[74] (0.16 g, 1.82 mmol) in pyridine (4 mL) was added dropwise at rt. Then the mixture was refluxed for 3 h, cooled to rt, poured onto ice−water (200 mL), acidified with HCl (2 mol/L), and extracted with CH_2Cl_2 (3 × 50 mL). The combined organic phase was washed with water (2 × 200 mL) and dried over Na_2SO_4. The solution was evaporated, and the product was purified by chromatography (CH_2Cl_2/ethyl acetate, 9/1) to afford light yellow needles: 25% yield; mp 171−173 °C; FTIR 1599, 1571 cm^{-1}; ^1H NMR ($CDCl_3$) δ 8.34 (s, 1H), 7.89 (dd, ^3J = 8.61 Hz, ^4J = 0.78 Hz, 1H), 7.41 (t, J = 8.60 Hz, 1H), 6.91 (d, J = 7.44 Hz, 1H), 4.15 (s, 3H), 4.06 (s, 3H), 4.05 (s, 3H), 2.86 (q, 2H), 1.42 (t, 3H); MS m/z 370 (87, $M^{\bullet+}$), 355 (100. Anal. ($C_{19}H_{18}N_2O_4S$) C, H, N.

Analogously, compound **33b** was prepared from **27a**. See the Supporting Information for details.

Keratinocyte Culture and Determination of Cell Proliferation. HaCaT keratinocytes[43] were cultured, and the cell proliferation assay was performed as described previously.[36] After 48 h of incubation, cell growth was determined by enumerating the dispersed cells by phase contrast microscopy. Inhibition was calculated by the comparison of the mean values of the test compound (N = 3) with the control (N = 6−8) activity: (1 − test compound/control) × 100. Inhibition was statistically significant compared to that of the control (Student's t test, $P < 0.05$). IC_{50} values were obtained by nonlinear regression.

Lactate Dehydrogenase Release. The assay was performed as described.[48,49] HaCaT cells were incubated with the test compounds (2 μM) for 4 h at 37 °C. Extracellular LDH activity was measured using the UV method with pyruvate and NADH and is expressed in milliunits per milliliter. Appropriate controls with the vehicle were performed ($P < 0.01$, N = 3, SD < 10%). Brij 35 (polyoxyethylene glycol dodecyl ether)/ultrasound was the positive control.

Superoxide Generation Assays (Enzymatic One-Electron Reduction, Enzymatic Two-Electron Reduction). Both assays were performed exactly as previously described in full detail.[23]

Intracellular Superoxide Generation. To detect intracellular superoxide levels,[75] HaCaT keratinocytes were cultivated as described.[36] The cells were grown in Dulbecco's modified Eagle's medium (DMEM; no. E15-810, PAA) in six-well plates (2.5 × 10^5 cells/mL) supplemented with 10% fetal bovine serum, penicillin (100 units/mL), and streptomycin (100 μg/mL) in a CO_2 incubator for 24 h at 37 °C. Then the medium was replaced, and the cells were treated for 18 h (chronic treatment) with the test compound (5 μM, DMSO; the final concentration of DMSO in the culture medium was 0.1%) in a CO_2 incubator for 24 h at 37 °C. Also, acute treatment experiments (30 min, 50 μM test compound) were carried out. In a separate set of experiments, keratinocytes were preincubated with the NQO-1 inhibitor dicoumarol[61,67] (5 μmol/L) for 25 min before the treatment test compounds were added. After incubation with the test compound, DHE (10 μmol/mL) was added, the cell culture plates were treated on a shaker (200 rpm) for 5 min, and the cells were allowed to load DHE for an additional 25 min under incubation conditions. Then the

dx.doi.org/10.1021/jm500754d | J. Med. Chem. XXXX, XXX, XXX−XXX

medium was removed, and the cells were washed with PBS (0.5 mL/well), trypsinized (0.3 mL/well) for 7 min, treated with FACS buffer (FACSFlow, BD Biosciences, no. 342003, 0.7 mL), centrifuged (1000*g*), and resuspended in FACS buffer (0.5 mL). Experiments with DHE were performed in the dark. Controls were performed with no DHE and with no test compound (DMSO alone). DHE-treated keratinocytes were immediately evaluated by flow cytometric measurements, which were performed at different time points (0, 10, 20, 30, 60, and 90 min). Keratinocytes were kept in the CO_2 incubator at 37 °C between measurements. All experiments were run in triplicate.

Flow Cytometry. The fluorescence of the oxidized product 2-OH-E^+ was monitored on a FACSCalibur (Becton Dickinson) flow cytometer equipped with an argon laser (λ = 488 nm) as a light source, and the data were collected in the FL2 channel (λ = 550−600 nm, 42 nm bandpass). For each sample, 10 000 live cells were examined, and dead cells were gated out for analysis. The recorded histograms were analyzed using the software CellQuest Pro and were compared with the histograms of untreated control cells. Data are expressed as the mean fluorescence intensity (MFI).

Flow Cytometric Detection of 7-Aminoactinomycin D (7-AAD) Positive Cells. Cells were grown and exposed to test compounds or preincubated with dicoumarol exactly as described above. Evaluation of apoptosis[68] was performed using 7-AAD (10 μg/mL) according to the manufacturer's instructions. At least 10 000 cells were acquired, and data were collected in the FL3 channel and analyzed by CellQuest Pro.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Supplementary chemical data of compounds **18c**, **19c**, **20**, **20c**, **22a**−**22r**, **22t**, **23**, **24a**−**24m**, **25a**−**25m**, **26a**−**26m**, **27c**−**27e**, **28a**−**28e**, **29a**−**29c**, **30**−**32**, **33b**, **34a**, **34b**, **35a**, **35b**, and **37**−**40**, X-ray structure of **8a**, and analytical data of all test compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Phone: +49-251-8333324. E-mail: kmuller@uni-muenster.de.

Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS USED

7-AAD, 7-aminoactinomycin D; CPR, NADPH−cytochrome P450 oxidoreductase; DCC, *N,N′*-dicyclohexylcarbodiimide; DHE, dihydroethidium; DMAP, 4-(dimethylamino)pyridine; DTPA, diethylenetriaminepentaacetic acid; NQO-1, NAD(P)H:quinone oxidoreductase 1; ROS, reactive oxygen species; rt, room temperature; SAR, structure−activity relationship; SOD, superoxide dismutase

■ REFERENCES

(1) Burnett, A. R.; Thomson, R. H. Naturally occuring quinones. Part X. The quinoid constituents of *Tabebuia avellanedae* (Bignoniaceae). *J. Chem. Soc. C* **1967**, 2100−2104.

(2) Rao, M. M.; Kingston, D. G. I. Plant anticancer agents. XII. Isolation and structure elucidation of new cytotoxic quinones from *Tabebuia cassinoides*. *J. Nat. Prod.* **1982**, *45*, 600−604.

(3) Epifano, F.; Genovese, S.; Fiorito, S.; Mathieu, V.; Kiss, R. Lapachol and its congeners as anticancer agents: a review. *Phytochem. Rev.* **2014**, *13*, 37−49.

(4) Jones, K. *Pau d'arco: Immune Power from the Rain Forest*; Healing Arts Press: Rochester, VT, 1995.

(5) Gómez Castellanos, J. R.; Prieto, J. M.; Heinrich, M. Red lapacho (*Tabebuia impetiginosa*)—a global ethnopharmacological commodity? *J. Ethnopharmacol.* **2009**, *121*, 1−13.

(6) Arenas, P. Medicine and magic among the Maka Indians of the Paraguayan Chaco. *J. Ethnopharmacol.* **1987**, *21*, 279−295.

(7) Bastien, J. W. Pharmacopeia of Qollahuaya Andeans. *J. Ethnopharmacol.* **1983**, *8*, 97−111.

(8) Hartwell, J. L. Plants used against cancer. A survey. *Lloydia* **1968**, *31*, 71−170.

(9) Queiroz, M. L. S.; Valadares, M. C.; Torello, C. O.; Ramos, A. L.; Oliveira, A. B.; Rocha, F. D.; Arruda, V. A.; Accorci, W. R. Comparative studies of the effects of *Tabebuia avellanedae* bark extract and *β*-lapachone on the hematopoietic response of tumour-bearing mice. *J. Ethnopharmacol.* **2008**, *117*, 228−235.

(10) de Castro, S. L.; Emery, F. S.; da Silva, E. N., Jr. Synthesis of quinoidal molecules: strategies towards bioactive compounds with an emphasis on lapachones. *Eur. J. Med. Chem.* **2013**, *69*, 678−700.

(11) Fiorito, S.; Epifano, F.; Bruyère, C.; Mathieu, V.; Kiss, R.; Genovese, S. Growth inhibitory activity for cancer cell lines of lapachol and its natural and semi-synthetic derivatives. *Bioorg. Med. Chem. Lett.* **2014**, *24*, 454−457.

(12) Eyong, K. O.; Kumar, P. S.; Kuete, V.; Folefoc, G. N.; Nkengfack, E. A.; Baskaran, S. Semisynthesis and antitumoral activity of 2-acetylfuranonaphthoquinone and other naphthoquinone derivatives from lapachol. *Bioorg. Med. Chem. Lett.* **2008**, *18*, 5387−5390.

(13) Takano, A.; Hashimoto, K.; Ogawa, M.; Koyanagi, J.; Kurihara, T.; Wakabayashi, H.; Kikuchi, H.; Nakamura, Y.; Motohashi, N.; Sakagami, H.; Yamamoto, K.; Tanaka, A. Tumor-specific cytotoxicity and type of cell death induced by naphtho[2,3-*b*]furan-4,9-diones and related compounds in human tumor cell lines: relationship to electronic structure. *Anticancer Res.* **2009**, *29*, 455−464.

(14) Wu, C.; Johnson, R. K.; Mattern, M. R.; Wong, J. C.; Kingston, D. G. I. Synthesis of furanonaphthoquinones with hydroxyamino side chains. *J. Nat. Prod.* **1999**, *62*, 963−968.

(15) Yamashita, M.; Kaneko, M.; Tokuda, H.; Nishimura, K.; Kumeda, Y.; Iida, A. Synthesis and evaluation of bioactive naphthoquinones from the Brazilian medicinal plant, *Tabebuia avellanedae*. *Bioorg. Med. Chem.* **2009**, *17*, 6286−6291.

(16) Jiménez-Alonso, S.; Guasch, J.; Estévez-Braun, A.; Ratera, I.; Veciana, J.; Ravelo, A. G. Electronic and cytotoxic properties of 2-amino-naphtho[2,3-*b*]furan-4,9-diones. *J. Org. Chem.* **2011**, *76*, 1634−1643.

(17) Gomez-Monterrey, I.; Campiglia, P.; Aquino, C.; Bertamino, A.; Granata, I.; Carotenuto, A.; Brancaccio, D.; Stiuso, P.; Scognamiglio, I.; Rusciano, M. R.; Maione, A. S.; Illario, M.; Grieco, P.; Maresca, B.; Novellino, E. Design, synthesis, and cytotoxic evaluation of acyl derivatives of 3-aminonaphtho[2,3-*b*]thiophene-4,9-dione, a quinone-based system. *J. Med. Chem.* **2011**, *54*, 4077−4091.

(18) Müller, K.; Sellmer, A.; Wiegrebe, W. Potential antipsoriatic agents: lapacho compounds as potent inhibitors of HaCaT cell growth. *J. Nat. Prod.* **1999**, *62*, 1134−1136.

(19) Müller, K.; Reindl, H.; Breu, K. Antipsoriatic anthrones with modulated redox properties. 5. Potent inhibition of human keratinocyte growth, induction of keratinocyte differentiation, and reduced membrane damage by novel 10-arylacetyl-1,8-dihydroxy-9(10*H*)-anthracenones. *J. Med. Chem.* **2001**, *44*, 814−821.

(20) Zuse, A.; Schmidt, P.; Baasner, S.; Böhm, K. J.; Müller, K.; Gerlach, M.; Günther, E. G.; Unger, E.; Prinz, H. Sulfonate derivatives of naphtho[2,3-*b*]thiophen-4(9*H*)-one and 9(10*H*)-anthracenone as highly active antimicrotubule agents. Synthesis, antiproliferative activity, and inhibition of tubulin polymerization. *J. Med. Chem.* **2007**, *50*, 6059−6066.

(21) Putic, A.; Stecher, L.; Prinz, H.; Müller, K. Structure−activity relationship studies of acridones as potential antipsoriatic agents. 1. Synthesis and antiproliferative activity of simple N-unsubstituted 10*H*-acridin-9-ones against human keratinocyte growth. *Eur. J. Med. Chem.* **2010**, *45*, 3299−3310.

(22) Prinz, H.; Chamasmani, B.; Vogel, K.; Böhm, K. J.; Aicher, B.; Gerlach, M.; Günther, E. G.; Amon, P.; Ivanov, I.; Müller, K. *N*-Benzoylated phenoxazines and phenothiazines: synthesis, antiproliferative activity and inhibition of tubulin polymerization. *J. Med. Chem.* **2011**, 4247−4263.

(23) Reichstein, A.; Vortherms, S.; Bannwitz, S.; Tentrop, J.; Prinz, H.; Müller, K. Synthesis and structure−activity relationships of lapacho analogues. 1. Suppression of human keratinocyte hyperproliferation by 2-substituted naphtho[2,3-*b*]furan-4,9-diones, activation by enzymatic one- and two-electron reduction, and intracellular generation of superoxide. *J. Med. Chem.* **2012**, *55*, 7273−7284.
(24) Harris, D. R. Old wine in new bottles: the revival of anthralin. *Cutis* **1998**, *62*, 201−203.
(25) Inzinger, M.; Heschl, B.; Weger, W.; Hofer, A.; Legat, F. J.; Gruber-Wackernagel, A.; Tilz, H.; Salmhofer, W.; Quehenberger, F.; Wolf, P. Efficacy of psoralen plus ultraviolet A therapy vs. biologics in moderate to severe chronic plaque psoriasis: retrospective data analysis of a patient registry. *Br. J. Dermatol.* **2011**, *165*, 640−645.
(26) Lowes, M. A.; Bowcock, A. M.; Krueger, J. G. Pathogenesis and therapy of psoriasis. *Nature* **2007**, *445*, 866−873.
(27) Schroeder, H. E.; Weinmayr, V. The synthesis of thiophanthraquinones from thenoyl and thenylbenzoic acids. *J. Am. Chem. Soc.* **1952**, *74*, 4357−4361.
(28) Weinmayr, V. *o*-(2-Thenoyl)-benzoic acids and thiophanthraquinones. *J. Am. Chem. Soc.* **1952**, *74*, 4353−4357.
(29) Müller, K.; Sellmer, A.; Salvesen, J. Lapacho compounds, their preparation, and methods of use for the treatment of cell proliferative disorders. PCT Int. Appl. WO 2004026253 A2 20040401, 2004.
(30) Prinz, H.; Wiegrebe, W.; Müller, K. Synthesis of anthracenones. 1. Sodium dithionite reduction of *peri*-substituted anthracenediones. *J. Org. Chem.* **1996**, *61*, 2853−2856.
(31) Kraus, G. A.; Man, T. O. An improved reductive methylation procedure for quinones. *Synth. Commun.* **1986**, *16*, 1037−1042.
(32) Tedjamulia, M. L.; Tominaga, Y.; Castle, R. N.; Lee, M. L. The synthesis of dinaphthothiophenes. *J. Heterocycl. Chem.* **1983**, *20*, 1143−1148.
(33) Hoover, J. R. E.; Day, A. R. Preparation of some imidazole derivatives of 1,4-naphthoquinone. *J. Am. Chem. Soc.* **1954**, *76*, 4148−4152.
(34) Fries, K.; Walter, R.; Schilling, K. Über tricyclische Verbindungen, in denen Naphtalin mit einem Heterocyclus anelliert ist. *Liebigs Ann. Chem.* **1935**, *516*, 248−285.
(35) Zellner, H.; Zellner, G.; Köppl, F.; Dirnberger, J. Reaktion von Brenztraubensäure mit o-Diaminen, 3. Mitt.: Synthesen von 2-(α-Keto-alkyl)-benzimidazolen. *Monatsh. Chem.* **1967**, *98*, 643−665.
(36) Müller, K.; Leukel, P.; Ziereis, K.; Gawlik, I. Antipsoriatic anthrones with modulated redox properties. 2. Novel derivatives of chrysarobin and isochrysarobin—antiproliferative activity and 5-lipoxygenase inhibition. *J. Med. Chem.* **1994**, *37*, 1660−1669.
(37) Marschalk, C.; Koenig, F.; Ouroussoff, N. Nouvelle méthode d'introduction des chaines latérales dans le noyau anthraquinonique. *Bull. Soc. Chim. Fr.* **1936**, *3*, 1545−1575.
(38) Krohn, K. Synthesis of anthracyclinones by electrophilic and nucleophilic addition to anthraquinones. *Tetrahedron* **1990**, *46*, 291−318.
(39) Koyanagi, J.; Yamamoto, K.; Nakayama, K.; Tanaka, A. A new synthetic route to 2-substituted naphtho[2,3-*b*]furan-4,9-dione. *J. Heterocycl. Chem.* **1997**, *34*, 407−412.
(40) Neises, B.; Steglich, W. 4-Dialkylaminopyridines as acylation catalysts. 5. Simple method for esterification of carboxylic acids. *Angew. Chem., Int. Ed. Engl.* **1978**, *17*, 522−524.
(41) Borg, S.; Estenne-Bouhtou, G.; Luthman, K.; Csöregh, I.; Hesselink, W.; Hacksell, U. Synthesis of 1,2,4-oxadiazole-, 1,3,4-oxadiazole-, and 1,2,4-triazole-derived dipeptidomimetics. *J. Org. Chem.* **1995**, *60*, 3112−3120.
(42) Pol, A.; Bergers, M.; Schalkwijk, J. Comparison of antiproliferative effects of experimental and established antipsoriatic drugs on human keratinocytes, using a simple 96-well-plate assay. *In Vitro Cell. Dev. Biol.: Anim.* **2003**, *39*, 36−42.
(43) Boukamp, P.; Petrussevska, R. T.; Breitkreutz, D.; Hornung, J.; Markham, A.; Fusenig, N. E. Normal keratinization in a spontaneously immortalized aneuploid human keratinocyte cell line. *J. Cell Biol.* **1988**, *106*, 761−771.
(44) George, S. E.; Anderson, R. J.; Cunningham, A.; Donaldson, M.; Groundwater, P. W. Evaluation of a range of anti-proliferative assays for the preclinical screening of anti-psoriatic drugs: a comparison of colorimetric and fluorimetric assays with the thymidine incorporation assay. *Assay Drug Dev. Technol.* **2010**, *8*, 384−395.
(45) Müller, K.; Prinz, H.; Gawlik, I.; Ziereis, K.; Huang, H.-S. Simple analogues of anthralin: unusual specificity of structure and antiproliferative activity. *J. Med. Chem.* **1997**, *40*, 3773−3780.
(46) Chen, X.; Wang, W. The use of bioisosteric groups in lead optimization. *Annu. Rep. Med. Chem.* **2003**, *38*, 333−346.
(47) Boström, J.; Hogner, A.; Llinàs, A.; Wellner, E.; Plowright, A. T. Oxadiazoles in medicinal chemistry. *J. Med. Chem.* **2012**, *55*, 1817−1830.
(48) Bonnekoh, B.; Farkas, B.; Geisel, J.; Mahrle, G. Lactate dehydrogenase release as an indicator of dithranol-induced membrane injury in cultured human keratinocytes. *Arch. Dermatol. Res.* **1990**, *282*, 325−329.
(49) Müller, K.; Huang, H.-S.; Wiegrebe, W. Antipsoriatic anthrones with modulated redox properties. 3. 10-Thio-substituted 1,8-dihydroxy-9(10*H*)-anthracenones as inhibitors of keratinocyte growth, 5-lipoxygenase, and the formation of 12(*S*)-HETE in mouse epidermis. *J. Med. Chem.* **1996**, *39*, 3132−3138.
(50) Bolton, J. L.; Trush, M. A.; Penning, T. M.; Dryhurst, G.; Monks, T. J. Role of quinones in toxicology. *Chem. Res. Toxicol.* **2000**, *13*, 135−160.
(51) Powis, G. Free radical formation by antitumor quinones. *Free Radical Biol. Med.* **1989**, *6*, 63−101.
(52) Brunmark, A.; Cadenas, E. Redox and addition chemistry of quinoid compounds and its biological implications. *Free Radical Biol. Med.* **1989**, *7*, 435−447.
(53) Song, Y.; Buettner, G. R. Thermodynamic and kinetic considerations for the reaction of semiquinone radicals to form superoxide and hydrogen peroxide. *Free Radical Biol. Med.* **2010**, *49*, 919−962.
(54) Doering, M.; Ba, L. A.; Lilienthal, N.; Nicco, C.; Scherer, C.; Abbas, M.; Zada, A. A. P.; Coriat, R.; Burkholz, T.; Wessjohann, L.; Diederich, M.; Batteux, F.; Herling, M.; Jacob, C. Synthesis and selective anticancer activity of organochalcogen based redox catalysts. *J. Med. Chem.* **2010**, *53*, 6954−6963.
(55) da Silva, E. N., Jr.; Cavalcanti, B. C.; Guimaraes, T. T.; Pinto, M. d. F. R.; Cabral, I. O.; Pessoa, C.; Costa-Lotufo, L. V.; de Moraes, M. O.; de Andrade, C. K. Z.; dos Santos, M. R.; de Simone, C. A.; Goulart, M. O. F.; Pinto, A. V. Synthesis and evaluation of quinonoid compounds against tumor cell lines. *Eur. J. Med. Chem.* **2011**, *46*, 399−410.
(56) Lizzi, F.; Veronesi, G.; Belluti, F.; Bergamini, C.; López-Sánchez, A.; Kaiser, M.; Brun, R.; Krauth-Siegel, R. L.; Hall, D. G.; Rivas, L.; Bolognesi, M. L. Conjugation of quinones with natural polyamines: toward an expanded antitrypanosomatid profile. *J. Med. Chem.* **2012**, *55*, 10490−10500.
(57) Sies, H. Biochemistry of oxidative stress. *Angew. Chem., Int. Ed. Engl.* **1986**, *25*, 1058−1071.
(58) Halliwell, B. Oxidative stress and cancer: have we moved forward? *Biochem. J.* **2007**, *401*, 1−11.
(59) Tarpey, M. M.; Wink, D. A.; Grisham, M. B. Methods for detection of reactive metabolites of oxygen and nitrogen: in vitro and in vivo considerations. *Am. J. Physiol.: Regul., Integr. Comp. Physiol.* **2004**, *286*, R431−R444.
(60) Halliwell, B. Superoxide-dependent formation of hydroxyl radicals in the presence of iron chelates. Is it a mechanism for hydroxyl radical production in biochemical systems? *FEBS Lett.* **1978**, *92*, 321−326.
(61) Thor, H.; Smith, M. T.; Hartzell, P.; Bellomo, G.; Jewell, S. A.; Orrenius, S. The metabolism of menadione (2-methyl-1,4-naphthoquinone) by isolated hepatocytes. A study of the implications of oxidative stress in intact cells. *J. Biol. Chem.* **1982**, *257*, 12419−12425.
(62) Buffinton, G. D.; Öllinger, K.; Brunmark, A.; Cadenas, E. DT-diaphorase-catalysed reduction of 1,4-naphthoquinone derivatives and

glutathionyl-quinone conjugates. Effect of substituents on autoxidation rates. *Biochem. J.* **1989**, *257*, 561−571.
(63) Zhao, H.; Kalivendi, S.; Joseph, J.; Zhang, H.; Joseph, J.; Nithipatikom, K.; Vásquez-Vivar, J.; Kalyanaraman, B. Superoxide reacts with hydroethidine but forms a fluorescent product that is distinctly different from ethidium: potential implications in intracellular fluorescence detection of superoxide. *Free Radical Biol. Med.* **2003**, *34*, 1359−1368.
(64) Zhao, H.; Joseph, J.; Fales, H. M.; Sokoloski, E. A.; Levine, R. L.; Vasquez-Vivar, J.; Kalyanaraman, B. Detection and characterization of the product of hydroethidine and intracellular superoxide by HPLC and limitations of fluorescence. *Proc. Natl. Acad. Sci. U.S.A.* **2005**, *102*, 5727−5732.
(65) Zielonka, J.; Vasquez-Vivar, J.; Kalyanaraman, B. Detection of 2-hydroxyethidium in cellular systems: a unique marker product of superoxide and hydroethidine. *Nat. Protoc.* **2008**, *3*, 8−21.
(66) De Iuliis, G. N.; Wingate, J. K.; Koppers, A. J.; McLaughlin, E. A.; Aitken, R. J. Definitive evidence for the nonmitochondrial production of superoxide anion by human spermatozoa. *J. Clin. Endocrinol. Metab.* **2006**, *91*, 1968−1975.
(67) Tan, A. S.; Berridge, M. V. Evidence for NAD(P)H:quinone oxidoreductase 1 (NQO1)-mediated quinone-dependent redox cycling via plasma membrane electron transport: a sensitive cellular assay for NQO1. *Free Radical Biol. Med.* **2010**, *48*, 421−429.
(68) Zembruski, N. C. L.; Stache, V.; Haefeli, W. E.; Weiss, J. 7-Aminoactinomycin D for apoptosis staining in flow cytometry. *Anal. Biochem.* **2012**, *429*, 79−81.
(69) Zani, C. L.; Chiari, E.; Krettli, A. U.; Murta, S. M.; Cunningham, M. L.; Fairlamb, A. H.; Romanha, A. J. Anti-plasmodial and anti-trypanosomal activity of synthetic naphtho[2,3-*b*]thiophen-4,9-quinones. *Bioorg. Med. Chem.* **1997**, *5*, 2185−2192.
(70) Watanabe, T.; Miyagi, C.; Murakami, Y. Synthetic studies on indoles and related compounds. XXXI. Chemical confirmation of the synthetic route for the benz[*f*]indole skeleton and its application. *J. Heterocycl. Chem.* **1993**, *30*, 217−224.
(71) de Oliveira, C. G. T.; Ferreira, V. F.; Freitas, C.; Carballido, J. M. Synthesis and antimicrobial evaluation of oxazole-1,4-naphthquinones. *Heterocycl. Commun.* **2002**, *8*, 199−204.
(72) Boggust, W. A.; Cocker, W.; Schwarz, J. C. P.; Stuart, E. R. Some naphthothiazoles. *J. Chem. Soc.* **1950**, 680−682.
(73) Krapcho, A. P.; Petry, M. E.; Hacker, M. P. Heterosubstituted anthracene-9,10-dione analogues. The synthesis and antitumor evaluation of 5,8-bis[(aminoalkyl)amino]naphtho[2,3-*b*]thiophene-4,9-diones. *J. Med. Chem.* **1990**, *33*, 2651−2655.
(74) Moloney, G. P.; Martin, G. R.; Mathews, N.; MacLennan, S.; Dodsworth, S.; Sang, P. Y.; Knight, C.; Maxwell, M.; Glen, R. C. Synthesis and serotonergic activity of 2-oxadiazolyl-5-substituted-*N*,*N*-dimethyltryptamines: novel antagonists for the vascular 5-HT_{1B}-like receptor. *J. Chem. Soc., Perkin Trans. 1* **1999**, 2725−2733.
(75) Mukhopadhyay, P.; Rajesh, M.; Yoshihiro, K.; Haskó, G.; Pacher, P. Simple quantitative detection of mitochondrial superoxide production in live cells. *Biochem. Biophys. Res. Commun.* **2007**, *358*, 203−208.