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A laccase-catalysed one-pot synthesis of aminonaphthoquinones and their anticancer activity

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ABSTRACT

Nuclear monoamination of a 1,4-naphthohydroquinone with primary aromatic amines was catalysed by the commercial laccase, Novozym 51003, from Novozymes to afford aminonaphthoquinones. The synthesis was accomplished by reacting a mixture of the primary amine and 1,4-naphthohydroquinone in succinate-lactate buffer and a co-solvent, dimethylformamide, under mild reaction conditions in a vessel open to air at pH 4.5 and pH 6.0.

Anticancer screening showed that the aminonaphthoquinones exhibited potent cytostatic effects particularly against the UACC62 (melanoma) cancer cell line (GI_{50} = 3.98–7.54 μM). One compound exhibited potent cytostatic effects against both the TK10 (renal) and the UACC62 (melanoma) cancer cell line. The cytostatic effects of this compound (GI_{50} = 8.38 μM) against the TK10 cell line was almost as good as that of the anticancer agent, etoposide (GI_{50} = 7.19 μM). Two compounds exhibited potent cytostatic effects against both the UACC62 (melanoma) and the MCF7 (breast) cancer cell lines. The total growth inhibition (TGI) of most of the compounds was better than that of etoposide against the UACC62 cell line. Three compounds (TGI = 7.17–7.94 μM) exhibited potent cytostatic effects against the UACC62 cell line which was 7 to 8-fold better than that of etoposide (TGI = 52.71 μM).

The results are encouraging for further study of the aminonaphthoquinones for potential application in anticancer therapy.

1. Introduction

1,4-Naphthoquinone derivatives have exhibited an interesting variety of biological responses such as antiallergic,[1–3] antibacterial,[4,5] antifungal,[4–7] anti-inflammatory,[1–4,7] antithrombotic,[8,9] antiplatelet,[1–3,10–13] antiviral,[4,14,10,15] apoptosis,[16–18] lipoxygenase,[19,20] radical scavenging[21] and anti-ringworm[4] activities. There have also been reports on their anticancer activity.[5,6,10–12] Human DNA topoisomerase I and II are known to be inhibited by 1,4-naphthoquinone derivatives.[12,21–26] These 1,4-naphthoquinone derivatives can also produce reactive oxygen species (ROS) such as semiquinone and hydroxyl radicals by enzymatic reduction (i.e. NADPH-cytochrome P 450 reductase).[26–29] It is the hydroxyl radical that is the cause of DNA strand breaks.[30]

The aminoquinone moiety is prevalent in several drugs that are in use such as the commercial anti-neoplastic agents actinomycin[29] and streptonigrin[29] (Fig. 1). The antibiotics, mitomycin[31] and rifamycin,[32] are also based on an aminoquinone (Fig. 1).

It must be noted that the amino group is in the *ortho* position to the ketone in mitomycin C, streptonigrin,[29] actinomycin[32] (and the structurally related aurantins[33]) as well as in the *ansa*-antibiotics rifamycin[32] and geldanamycin.[34] The position of the amine group may be a requirement for the biological activity exhibited by these compounds which has inspired research into new routes to the synthesis of aminoquinones.

In 2008 7.6 million deaths (13% of all deaths) were attributed to cancer which is the leading cause of death worldwide. Cancer in the breast, colon, lung, stomach, and liver cause the most deaths each year. Deaths from cancer are projected to continue to rise worldwide with an estimated 13.1 million deaths predicted for 2030.[35]

Chemotherapy is the primary treatment for cancer and has been hampered by the development of drug resistant and multidrug resistant tumours. Multidrug resistance is the principal mechanism by which many cancers develop resistance to chemotherapy drugs and is a major factor in the failure of many forms of chemotherapy.[36] One of the largest protein families in biology is the ATP-binding cassette (ABC), a superfamily of proteins.[37] Their role in resistance to chemotherapy drugs has been known for more than three decades.[38] Fifteen family members of these ABC proteins

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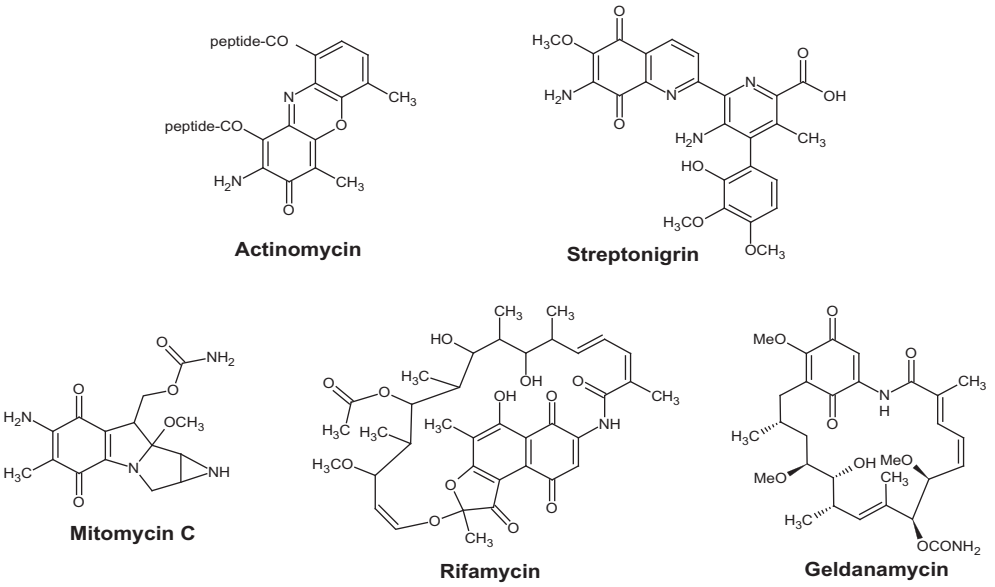


Figure 1. Drugs having an aminoquinone moiety.

can function as drug-efflux pumps and these have been implicated as potentially conferring resistance.[39,40]

Only three of these account for the most observed multidrug resistance (MDR) in humans. These are P-glycoprotein (Pgp/MDR1/ANCB1) and the MDR–associated protein (MRP)1 (ABCC1), and the breast cancer resistance protein (ABCG2).[41] The drug efflux pumps, located in the tumour-cell plasma membrane, actively expel chemotherapy drugs from the interior thus allowing tumour cells to avoid the toxic effects of the drug.[36] There is thus a need for new compounds that are effective in treating drug resistant and multidrug resistant tumours.

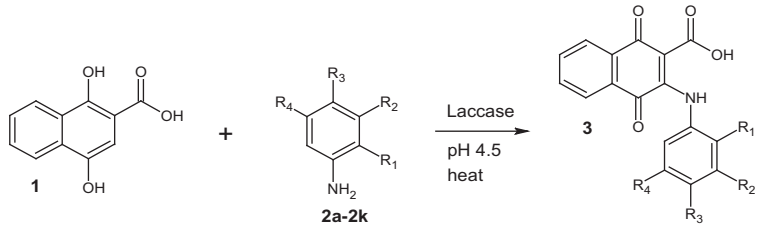
Laccase (*p*-diphenol: oxygen oxidoreductase, EC 1.10.3.2) belongs to the group of copper-containing oxidases called oxidoreductases.[42,43] It catalyses the reduction of molecular oxygen to water and by-pass hydrogen peroxide formation.[42,43] This blue multi-copper oxidase is able to catalyse the oxidation of various low-molecular weight compounds, including: benzenediols, aminophenols, polyphenols, polyamines, and lignin-related molecules.[44] The demand for green chemical processes has inspired interest in the application of enzymes to address modern synthetic organic chemistry challenges. The successful application of laccase in organic synthesis has resulted in several literature reports.[45,46,49] Amongst these there have also been reports on amination of *p*-hydroquinones with primary aromatic amines.[47]

Our goal was to synthesise aminonaphthoquinones having the amine moiety in the *ortho* position to the ketone of the quinone ring. We report here on the synthesis of aminonaphthoquinones using a commercial laccase (Novozym 51003) from Novozymes and also on the anticancer screening results of the synthesised compounds. This report is, to the best of our knowledge, the first on amination of 1,4-dihydroxy-2-naphthoic acid using laccase and also the first report on the anticancer activity of the synthesised compounds. We have previously reported on the synthesis of diaminobenzoquinones from the reaction between *p*-hydroquinones and primary aryl and alkyl amines using a commercial laccase.[48]

2. Results and discussion

2.1. Synthesis

Novozymes has a few laccases available on the market in different preparations. Novozym 51003 is a robust, stable, fungal laccase



2a	2b	2c	2d	2e	2f	2g	2h	2i	2j	2k
R^1 = H	R^1 = H	R^1 = H	R^1 = H	R^1 = H	R^1 = H	R^1 = Cl	R^1 = H	R^1 = H	R^1 = F	R^1 = F
R^2 = H	R^2 = H	R^2 = H	R^2 = H	R^2 = H	R^2 = Cl	R^2 = H	R^2 = H	R^2 = F	R^2 = H	R^2 = H
R^3 = H	R^3 = $CH(CH_3)_2$	R^3 = EtOH	R^3 = H	R^3 = Cl	R^3 = H	R^3 = H	R^3 = F	R^3 = H	R^3 = H	R^3 = Cl
R^4 = H	R^4 = H	R^4 = H	R^4 = CN	R^4 = H	R^4 = H	R^4 = H	R^4 = H	R^4 = H	R^4 = H	R^4 = H

Scheme 1. Laccase-catalysed synthesis of aminonaphthoquinones.

Table 1
The **s**ynthesised aminonaphthoquinones (yield in parentheses) at pH 4.5 using Methods A–D

Entry	Hydroquinone	Amine (equiv)	Reaction Time (h)	Method	Product
1	1	**2a** (2)	48	D	**4** (77%)
2	1	**2b** (2)	26	A	**5** (70%)
3	1	**2b** (2)	48	D	**5** (47%)
4	1	**2c** (2)	27	A	**6** (51%)
5	1	**2c** (2)	72	B	**6** (64%)
6	1	**2d** (2)	24	C	**7** (32%)
7	1	**2f** (2)	24	C	**8** (52%)

Method A–2.0 mL (2 220 U) Novozym 51003, 2 equiv amine (1.2 mmol), 1,4-dihydroxy-2-naphthoic acid (0.6 mmol), 2.0 mL water, 2.0 mL succinate-lactate buffer (1.0 M, pH 4.5), 1.0 mL DMF, 35 °C.
Method B–4.0 mL (4 440 U) Novozym 51003, 2 equiv amine (1.2 mmol), 1,4-dihydroxy-2-naphthoic acid (0.6 mmol), 2.0 mL water, 2.0 mL succinate-lactate buffer (1.0 M, pH 4.5), 1.0 mL DMF, 35 °C.
Method C–1.0 mL (1 110 U) Novozym 51003, 2 equiv amine (1.2 mmol), 1,4-dihydroxy-2-naphthoic acid (0.6 mmol), 2.0 mL water, 2.0 mL succinate-lactate buffer (1.0 M, pH 4.5), 1.0 mL DMF, 35 °C.
Method D–3.5 mL (3 885 U) Novozym 51003, 2 equiv amine (1.2 mmol), 1,4-dihydroxy-2-naphthoic acid (0.6 mmol), 2.0 mL water, 2.0 mL succinate-lactate buffer (1.0 M, pH 4.5), 1.0 mL DMF, 35 °C.

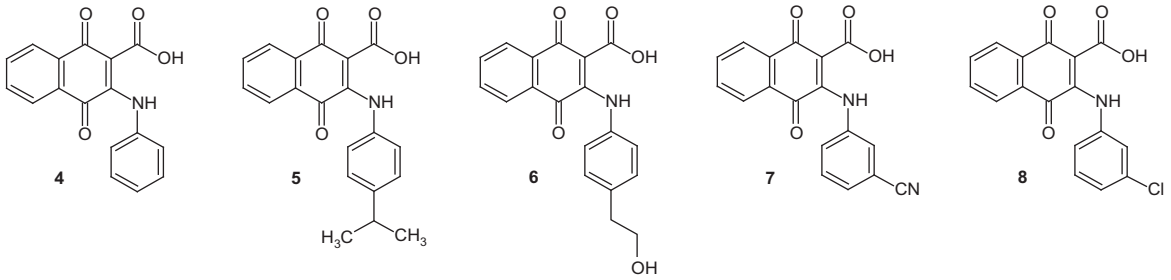


Figure 2. Aminonaphthoquinones synthesised at pH 4.5.

from the thermophilic ascomycete *Myceliophthora thermophila* used for lignin modification within pulps and effluents. It is produced by submerged fermentation of genetically modified *Aspergillus* sp. with molecular weight of 56,000 Da. The goal of our investigation was to determine whether this commercial laccase could be used to catalyse C–N bond formation to afford aminonaphthoquinones. The reaction that was investigated was that between the hydroquinone **1** and the primary amines **2a–k** to form **3** as shown in Scheme 1.

The 1,4-naphthoquinone with a carboxyl group was chosen because the carbon at the 3-position would be a good electrophile due to the electron withdrawing effect of the carboxyl group in the 2-position. This would promote C–N bond formation and thus allow access to the aminonaphthoquinones. The carboxyl group would also increase the solubility of the 1,4-naphthohydroquinone in an aqueous medium in addition to providing another point where structural modification can be done, for example, by amidation and esterification for the synthesis of derivatives. Only primary amines were used in this investigation and no reactions were attempted with secondary, tertiary or cyclic amines.

The reactions were conducted using the laccase, Novozym 51003, in succinate-lactate buffer (pH 4.5) and a co-solvent, DMF. The latter was added to aid the dissolution of the substrates. For these reactions 2 equiv of the amine was used since this would promote the formation of the aminonaphthoquinone. The enzyme was added at different time intervals to the reaction mixture to ensure fresh enzyme and to circumvent the possibility of denaturing all at once. The results of the investigation are shown in Table 1 below.

The highest yield was obtained for **4** (77%, entry 1) and the lowest for **7** (32%, entry 6). Method A is better for the synthesis of **5** because a higher yield (70%, entry 2) was obtained which was 23% higher than that obtained using Method D (47%, entry 3). A longer reaction time in Method D did not increase the yield for **5**. A 13% higher yield for **6** (64%, entry 5) was obtained using Method B compared to that obtained using Method A (51%, entry 4), a longer reaction time was in this case better for product formation. A characteristic dark-purple, red or brown color of the product was indicative of the formation of the aminonapthoquinone and simplified product isolation. The phenolic hydroxyl groups were not observed and the amine proton was not observed for all of the synthesised compounds. Signals characteristic of the carbonyl carbons of the quinones are observed in the 178–186 ppm range of the ^{13}C NMR spectrum. The structures of the **s**ynthesised aminonaphthoquinones are shown in Figure 2 below.

Since the yields of some of the products were low, it was decided to investigate whether the aminonaphthoquinones could be synthesised at pH 6.0 and also whether the yields could be improved. The results of the investigation using Method E are shown in Table 2 below.

From the results it can be seen that the aminonaphthoquinones can also be synthesised at pH 6.0. The highest yield was obtained for **4** (85%, entry 1) and the lowest for **9** (25%, entry 3). The yield for **4** is 8% more than that obtained at pH 4.5 using Method E (77%, Table 1, entry 1). The yield for **7** (33%, Table 2, entry 2) is sim-

Table 2
The **s**ynthesised aminonaphthoquinones (yield in parentheses) using Novozym 51003 in aqueous DMF at pH 6.0

Entry	Hydroquinone	Amine (2 equiv)	Product time (h)	Reaction
1	1	**2a**	48	**4** (85%)
2	1	**2d**	48	**7** (33%)
3	1	**2e**	48	**9** (25%)
4	1	**2g**	48	**10** (29%)
5	1	**2h**	48	**11** (49%)
6	1	**2i**	48	**12** (28%)
7	1	**2j**	48	**13** (40%)
8	1	**2k**	48	**14** (31%)

Method E–2.5 mL (2 775 U) Novozym 51003, 2 equiv amine, 1,4-dihydroxy-2-naphthoic acid (0.6 mmol), 1.0 mL DMF, 3.0 mL sodium phosphate buffer (0.01 M, pH 6.0).

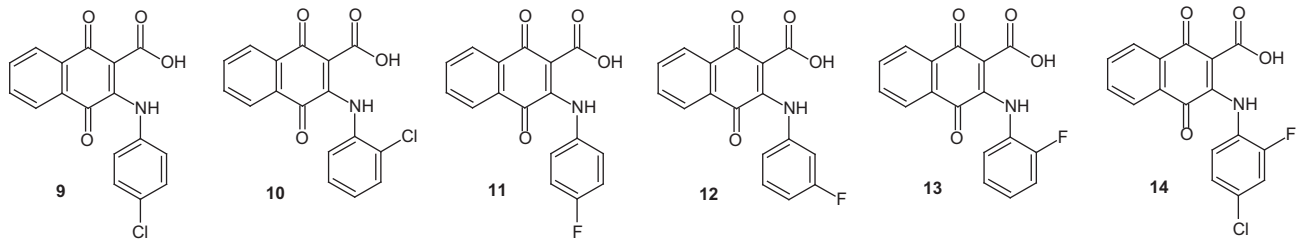


Figure 3. Aminonaphthoquinones synthesised at pH 6.0.

ilar to that obtained using Method A (32%, Table 1, entry 6). The additional aminonaphthoquinones are shown in Figure 3.

Cyanide and halides are known to inhibit laccase.[45,50] The low yield for **7** could be due to the nitrile moiety, in both **2d** and **7,** inhibiting laccase. This can occur by coordination of the nitrogen atom of the nitrile moiety to the copper atoms within the laccase active site. The possibility exists that the presence of halides such as chloro and fluoro may also inhibit laccase and may account for the low yields of the halogenated aminonaphthoquinones.

The optimal pH of most laccases is known to be between 3.5 and 5.0.[51] By conducting the reactions at pH 6.0 the catalytic activity of the commercial laccase could have been reduced resulting in lower conversion of 1,4-dihydroxy-2-naphthoic acid **1** to the corresponding naphthoquinone intermediate. Lower concentrations of the intermediate could also account for the generally lower yields of the aminonaphthoquinones at pH 6.0.

A proposed mechanism for the formation of the aminonaphthoquinones is shown in Figure 4.

The role of laccase is simply that of an oxidant, two laccase oxidations occur before the aminonaphthoquinone is formed.

Literature reports on nuclear amination by chemical methods are limited. This is due to the susceptibility of the amino group to oxidation and hydrolysis. A chemical method for achieving nuclear amination on a 1,4-benzohydroquinone was reported by Chakraborty.[52] This was accomplished by first halogenating (iodinating) the 1,4-benzohydroquinone followed by coupling to the primary amine using a palladium catalyst and a triphenylphosphine ligand while refluxing under argon.[52] Some well-known chemical oxidants such as cupric acetate, silver (I) oxide and sodium iodate have also been used to achieve nuclear amination of 1,4-hydrobenzoquinones with primary aromatic amines.[53] Amongst these sodium iodate can be used to accomplish amination more conveniently.[53]

A chemical method which leads to aminonaphthoquinones has been reported by Benites et al.[54] Amination of 1,4-naphthoquinone and 2,3-dichloro-1,4-naphthoquinone with a variety of aryl- and alkylamines was achieved using a Lewis acid catalyst, $CeCl_3{\cdot}7H_2O$ in ethanol at room temperature.[54]

Similar synthetic approaches to that reported by Chakraborty[52] and Benites et al.[54] would have to be attempted to access the aminonaphthoquinones. Our method allows access to aminonaphthoquinones in a one-pot synthesis directly from 1,4-dihydroxy-2-naphthoic acid **1**. We have thus eliminated the use of a chemical oxidant, an iodinated intermediate, a palladium catalyst and a phosphine ligand in the one approach, and in the other the use of a Lewis acid catalyst ($CeCl_3.7H_2O$) and a chlorinated intermediate.

2.2. Anticancer evaluation

Screening was conducted against TK10 (renal), UACC62 (melanoma), MCF7 (breast) and HeLa (Human Negroid Cervix Epitheloid Adenocarcinoma, ECACC no. 93021013) cancer cell lines using the sulforhodamine B (SRB) assay to determine the growth inhibitory effects of the compounds.[55] The TK10 (renal), UACC62 (melanoma) and MCF7 (breast) cell lines have been used routinely at the U.S. National Cancer Institute for screening for new anticancer agents and were derived from tumours that have different sensitivities to chemotherapeutic drugs.[56] Etoposide, an anticancer agent, was used as a positive control. It is known to be an inhibitor of topoisomerase, particularly topoisomerase II and aids in DNA unwinding which causes the DNA strands to break.[57] Three parameters were determined during the screening process such as 50% cell growth inhibition (GI_{50}), total cell growth inhibition (TGI) and the lethal concentration that kills 50% of cells (LC_{50}). The results are shown in Table 3.

Several compounds exhibited potent growth inhibitory activity as seen from the results in Table 3. The GI_{50} concentrations of the compounds were compared to that of etoposide.

Only 5 exhibited potent activity (GI_{50} = 8.38 μM, entry 2) against the TK10 cell line which was almost as good as that of

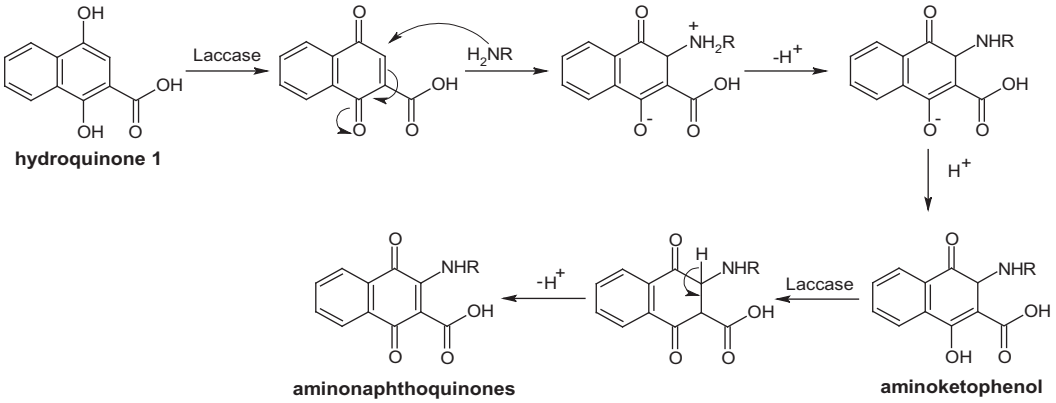


Figure 4. A proposed mechanism for the formation of the aminonaphthoquinones.

Table 3
In vitro anticancer screening of the compounds against TK10 (renal), UACC62 (melanoma), MCF7 (breast) and HeLa cancer cells expressed as GI_{50}, TGI and LC_{50} values (μM)

Entry	Compd	Tk10 (renal)				UACC62 (melanoma)				MCF7 (breast)				HeLa			
		GI_{50}	Activity	TGI	Activity	LC_{50}	GI_{50}	Activity	TGI	Activity	LC_{50}	GI_{50}	Activity	TGI	Activity	LC_{50}	GI_{50}	Activity	TGI	Activity	LC_{50}
1	4	74.09	w	0.1	i	>100	6.66	p	30.69	w	73.11	42.56	w	>100	i	>100	49.17	w	97.25	w	>100
2	5	8.38	p	63.55	w	>100	5.19	p	10.81	m	58.11	37.67	w	>100	i	>100	38.26	w	73.04	w	>100
3	6	91.1	w	>100	i	>100	7.17	p	35.32	w	72.02	54.91	w	>100	i	>100	72.5	w	>100	i	>100
4	7	23.68	m	76.22	w	>100	3.99	p	7.17	p	19.3	15.11	m	53.75	w	92.39	15.95	m	49.14	w	82.33
5	8	35.85	w	>100	i	>100	16.98	m	49.08	w	81.18	43.2	w	>100	i	>100	37.31	w	7.068	w	>100
6	9	12.95	mw	79.02	w	>100	3.98	p	7.83	p	55.1	17.19	m	55.1	w	93.01	24.75	m	55.44	w	86.14
7	10	80.74	m	>100	i	>100	31.03	m	65.85	w	>100	63.49	w	>100	i	>100	28.03	m	60.28	w	90.23
8	11	46.55	w	>100	i	>100	7.54	p	35.03	w	72.14	38.09	w	98.93	w	>100	36.19	w	67.31	w	98.42
9	12	24.68	m	>100	i	>100	5.28	p	13.65	m	59.41	9.08	p	48.43	w	91.05	28.03	m	60.28	w	92.53
10	13	36.85	w	>100	i	>100	5.67	p	23.31	m	65.41	9.84	p	50.78	w	92.15	47.54	w	96.99	w	>100
11	14	13.4	m	93.27	w	>100	4.17	p	7.94	p	40.4	21.4	m	54.93	w	88.46	25.18	m	55.52	w	85.85
12	Etoposide	7.19	p	49.74	w	>100	0.89	p	52.71	w	>100	0.56	p	>100	i	>100	3.56	p	40.18		87.54

Compound = cpd: Inactive, i: GI_{50} or TGI >100 μM; Weak Activity, w: >30 μM GI_{50} or TGI <100 μM; Moderate Activity, m: <30 μM GI_{50} or TGI >10 μM; Potent Activity, p: GI_{50} or TGI <10 μM; Value >100 indicates absence of activity.

etoposide (GI_{50} = 7.19 μM, entry 12). The other compounds had medium to weak activity.

Potent growth inhibitory activity (GI_{50} = 3.98–7.54 μM) was observed for almost all the compounds against the UACC62 cell line. The best activity was for 9 (GI_{50} = 3.98 μM, entry 6) and 7 (GI_{50} = 3.99 μM, entry 4) but it was not as good as that of etoposide (GI_{50} = 0.89 μM, entry 12).

Screening against the MCF7 cell line showed that only the two fluorinated compounds, **12** (GI_{50} = 9.08 μM, entry 9) and **13** (GI_{50} = 9.84 μM, entry 10) exhibited potent growth inhibitory activity which was not as good as that of etoposide (GI_{50} = 0.56 μM, entry 12). The activities of the other compounds were moderate to weak.

The aminonaphthoquinones exhibited moderate to weak activity against the HeLa cell line.

A comparison of the TGI concentrations of the compounds with etoposide was also done. Most of the compounds were inactive and a few exhibited weak activity against the TK10 cell line. Compounds **7** (TGI = 7.17 μM, entry 4), **9** (TGI = 7.83 μM, entry **6**) and **14** (TGI = 7.94 μM, entry 11) all exhibited potent activity against the UACC62 cell line. The activities of these compounds were 7- to 8-fold better than that of etoposide (TGI = 52.71 μM, entry 12) and the best activity was exhibited by **7**. Most of the other compounds exhibited moderate to weak activities (TGI = 10.81–49.08 μM) which was better than etoposide.

Screening against the MCF7 cell line showed that about half the number of compounds showed weak activity (TGI = 48.43–98.93 μM) while other compounds were inactive like etoposide. The best activity was exhibited by **12** (TGI = 48.43 μM, Entry 9). Benites et al. also reported on the anticancer activity of a series of aminonaphthoquinones.[54] Potent activity was observed against the MCF7 cell line for some of the aminonaphthoquinones.[54]

The TGIs of the aminonaphthoquinones were weaker than that of etoposide against the HeLa cancer cell line.

The LC_{50} concentrations of the compounds were compared to that of etoposide to get an indication of the cytotoxic effects of these compounds against the three cell lines. The compounds were inactive like etoposide against the TK10 cell line.

Almost all of the compounds were more lethal (TGI = 19.13–81.18 μM) against the UACC62 cell line than etoposide which was inactive (LC_{50} >100 μM).

Five compounds, **7, 9**, **12, 13** and **14**, exhibited more lethal cytotoxic effects (TGI = 88.46–93.01 μM) against the MCF7 cell line than that of etoposide which was inactive (LC_{50} >100 μM).

Only compounds **7, 9** and **14** (TGI = 82.33, 86.14 and 85.85 μM, respectively) were slightly more lethal than etoposide (TGI = 87.54 μM, Entry 12) against the HeLa cell line.

Compound **5** having the isopropyl substituent on the phenyl ring exhibited potent activity against both the TK10 and UACC62 cell lines. The two fluorinated compounds, **12** and **13**, exhibited potent growth inhibitory activity against both the UACC62 and MCF7 cell lines. Compound **7**, having a nitrile substituent in the *meta* position on the phenyl ring, and **9** having a chloro substituent in the *para* position on the phenyl ring, were the most potent against the UACC62 cell line. The compounds exhibited better cytostatic (lower TGI concentrations) and cytotoxic effects (lower LC_{50} concentrations) than etoposide against the UACC62 cell line. Overall, the aminonaphthoquinones were most effective against the UACC62 cell line in spite of the HeLa cell line being a more sensitive one.

The aminonaphthoquinones were most effective against the UACC62 (melanoma) cancer cell line exhibiting potent activity against it. Only compound **5** exhibited potent cytostatic effects against both the TK10 (renal) and the UACC62 (melanoma) cancer cell lines. The activity of **5** (GI_{50} = 8.38 μM) against the TK10 cell line was almost as good as that of the known drug, etoposide (GI_{50} = 7.19 μM). Two compounds, **12** and **13**, exhibited potent

cytostatic effects against both the UACC62 (melanoma) and the MCF7 (breast) cancer cell lines. The aminonaphthoquinones also exhibited better TGI than that of etoposide against the UACC62 cell line of which **7** (TGI = 7.17 μM), **9** (TGI = 7.83 μM) and **14** (TGI = 7.94 μM) all exhibited potent cytostatic effects 7- to 8-fold better than that of etoposide (TGI = 52.71 μM).

3. Conclusions

A new biocatalytic method was developed for the synthesis of aminonaphthoquinones by using laccase, a non-hazardous oxidising agent. The commercial laccase, Novozym 51003, can be used to access aminonaphthoquinones at both pH 4.5 and at pH 6.0 in a one-pot synthesis from the reaction of 1,4-dihydroxy-2-naphthoic acid and a primary amine. The formation of the product is affected by factors such as the solubility of the substrates, pH of the reaction mixture, reaction temperature in addition to the nucleophillicity and number of equivalents of the amine. There is potential for other laccases, particularly those with a higher activity and better substrate specificity, to be employed for the synthesis of aminonaphthoquinones. The aminonaphthoquinones were most effective against the UACC62 (melanoma) cancer cell line exhibiting potent cytostatic effects. These results are encouraging for further studies of the aminonaphthoquinones for their application in anticancer therapy. Future work will entail further synthesis of aminonaphthoquinones and structural modification of compounds exhibiting potent activity to enhance their anticancer activity.

4. Experimental

4.1. General

Proton nuclear magnetic resonance (^{1}H NMR) spectra were recorded on a 200 MHz Varian Gemini spectrometer and also on a 400 MHz Varian Unity Plus spectrometer. Carbon-13 nuclear magnetic resonance (^{13}C NMR) spectra were recorded on the same instruments at 50 MHz and 100 MHz. Chemical shifts are reported in ppm relative to the solvent peaks. High-resolution mass spectra were recorded on a Waters HPLC coupled to a Synapt HDMS mass spectrometer. Reactions were monitored by thin layer chromatography (TLC) on aluminium-backed Merck silica gel 60 F_{254} plates. Gravity column chromatography was done using Merck silica gel 60 (70–230 mesh). Melting points were determined using a Glassco melting point apparatus and are uncorrected.

4.2. Materials

All chemicals were reagent grade materials.

4.2.1. Substrates

The 1,4-naphthohydroquinone-2-carboxylic acid, primary amines and were obtained from Sigma–Aldrich South Africa.

4.2.2. Enzymes

The laccase, Novozym 51003 (1,110.00 U/g) from the ascomycete *M. thermophila* was obtained from Novozymes SA.

4.3. Synthetic methods

The following methods were used for the synthesis of the aminonaphthoquinones.

4.3.1. Method A

Novozym 51003 (1.0 mL) was added to a mixture of 1,4-dihydroxy-2-naphthoic acid (0.6 mmol), amine (1.2 mmol, 2 equiv), succinate-lactate buffer (2.0 mL, 1.0 M, pH 4.5), water (2.0 mL) and DMF (1.0 mL) at 35 °C. More enzyme (1.0 mL) was added after 6 h. After heating the reaction mixture was transferred to a separating funnel to which water (25 mL) was added. The mixture was extracted with EtOAc, the solvent evaporated and the residue purified by flash chromatography. The product was then washed with a 25% Et_2O/hexane solution.

4.3.2. Method B

Novozym 51003 (1.0 mL) was added to a mixture of 1,4-dihydroxy-2-naphthoic acid (0.6 mmol), amine (1.2 mmol, 2 equiv), succinate-lactate buffer (2.0 mL, 1.0 M, pH 4.5), water (2.0 mL) and DMF (1.0 mL) at 35 °C. More enzyme (1.0 mL) was added after 2, 4 and 24 h. After heating the reaction mixture was transferred to a separating funnel to which water (25 mL) was added. The mixture was extracted with EtOAc, the solvent evaporated and the residue purified by flash chromatography. The product was then washed with a 25% Et_2O/hexane solution.

4.3.3. Method C

Novozym 51003 (1.0 mL) was added to a mixture of 1,4-dihydroxy-2-naphthoic acid (0.6 mmol), amine (1.2 mmol, 2 equiv), succinate-lactate buffer (2.0 mL, 1.0 M, pH 4.5), water (2.0 mL) and DMF (1.0 mL) at 35 °C. After heating the reaction mixture was transferred to a separating funnel to which water (25 mL) was added. The mixture was extracted with EtOAc, the solvent evaporated and the residue purified by flash chromatography. The product was then washed with a 25% Et_2O/hexane solution.

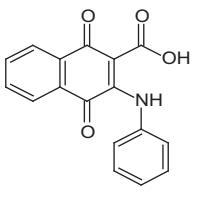
4.3.4. Method D

Novozym 51003 (1.5 mL) was added to a mixture of 1,4-dihydroxy-2-naphthoic acid (0.6 mmol), amine (1.2 mmol, 2 equiv), succinate-lactate buffer (2.0 mL, 1.0 M, pH 4.5), water (2.0 mL) and DMF (1.0 mL) at 35 °C. More enzyme (1.0 mL) was added after heating for 1.5 h and 3 h. After heating the reaction mixture was transferred to a separating funnel to which water (25 mL) was added. The mixture was extracted with EtOAc, the solvent evaporated and the residue purified by flash chromatography. The product was then washed with a 25% Et_2O/hexane solution.

4.3.5. Method E

Novozym 51003 (1.0 mL) was added to a mixture containing amine (1.8 mmol, 2 equiv), 1,4-dihydroxy-2-naphthoic acid (0.9 mmol), sodium phosphate buffer (3.0 mL, 0.01 M, pH 6.0) and DMF (1.0 mL) while stirring at 40 °C. After heating for 2 h more enzyme (1.0 mL) was added. More enzyme (0.5 mL) was again added after 24 h. After heating for 48 h the reaction mixture was transferred to a separating funnel to which water (25 mL) was added. The mixture was extracted with EtOAc, the solvent evaporated and the residue purified by flash chromatography. The product was then washed with a 25% Et_2O/hexane solution.

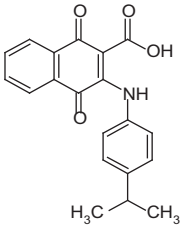
4.3.6. 1,4-Dioxo-3-(phenylamino)-1,4-dihydronaphthalene-2-carboxylic acid 4



4.3.6.1. Method D. Purification by flash chromatography (silica: EtOAc/hexane, 1:6, 1:4, 1:2 and EtOAc) to afford a brown solid (0.1394 g, 77%).

4.3.6.2. Method E. Heating time = 48 h. Purification by flash chromatography (silica: DCM/hexane, 1:2, 1:1; DCM) to afford a dark brown solid (0.2208 g, 85%). (**M+H$^+$** Found: 294.0771. $C_{17}H_{12}NO_4$ requires M+H, 294.0766). R_f = 0.35 (EtOAc/hexane, 1:2). Mp = 177–178 °C. ^{1}H NMR (200 MHz, $CDCl_3$): δ = 7.16 (2H, d J = 8.0 Hz, ArH), 7.40 (3H, m, ArH), 7.65–7.88 (2H, m, ArH), 7.92 (1H, d J = 7.4 Hz, ArH), 8.24 (1H, d J = 7.6 Hz, ArH) and 13.12; ^{13}C NMR (100 MHz, $CDCl_3$): δ = 100.2, 124.5, 126.9, 127.1, 127.7, 129.3, 131.0, 131.9, 133.8, 135.6, 138.4, 153.9, 171.3, 180.1 and 184.5.

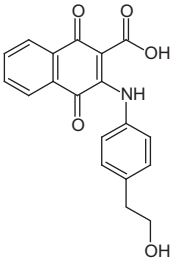
4.3.7. 1,4-dioxo-3-{[4-(propan-2-yl)phenyl]amino}-1,4-dihydronaphthalene-2-carboxylic acid 5



4.3.7.1. Method A. Heating time = 26 h. Purification by flash chromatography (silica: $CHCl_3$; MeOH/$CHCl_3$, 1: 100 and 1:50) to afford a dark-brown solid (0.1394 g, 70%). (**M−H$^+$** Found: 334.1082. $C_{20}H_{16}NO_4$ requires M−H, 334.1082). R_f = 0.56 (DCM). Mp = 148–150 °C. ^{1}H NMR (200 MHz, $CDCl_3$): δ = 1.29 (6H, m, 2 × CH_3), 2.88–3.10 (1H, s, CH), 7.09 (2H, d J 8.2 Hz, ArH), 7.28 (2H, d J 8.40 Hz, ArH), 7.65–7.90 (2H, m, ArH), 7.95 (1H, d J 7.0 Hz, ArH), 8.25 (1H, d J 7.6 Hz, ArH), and 13.1 (1H, br s, CO_2H); ^{13}C NMR (50 MHz, $CDCl_3$): δ = 24.1, 34.0, 100.5, 124.3, 126.9, 127.0, 127.3, 131.1, 132.1, 133.7, 135.5 and 136.0.

4.3.7.2. Method D. Heating time = 48 h. Purification by flash chromatography (silica: EtOAc/hexane, 1: 10, 1:6, 1:4, 1:2; EtOAc) to afford a dark-brown solid (0.0940 g, 47%).

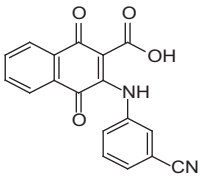
4.3.8. 3-{[4-(2-Hydroxyethyl)phenyl]amino}-1,4-dioxo-1,4-dihydronaphthalene-2-carboxylic acid 6



4.3.8.1. Method A. Heating time = 27 h. Purification by flash chromatography (silica: EtOAc/hexane, 1:2, 1:1; EtOAc; EtOAc/MeOH, 19.9:0.1 and 19.5:0.5) to afford a dark-brown solid (0.1027 g, 51%).

4.3.8.2. Method B. Heating time = 72 h. Purification by flash chromatography (silica: EtOAc/hexane, 1:2, 1:1; EtOAc; EtOAc/MeOH, 19.9:0.1 and 19.5:0.5) to afford a dark-brown solid (0.1305 g, 64%). (**M−H$^+$** Found: 336.0865. $C_{19}H_{14}NO_5$ requires M−H, 336.0872). R_f = 0.24 (EtOAc/hexane, 1:1). Mp = 157–159 °C. ^{1}H NMR (200 MHz, $CDCl_3$): δ = 1.68 2H, br s, NH and OH), 2.92 (2H, t J = 6.4 and 6.8 Hz, CH_2), 3.91 (2H, t J = 6.4 and 6.6 Hz, CH_2), 7.11 (2H, d J = 8.2 Hz, ArH), 7.29 (2H, d J = 7.8 Hz, ArH), 7.64–7.87 (2H, m, ArH), 7.91 (1H, m, ArH), 8.23 (1H, d J = 7.8 Hz, ArH) and 13.10 (1H, br s, CO_2H); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 38.7, 63.4, 100.2, 124.6, 126.9, 127.0, 130.0, 131.0, 131.9, 133.8, 135.6, 136.8, 138.3, 153.8, 171.3, 180.2 and 184.4.

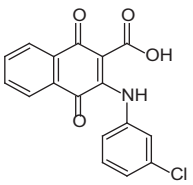
4.3.9. 3-[(3-Cyanophenyl)amino]-1,4-dioxo-1,4-dihydronaphthalene-2-carboxylic acid 7



4.3.9.1. Method C. Heating time = 24 h. Purification by flash chromatography (silica: EtOAc/hexane, 1: 5 1:4, 1:3 and 1:2) to afford a dark-brown solid (0.0607 g, 32%). (**M-H$^+$** Found: 317.0540. $C_{18}H_9N_2O_4$ requires M-H, 317.0562). R_f = 0.18 (EtOAc/hexane, 1:2). Mp = 214–216 °C.

4.3.9.2. Method E. Heating time = 48 h. Purification by flash chromatography (silica: EtOAc/hexane, 1:2, 1:1; EtOAc; MeOH/EtOAc, 1:50, 1:30) to afford an orange-brown solid (0.0982 g, 33%). (**M+H$^+$** Found: 319.0739. $C_{18}H_{11}N_2O_4$ requires M+H, 319.0719). R_f = 0.21 (EtOAc/hexane, 1:1). Mp = 296–297 °C. ^{1}H NMR (200 MHz, $CDCl_3$): δ = 1.62 (1H, br s, NH), 7.39–7.88 (6H, m, ArH), 7.94 (1H, d J = 7.6 Hz, ArH), 8.25 (1H, d J 7.6 Hz, ArH) and 13.10 (1H, br s, CO_2H); ^{13}C NMR (50 MHz, $CDCl_3$): δ = 101.0, 113.6, 117.7, 127.1, 127.3, 128.4, 130.3, 130.7, 131.0, 131.6, 134.2, 136.0, 139.6, 153.5, 171.1, 180.0 and 184.9.

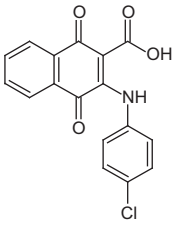
4.3.10. 3-[(3-Chlorophenyl)amino]-1,4-dioxo-1,4-dihydronaphthalene-2-carboxylic acid 8



4.3.10.1. Method C. Heating time = 24 h. Purification by flash chromatography (silica: MeOH/$CHCl_3$, 1: 49 1:39 and 1:19) to afford a dark-brown solid (0.1046 g, 52%). (**M−H$^+$** Found: 326.0226. $C_{17}H_9NO_4Cl$ requires M−H, 326.0220). R_f = 0.34 (EtOAc/hexane, 1:3). Mp = 203–205 °C. ^{1}H NMR (200 MHz, $CDCl_3$): δ = 1.57 (1H,

br s, NH), 7.07 (1H, m, ArH), 7.19 (1H, s, ArH), 7.35 (2H, d *J* = 4.4 Hz, ArH), 7.65–8.00 (3H, m, ArH), 8.24 (1H, d *J* 7.4 Hz, ArH), and 13.10 (1H, br s, CO_2H); ^{13}C NMR (50 MHz, $CDCl_3$): δ = 123.0, 124.3, 125.0, 127.0, 127.2, 127.9, 130.3, 130.9, 131.7, 134.0, 123.9, 135.8, 139.6, 153,7, 167.1, 171.2, 179.9 and 184.7.

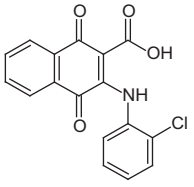
4.3.10.2. 2-Acetyl-3-[(4-chlorophenyl)amino]naphthalene-1,4-dione 9.



4.3.11. Method E

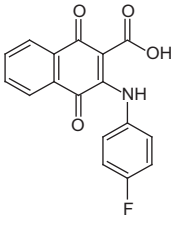
Heating time = 48 h. Purification by flash chromatography (silica: DCM/hexane, 1:2, 1:1; DCM) to afford a brown solid (0.0737 g, 25%). (**M**−**H**$^+$ Found: 328.0386. $C_{17}H_{11}NO_4Cl$ requires M−H, 328.0377). R_f = 0.23 (DCM). Mp = 259–262 °C. ^1H NMR (200 MHz, DMSO-d_6): δ = 7.33 (2H, d *J* = 8.8 Hz, ArH), 7.42 (2H, d *J* = 8.8 Hz, ArH), 7.82 (1H, t *J* = 6.8 and 7.6 Hz, ArH), 7.91 (1H, t *J* = 7.20 and 7.6 Hz, ArH), 7.98 (1H, d *J* = 7.2 Hz, ArH), 8.07 (1H, d *J* 7.2 Hz, ArH); ^{13}C NMR (50 MHz, $CDCl_3$): δ = 125.8, 126.3, 126.4, 128.5, 130.5, 130.6, 131.8, 133.4, 135.2, 137.6, 148.7, 167.7, 180.7 and 182.3.

4.3.12. 3-[(2-Chlorophenyl)amino]-1,4-dioxo-1,4-dihydronaphthalene-2-carboxylic acid 10



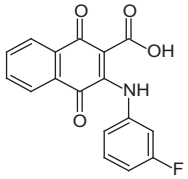
4.3.12.1. Method E. Heating time = 48 h. Purification by flash chromatography (silica: DCM/hexane, 1:2, 1:1.5; 1:1; DCM) to afford a brown solid (0.0853 g, 29%). (**M+H**$^+$ Found: 328.0361. $C_{17}H_{11}NO_4Cl$ requires M+H, 328.0377). R_f = 0.43 (EtOAc/hexane, 1:3). Mp = 189–191 °C. ^1H NMR (200 MHz, $CDCl_3$): δ = 7.20–7.55 (4H, m, ArH), 7.65–7.88 (2H, m, ArH), 7.93 (1H, d *J* = 7.6 Hz, ArH), 8.24 (1H, d *J* = 7.6 Hz, ArH) and 12.92 (1H, br s, CO_2H); ^{13}C NMR (50 MHz, $CDCl_3$): δ = 100.1, 126.4, 127.0, 127.1, 127.8, 128.8, 129.3, 130.2, 130.6, 131.8, 133.9, 135.6, 136.4, 138.2, 154.7, 171.1, 179.9 and 184.9.

4.3.13. 3-[(4-Fluorophenyl)amino]-1,4-dioxo-1,4-dihydronaphthalene-2-carboxylic acid 11



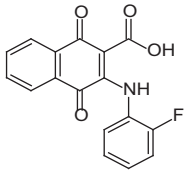
4.3.13.1. Method E. Heating time = 48 h. Purification by flash chromatography (silica: DCM/hexane, 1:2, 1:1.5, 1:1; DCM) to afford a brown solid (0.1358 g, 49%). (**M-H**$^+$ Found: 312.0682. $C_{17}H_{11}NO_4F$ requires M-H, 312.0672). R_f = 0.43 (EtOAc/hexane, 1:3). Mp = 235–237 °C. ^1H NMR (200 MHz, $CDCl_3$): δ = 7.05–7.19 (4H, m, ArH), 7.66–7.88 (2H, m, ArH), 7.91 (1H, d *J* = 7.6 Hz, ArH), 8.23 (1H, d *J* = 7.2 Hz, ArH) and 13.06 (1H, br s, CO_2H); ^{13}C NMR (50 MHz, $CDCl_3$): δ = 100.6, 116.4, 116.9, 126.6, 126.8, 127.3, 127.4, 131.2, 132.1, 134.2, 134.7, 136.0, 154.1, 159.5, 164.4, 171.6, 180.4 and 184.9.

4.3.14. 3-[(3-Fluorophenyl)amino]-1,4-dioxo-1,4-dihydronaphthalene-2-carboxylic acid 12



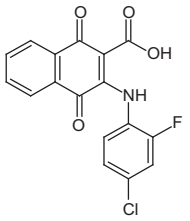
4.3.14.1. Method E. Heating time = 48 h. Purification by flash chromatography (silica: DCM/hexane, 1:2, 1:1; DCM) to afford a dark-brown solid (0.0797 g, 28%). (**M-H**$^+$ Found: 312.0655. $C_{17}H_{11}NO_4F$ requires M-H, 312.0655). R_f = 0.37 (EtOAc/hexane, 1:3). Mp = 194–195 °C. ^1H NMR (200 MHz, $CDCl_3$): δ = 6.94 (2H, t *J* = 8.2 and 11.4 Hz, ArH), 7.08 (1H, t *J* = 8.0, and 6.8 Hz, ArH), 7.39 (2H, q *J* = 8.0 and 6.2 Hz, ArH), 7.65–7.89 (2H, m, ArH), 7.93 (1H, d *J* = 6.6 Hz, ArH), 8.23 (1H, d *J* = 7.4 Hz, ArH) and 13.06 (1H, br s, CO_2H); ^{13}C NMR (50 MHz, $CDCl_3$): δ = 100.6, 112.2, 112.7, 114.6, 115.0, 120.5, 120.6, 127.0, 127.1, 130.5, 130.6, 130.9, 131.8, 134.0, 135.7, 138.2, 139.8, 140.1, 153.8, 160.3, 165.3, 171.2, 180.0 and 184.7.

4.3.15. 3-[(2-Fluorophenyl)amino]-1,4-dioxo-1,4-dihydronaphthalene-2-carboxylic acid 13



4.3.15.1. Method E. Heating time = 48 h. Purification by flash chromatography (silica: DCM/hexane, 1:2, 1:1.5, 1:1; DCM) to afford a red-brown solid (0.1119 g, 40%). (**M**−**H**$^+$ Found: 312.0668. $C_{17}H_{11}NO_4F$ requires M−H, 312.0672). R_f = 0.43 (EtOAc/hexane, 1:3). Mp = 193–195 °C. ^1H NMR (200 MHz, $CDCl_3$): δ = 7.08 (4H, m, ArH), 7.66–7.89 (2H, m, ArH), 7.95 (1H, d *J* = 7.6 Hz, ArH), 8.23 (1H, m, ArH) and 12.86 (1H, br s, CO_2H); ^{13}C NMR (50 MHz, $CDCl_3$): δ = 100.4, 116.0, 116.4, 124.8, 124.9, 125.8, 126.9, 127.1, 127.8, 128.9, 129.0, 130.7, 131.8, 133.9, 135.6, 153.3, 154.4, 158.2, 171.1, 180.1 and 184.9.

4.3.16. 3-[(4-Chloro-2-fluorophenyl)amino]-1,4-dioxo-1,4-dihydronaphthalene-2-carboxylic acid 14



4.3.16.1. Method E. Heating time = 48 h. Purification by flash chromatography (silica: DCM/hexane, 1:6, 1:4; 1:3 and 1:2) to afford a red-brown solid (0.0947 g, 31%). (**M+H$^+$** Found: 346.0301. $C_{17}H_{10}NO_4ClF$ requires M+H, 346.0282). R_f = 0.29 (EtOAc/hexane, 1:1.5). Mp = 193–195 °C. ^{1}H NMR (200 MHz, $CDCl_3$): δ = 7.12–7.30 (3H, m, ArH), 7.68–7.90 (2H, m, ArH), 7.95 (1H, m, ArH), 8.23 (1H, m, ArH) and 12.82 (1H, br s, CO_2H); ^{13}C NMR (50 MHz, $CDCl_3$): δ = 101.0, 117.2, 117.7, 125.4, 125.5, 125.8, 126.8, 127.3, 127.4, 130.8, 132.0, 134.4, 136.0, 153.4, 154.4, 158.4, 171.4, 180.4 and 185.3.

4.4. In vitro anticancer activity evaluation

4.4.1. Assay background

The growth inhibitory effects of the compounds were tested in duplicate in a 3-cell line panel consisting of TK10 (renal), UACC62 (melanoma) and MCF7 (breast) cancer cells using the Sulforhodamine B (SRB) assay.[55] The SRB assay was developed by Skehan and colleagues to measure drug-induced cytotoxicity and cell proliferation. Its principle is based on the ability of the protein dye, sulforhodamine B (Acid Red 52), to bind electrostatically in a pH-dependent manner to protein basic amino acid residues of trichloroacetic acid-fixed cells. Under mild acidic conditions it binds to the fixed cellular protein, while under mild basic conditions it can be extracted from cells and solubilised for measurement. The SRB Assay is performed at CSIR in accordance with the protocol of the Drug Evaluation Branch, NCI, and the assay has been adopted for this screen.

4.4.2. Materials and method

The human cell lines TK10, UACC62 and MCF7 were obtained from the NCI in a collaborative research program between the CSIR and the NCI. Cell lines were routinely maintained as a monolayer cell culture at 37 °C, 5% CO_2, 95% air and 100% relative humidity in RPMI containing 5% fetal bovine serum, 2 mM L-glutamine and 50 μg/mL gentamicin.

For the screening experiment the cells (3–19 passages) were inoculated in a 96-well microtiter plate at plating densities of 7–10 000 cells/well and were incubated for 24 h. After 24 h one plate was fixed with TCA to represent a measurement of the cell population for each cell line at the time of drug addition (T0). The other plates with cells were treated with the experimental drugs which were previously dissolved in DMSO and diluted in medium to produce 5 concentrations (0.01–100 μM). Cells without drug addition served as control. The blank contains complete medium without cells. Etoposide was used as a reference standard.

The plates were incubated for 48 h after addition of the compounds. Viable cells were fixed to the bottom of each well with cold 50% trichloroacetic acid, washed, dried and dyed by SRB. Unbound dye was removed and protein-bound dye was extracted with 10 mM Tris base for optical density determination at a wavelength of 540 nm using a multiwell spectrophotometer. Optical density measurements were used to calculate the net percentage cell growth.

The optical density of the test well after a 48 h period of exposure to test drug is Ti, the optical density at time zero is T0, and the control optical density is C. Percentage cell growth is calculated as:

$[(Ti-T0)/(C-T0)] \times 100$ for concentrations at which $Ti \geqslant T0$
$[(Ti-T0)/T0] \times 100$ for concentrations at which $Ti < T0$.

The results of a five dose screening were reported as TGI (total growth inhibition). The TGI is the concentration of test drug where $100 \times (T-T0)/(C-T0) = 0$. The TGI signifies a cytostatic effect.

The biological activities were separated into 4 categories: inactive (GI_{50} or TGI >100 μM), weak activity (30 μM < GI_{50} or TGI <100 μM, moderate activity (10 μM < GI_{50} or TGI <30 μM and potent activity (GI_{50} or TGI <10 μM).

For each tested compound, three response parameters, GI_{50} (50% growth inhibition and signifies the growth inhibitory power of the test agent), TGI (which is the drug concentration resulting in total growth inhibition and signifies the cytostatic effect of the test agent) and LC_{50} (50% lethal concentration and signifies the cytotoxic effect of the test agent) were calculated for each cell line.

Acknowledgments

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References and notes

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