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 aganet, 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 aminonaphthoquinones exhibit moderate to weak cytotoxic effects against a normal HeLa cell line.

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

Keywords

Biocatalysis, laccase, oxidative enzymes, green chemistry, 1,4-naphthohydroquinones, aminonaphthoquinones, C-N bond formation, primary amines, monoamination, anticancer agents, cytostatic effects, cytotoxic effects, GI_{50}, TGI, LC_{50}.

1. Introduction

1,4-Naphthoquinone derivatives have exhibited an interesting variety of biological responses such as antiallergic,[1-3] antibacterial,[4,5] anticancer,[5-10] antifungal,[4-6,11] anti-inflammatory,[1-4,11] antithrombotic,[12,13] antiplatelet,[1-3,12-14] antiviral,[4,7,8,15] apoptosis,[16-18] lipoxygenase,[19,20] radical scavenging[21] and anti-ringworm[4] activities. Human DNA topoisomerase I and II are known to be inhibited by 1,4-naphthoquinone derivatives.[10,21-26] The latter 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.

The aminoquinone moiety is prevalent in several drugs that are in use such as the commercial anti-neoplastic agents actinomycin[29] and streptonigrin[29] (Figure 1). The antibiotics, mitomycin[30] and rifamycin[31] are also based on an aminoquinone (Figure 1).

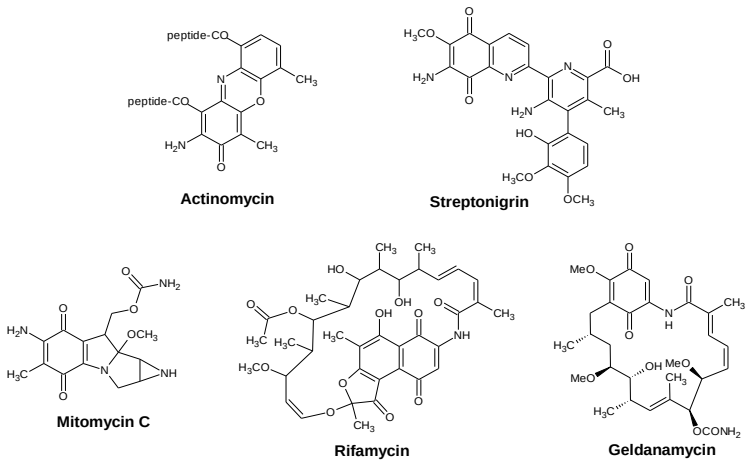


Figure 1. Drugs having an aminoquinone moiety.

It must be noted that the amino group is in the *ortho* position to the ketone in mitomycin C, streptonigrin,[29] actinomycin[31] (and the structurally related aurantins[32]) as well as in the *ansa*-antibiotics rifamycin[31] and geldanamycin.[33] 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.[34]

Chemotherapy is the primary treatment for cancer and has been hampered by the development of drug resistant and multi-drug resistant tumors. 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.[35] Two molecular "pumps" in tumor-cell membranes actively expel chemotherapy drugs from the interior thus allowing tumor cells to avoid the toxic effects of the drug or molecular processes within the nucleus or the cytoplasm.[35] P-glycoprotein and the multidrug resistance–associated protein (MRP) are the two pumps commonly found to confer chemoresistance in cancer. There is thus a need for new compounds that are effective in treating drug resistant and multidrug resistant tumors.

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[36] using laccase.

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 laccase 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.

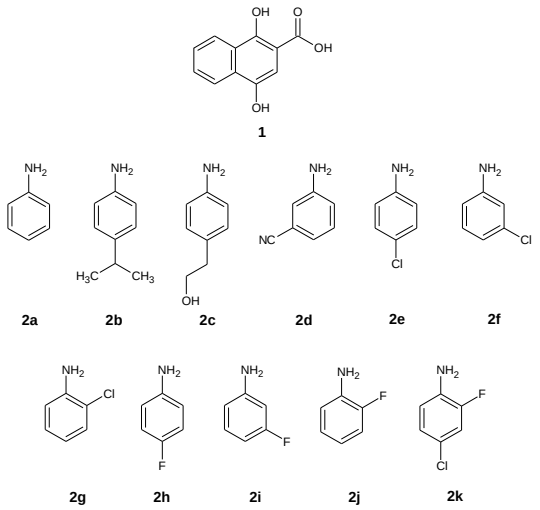
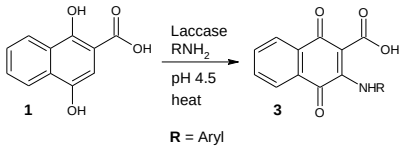


Figure 2. The hydroquinone and primary amines used in this study.

The 1,4-naphthohydroquinone **1** and primary amines **2a-k** used in this investigation are depicted in Figure 2. 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.



Scheme 1

These 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 2-3 equivalents 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 amine proton was observed, but no phenolic hydroxyl groups. Signals characteristic of the carbonyl carbons of the quinones are observed in the 178-186 ppm range of the ^{13}C NMR spectrum.

Table 1. The **s**ynthesised aminonaphthoquinones (Yield in Parentheses) at pH 4.5 using Methods A to D.

Entry	Hydroquinone	Amine (eq)	Reaction Time (hours)	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 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, 35ºC.
Method B - 4.0 ml 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), 1.0mL DMF, 35ºC.
Method C - 1.0 ml Novozym 51003, 2 equiv amine (1.2 mmol), 1,4-dihydroxy-2-naphthoic acid (0.6 mmol), 2.0 mL water, 2.0mL succinate-lactate buffer (1.0 M, pH 4.5), 1.0 mL DMF, 35ºC.
Method D - 3.5 ml 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, 35ºC.

The structures of the **s**ynthesised aminonaphthoquinones are shown in Figure 3 below.

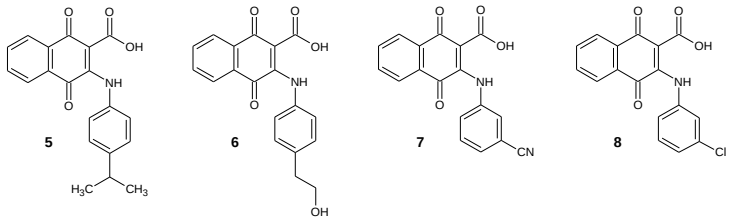


Figure 3. The structures of the aminonaphthoquinones synthesised at pH 4.5.

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.

Table 2. The **s**ynthesised aminonaphthoquinones (Yield in Parentheses) using Novozym 51003 in aqueous DMF at pH 6.0.

Entry	Hydroquinone	Amine (2eq)	Product Time (hours)	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 Novozym 51003, 2 equiv amine, 1,4-dihydroxy-2-naphthoic acid (0.6 mmol), 1.0mL DMF, 3.0 mL sodium phosphate buffer (0.01 M, pH 6.0).

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%. Entry 1, Table 1). The yield for **7** (33%, Entry 2, Table 2) is similar to that obtained using Method A (32%, Entry 6, Table 1). The additional aminonaphthoquinones are shown in Figure 4.

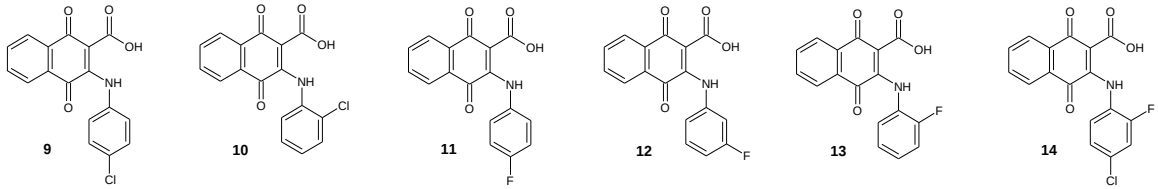


Figure 4. Aminonaphthoquinones synthesised at pH 6.0.

A proposed mechanism for the formation of the aminonaphthoquinones is shown in Figure 5.

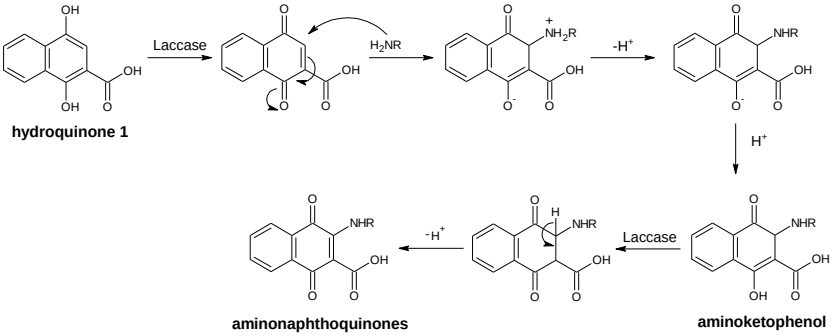


Figure 5. A proposed mechanism for the formation of the aminonaphthoquinones.

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.[37] 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.[37] 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.[38] Amongst these sodium iodate can be used to accomplish amination more conveniently.[38]

A similar synthetic approach to that reported by Chakraborty[37] would have to be attempted to access the aminonaphthoquinones. With our method the aminonaphthoquinones can be accessed in a one-pot synthesis directly from 1,4-dihydroxy-2-naphthoic acid **1**. We have eliminated the use of a chemical oxidant, an iodinated intermediate, a palladium catalyst and a phosphine ligand.

2.2 Anticancer and cytotoxicity evaluation

2.2.1 Anticancer evaluation

Screening was conducted against TK10 (renal), UACC62 (melanoma) and MCF7 (breast) cancer cell lines using the sulforhodamine B (SRB) assay to determine the growth inhibitory effects of the compounds.[39] These cell lines have been used routinely at the U.S. National Cancer Institute for screening for new anticancer agents and were derived from tumors that have different sensitivities to chemotherapeutic drugs.[40] Etoposide, an anti-cancer 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.[41] 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.

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

Entry	Cpd	TK10 (renal)					UACC62 (melanoma)					MCF7 (breast)				
		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	> 100	i	> 100	6.66	p	30.69	w	73.11	42.56	w	> 100	i	> 100
2	**5**	8.38	p	63.55	w	> 100	5.19	p	10.81	m	58.17	37.67	w	> 100	i	> 100
3	**6**	91.10	w	> 100	i	> 100	7.17	p	35.32	w	72.02	54.91	w	> 100	i	> 100
4	**7**	23.68	m	76.22	w	> 100	3.99	p	7.17	p	19.30	15.11	m	53.75	w	92.39
5	**8**	35.85	w	> 100	i	> 100	16.98	m	49.08	w	81.18	43.20	w	> 100	i	> 100
6	**9**	12.95	m	79.02	w	> 100	3.98	p	7.83	p	55.10	17.19	m	55.10	w	93.01
7	**10**	80.74	w	> 100	i	> 100	31.03	m	65.85	w	> 100	63.49	w	> 100	i	> 100
8	**11**	46.55	w	> 100	i	> 100	7.54	p	35.03	w	72.14	38.09	w	98.93	w	> 100
9	**12**	24.68	m	> 100	i	> 100	5.28	p	13.65	m	59.41	9.08	p	48.43	w	91.05
10	**13**	36.85	w	> 100	i	> 100	5.67	p	23.31	m	65.41	9.84	p	50.78	w	92.15
11	**14**	13.40	m	93.27	w	> 100	4.17	p	7.94	p	40.40	21.40	m	54.93	w	88.46
12	Etoposide	7.19	p	49.74	w	> 100	0.89	p	52.71	w	> 100	0.56	p	> 100	i	> 100

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.

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 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.

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 the other compounds were inactive like etoposide. The best activity was exhibited by **12** (TGI = 48.43 µM, Entry 9).

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).

Compound **5** having the isopropyl substituent on the phenyl ring exhibited potent activity against both the TK10 and UACC cell lines. The two fluorinated compounds, **12** and **13** exhibited potent growth inhibitory activity against both the UACC and MCF7cell 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 UACC 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.

2.2.2 Cytotoxicity evaluation

The sulforhodamine B (SRB) assay was used to evaluate the cytotoxic effects of selected compounds on a HeLa cell line.[39] Emetine was used as a positive control.

Table 4. In vitro cytotoxicity screening of the compounds against HeLa cells expressed as GI_{50}, TGI and LC_{50} values (µM).

Entry	Cpd	HeLa cells				
		GI_{50}	Activity	TGI	Activity	LC_{50}
1	**4**	49.17	w	97.25	w	> 100
2	**5**	38.26	w	73.04	w	> 100
3	**6**	72.50	w	> 100	i	> 100
4	**7**	15.95	m	49.14	w	82.33
5	**8**	37.31	w	70.68	w	> 100
6	**9**	24.75	m	55.44	w	86.14
7	**10**	28.03	m	60.28	w	90.23
8	**11**	36.19	w	67.31	w	98.42
9	**12**	28.03	m	60.28	w	92.53
10	**13**	47.54	w	96.99	w	> 100
11	**14**	25.18	m	55.52	w	85.85
12	Emetine	< 0.01	h	0.06	h	0.64

Compound = cpd; Low hazard, l: GI_{50} or TGI > 100 µM; Weak hazard, w: > 30 µM GI_{50} or TGI and < 100 µM; Moderate hazard, m: < 30 µM GI_{50} or TGI > 10 µM; High hazard, h: GI_{50} or TGI < 10 µM; Value > 100 indicates absence of activity.

50% of the growth inhibition (GI_{50}), the total growth inhibition (TGI) and the lethal concentration that kills 50% of cells (LC_{50}), were determined. The results of this investigation are shown in Table 4.

The compounds are moderate to weak hazards based on the GI_{50} values and weak hazards or inactive based on the TGI values. The lethal concentrations of these compounds are much higher (LC_{50} > 82.00 μM) than that of emetine which is a high hazard (LC_{50} = 0.64 μM, Entry 10) and thus they have much weaker cytotoxic effects.

When comparing the GI_{50}, TGI and LC_{50} concentrations of these compounds obtained against the cancer cell lines with that obtained against the HeLa cell line, it can be seen that much lower concentrations of the compounds are required to inhibit growth and also to kill the cancer cells, particularly the UACC62 cell line. This is evident for **4**-**7**, **9**, **11** and **13**-**14** which display a degree of selectivity for the UACC62 cell line over the HeLa cell line. Compound **6** had the best selectivity, it is about 10-fold more selective for the UACC62 cell line over the HeLa cell line.

The aminonaphthoquinones were most effective against the UACC62 (melanoma) cancer cell line exhibiting potent. 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). The cytotoxic effects of the aminonaphthoquinones were moderate to weak against a normal HeLa cell line.

3. Conclusions

A new green method of synthesis was developed for the synthesis of aminonaphthoquinones by using laccase, a non-hazardous oxidizing 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 while their cytotoxic effects were moderate to weak against a normal HeLa cell line. These results are encouraging for further studies of the aminonaphthoquinones for their application in anticancer therapy.

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 Ug^{-1}), was obtained from Novozymes SA.

4.3 Synthetic Methods

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

Method A

Novozym 51003 (1.0ml) was added to a mixture of 1,4-dihydroxy-2-naphthoic acid (0.6 mmol), amine (1.2 mmol, 2 equivalents), succinate-lactate buffer (2.0mL, 1.0M, pH 4.5), water (2.0mL) and DMF (1.0mL) at 35^{o}C. More enzyme (1.0mL) was added after 6h. 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.

Method B

Novozym 51003 (1.0ml) was added to a mixture of 1,4-dihydroxy-2-naphthoic acid (0.6 mmol), amine (1.2 mmol, 2 equivalents), succinate-lactate buffer (2.0mL, 1.0M, pH 4.5), water (2.0mL) and DMF (1.0mL) at 35^{o}C. More enzyme (1.0mL) was added after 2h, 4h and 24h. 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.

Method C

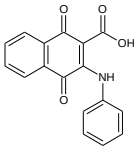
Novozym 51003 (1.0ml) was added to a mixture of 1,4-dihydroxy-2-naphthoic acid (0.6 mmol), amine (1.2 mmol, 2 equivalents), succinate-lactate buffer (2.0mL, 1.0M, pH 4.5),

water (2.0mL) and DMF (1.0mL) at 35^{o}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.

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 equivalents), succinate-lactate buffer (2.0 mL, 1.0 M, pH 4.5), water (2.0 mL) and DMF (1.0 mL) at 35^{o}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.

Method E
Novozym 51003 (1.0 mL) was added to a mixture containing amine (1.8 mmol, 2 equivalents), 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^{o}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.

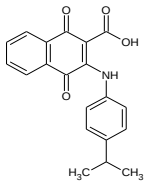
1,4-Dioxo-3-(phenylamino)-1,4-dihydronaphthalene-2-carboxylic acid 4



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

Method E
Heating time = 48h. Purification by flash chromatography (silica: DCM-Hexane, 1:2, 1:1; DCM) to afford a dark brown solid (0.2208g, 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^{o}C. ^{1}H NMR (200 MHz, $CDCl_3$): δ = 7.16 (2H, d *J* = 8.0Hz, ArH), 7.40 (3H, m, ArH), 7.65-7.88 (2H, m, ArH), 7.92 (1H, d *J* = 7.4Hz, ArH), 8.24 (1H, d *J* = 7.6Hz, 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.

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



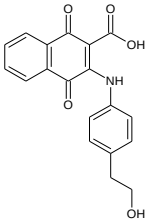
Method A

Heating time = 26h. Purification by flash chromatography (silica: $CHCl_3$; MeOH-$CHCl_3$, 1: 100 and 1:50) to afford a dark-brown solid (0.1394g, 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^{o}C. ^{1}H NMR (200 MHz, $CDCl_3$): δ = 1.29 (6H, m, 2 x 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, ArH), 8.25 (1H, d *J* 7.6, 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.

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 (0.094g, 47%).

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



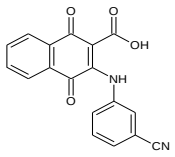
Method A

Heating time = 27h. 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 (0.1027g, 51%).

Method B

Heating time = 72h. 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 (0.1305g, 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^{o}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 6.8Hz, CH_2), 3.91 (2H, t *J* = 6.4 and 6.6Hz, CH_2), 7.11 (2H, d *J* = 8.2Hz, ArH), 7.29 (2H, d *J* = 7.8Hz, ArH), 7.64-7.87 (2H, m, ArH), 7.91 (1H, m, ArH), 8.23 (1H, d *J* = 7.8Hz, 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.

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



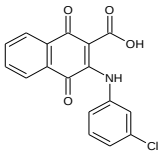
Method C

Heating time = 24h. Purification by flash chromatography (silica: EtOAc-Hexane, 1: 5 1:4, 1:3 and 1:2) to afford a dark-brown solid (0.0607g, 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^{o}C.

Method E

Heating time = 48h. Purification by flash chromatography (silica: EtOAc-Hexane, 1:2, 1:1; EtOAc; MeOH-EtOAc, 1:50, 1:30) to afford an orange-brown solid (0.0982g, 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 ^{o}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* = 7.6Hz, 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.

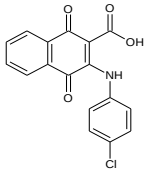
3-[(3-Chlorophenyl)amino]-1,4-dioxo-1,4-dihydronaphthalene-2-carboxylic acid 8



Method C

Heating time = 24h. Purification by flash chromatography (silica: MeOH-$CHCl_3$, 1: 49 1:39 and 1:19) to afford a dark-brown solid (0.1046g, 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^{o}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.

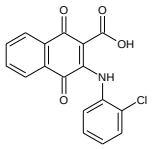
2-Acetyl-3-[(4-chlorophenyl)amino]naphthalene-1,4-dione 9



Method E

Heating time = 48h. Purification by flash chromatography (silica: DCM-Hexane, 1:2, 1:1; DCM) to afford a brown solid (0.0737g, 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^{o}C. ^{1}H 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.

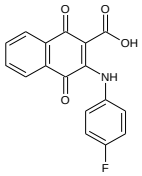
3-[(2-Chlorophenyl)amino]-1,4-dioxo-1,4-dihydronaphthalene-2-carboxylic acid 10



Method E

Heating time = 48h. Purification by flash chromatography (silica: DCM-Hexane, 1:2, 1:1.5; 1:1; DCM) to afford a brown solid (0.0853g, 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^{o}C. ^{1}H 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* = 7.6Hz, ArH), 8.24 (1H, d *J* = 7.6Hz, 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.

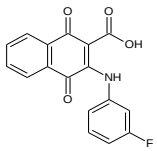
3-[(4-Fluorophenyl)amino]-1,4-dioxo-1,4-dihydronaphthalene-2-carboxylic acid 11



Method E

Heating time = 48h. Purification by flash chromatography (silica: DCM-Hexane, 1:2, 1:1.5, 1:1; DCM) to afford a brown solid (0.1358g, 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^{o}C. ^{1}H 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* = 7.6Hz, ArH), 8.23 (1H, d *J* = 7.2Hz, 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.

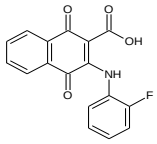
3-[(3-fluorophenyl)amino]-1,4-dioxo-1,4-dihydronaphthalene-2-carboxylic acid 12



Method E

Heating time = 48h. Purification by flash chromatography (silica: DCM-Hexane, 1:2, 1:1; DCM) to afford a dark-brown solid (0.0797g, 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^{o}C. ^{1}H NMR (200 MHz, $CDCl_3$): δ = 6.94 (2H, t *J* = 8.2 and 11.4Hz, ArH), 7.08 (1H, t *J* = 8.0, and 6.8Hz, ArH), 7.39 (2H, q *J* = 8.0 and 6.2Hz, ArH), 7.65-7.89 (2H, m, ArH), 7.93 (1H, d *J* = 6.6Hz, ArH), 8.23 (1H, d *J* = 7.4Hz, 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.

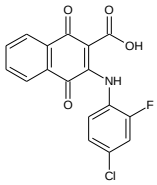
3-[(2-Fluorophenyl)amino]-1,4-dioxo-1,4-dihydronaphthalene-2-carboxylic acid 13



Method E

Heating time = 48h. Purification by flash chromatography (silica: DCM-Hexane, 1:2, 1:1.5, 1:1; DCM) to afford a red-brown solid (0.1119g, 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^{o}C. ^{1}H NMR (200 MHz, $CDCl_3$): δ = 7.08 (4H, m, ArH), 7.66-7.89 (2H, m, ArH), 7.95 (1H, d *J* = 7.6Hz, 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.

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



Method E

Heating time = 48h. Purification by flash chromatography (silica: DCM-Hexane, 1:6, 1:4; 1:3 and 1:2) to afford a red-brown solid (0.0947g, 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^{o}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

Assay background

The growth inhibitory effects of the compounds were tested in a 3-cell line panel consisting of TK10 (renal), UACC62 (melanoma) and MCF7 (breast) cancer cells using the Sulforhodamine B (SRB) assay.[39] 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.

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 37ºC, 5% CO_2, 95% air and 100% relative humidity in RPMI containing 5% fetal bovine serum, 2 mM L-glutamine and 50µg/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 (0.01-100µM). 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 10mM 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:

$$[(Ti-T0)/(C-T0)] \times 100 \text{ for concentrations at which } Ti \geq T0$$
$$[(Ti-T0)/T0] \times 100 \text{ for concentrations at which } Ti < T0.$$

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

The biological activities were separated into 4 categories: inactive (GI_{50} or TGI > 100µM), 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.

4.5. In vitro cytotoxicity activity evaluation

Assay background

The cytotoxic effects of the compounds were tested using the Sulforhodamine B (SRB) assay on a HeLa cell line. The SRB assay was developed by Skehan and colleagues to measure drug-induced cytotoxicity and cell proliferation.[39] The SRB Assay is performed at the CSIR in accordance with the protocol of the Drug Evaluation Branch, NCI, and the assay has been adopted for this screen.

Materials and method

The HeLa cell line (Human Negroid Cervix Epitheloid Adenocarcinoma, ECACC) was routinely maintained as a monolayer cell culture at 37ºC, 5% CO_2, 95% air and 100% relative humidity in EMEM 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 7 000 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 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 (0.01-100µM). Cells without drug addition served as control. The blank contains complete medium without cells. Emetine 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 10mM 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 48 h period of exposure to the 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:

$$[(Ti-T0)/(C-T0)] \times 100 \text{ for concentrations at which } Ti \geq T0$$
$$[(Ti-T0)/T0] \times 100 \text{ for concentrations at which } Ti < T0.$$

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

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

Acknowledgements

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References

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