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New insights into 3-(aminomethyl)naphthoquinones: evaluation of cytotoxicity, electrochemical behavior and search for structure-activity correlation

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ABSTRACT

Herein we describe the structure-activity relationship of a large library of Mannich bases (**MBs**) synthesized from 2-hydroxy-1,4-naphthoquinone. In general, the compounds have shown high to moderate activity against the HL-60 (promyelocytic leukaemia) cell line with IC_{50} = 1.1-19.2 μM. Our results suggest that the nature of the aryl moiety introduced in the structure of **MBs** by the aldehyde component is crucial to the cytotoxicity, and although the group originated from the primary amine has a lesser influence, aromatic ones were found to suppress the activity. Thus, **MBs** derived from salicylaldehydes or 2-pyridinecarboxaldehyde and aliphatic amines are the most active compounds. A satisfactory correlation of the E_{pIIc} *vs.* IC_{50} (μM) in dimethylsulfoxide was observed. The most cytotoxic **MBs** (**Series a-c**, derived from salicylaldehydes) showed the least negative E_{pIIc} values. Noteworthy, however, **Series d** (derived from 2-pyridinecarboxaldehyde) did not follow this correlation. They exhibited both the lowest IC_{50} and the most negative E_{pIIc} values, thus suggesting that other factors also influence the cytotoxicity of the **MBs**, such as lipophilicity, electronic distribution and hydrogen bonding.

Quinones are known for their wide range of pharmacological properties.[1] Naphthoquinones and anthraquinones, for instance, are active against parasites,[2] bacteria and fungi,[3] and their antitumor properties[4,5] have attracted increasing research interest. These compounds have been shown to act via oxidative stress, topoisomerases inhibition and DNA alkylation or intercalation, eventually causing strand breaks.[5,6]

3-(Aminomethyl)-2-hydroxy-1,4-naphthoquinones known as Mannich bases (**MBs**), constitute an interesting class of naphthoquinones that were first synthesized in the 40s by Leffer and co-workers who also described their antimalarial activity.[7] They are obtained by the condensation reaction of 2-hydroxy-1,4-naphthoquinone (lawsone), a non-enolizable aldehyde and a primary or secondary amine.[7,8] Methodologies for the synthesis of **MBs** derived from anilines and heterocyclic amines have also been reported using metal ions as catalysts.[9,10]

We have reported the bactericidal[11] and antiviral[12,13] profiles of novel **MBs**, which have shown very good activities. Furthermore, **MBs** derived from 2-pyridinecarboxaldehyde exhibited moderate to high activities against some tumor cell lines.[14] Their respective platinum(II) and platinum(IV) complexes were also evaluated and in some cases they were more active than cisplatin, one of the most commonly used drugs in chemotherapy.[14,15] It was also found that the **MBs** were more active than their respective platinum(II) complexes against most cell lines, even though the latter were found to interact strongly with DNA and induce DNA strand breaks *in vitro*.[16] The higher cytotoxicity of the **MBs** has been proposed to be due to deamination with formation of an *ortho*-quinone methide, which may cause DNA strand breaks and cell death.[16]

Continuing our efforts to obtain other **MB** derivatives with improved cytotoxicities, we have synthesized a series of novel 3-(R-amino-(R')-methyl)-2-hydroxy-1,4-naphthoquinones and

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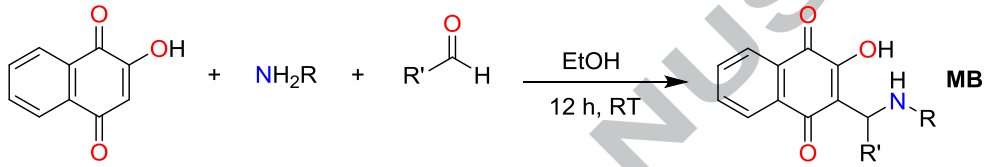
compared their cytotoxic activity against melanoma (MDA-MB-435), promyelocytic leukaemia (HL-60), colorectal adenocarcinoma (HCT-8) and glioblastoma (SF-295) with those of analogous compounds reported previously.[14] To look for possible correlations between their redox properties and cytotoxic activities, the electrochemical behavior of these molecules was evaluated in DMSO, a non-aqueous aprotic solvent, which has been suggested to mimic the cell membrane environment.[17]

The synthesis of the **MBs** was carried out following a procedure described previously and illustrated in Fig. 1A.[11] Except for **MBs 4a**, **5a**, **8a**, **4b**, **5b**, **4c**, **5c**, **4d-8d**, **9e**, **5g**, **4h** and **5h** the compounds are novel. The **MBs** were isolated as analytically pure orange powders with yields ranging from 33-94%. They are all stable in the solid state, but undergo slow decomposition in solution (MeOH, EtOH, $CHCl_3$ and DMSO) when left for long periods of time. This process is probably related to the deamination of the **MBs**, extensively discussed in the literature.[18] The compounds were formulated based on elemental analysis and their structures, confirmed by spectroscopic data (see Experimental, Supplementary data).

The ^{1}H NMR spectra show similar profiles. Hydrogens *H5-H8* are observed in the δ 8.0-7.0 range as dd or ddd for *H5* and *H8*, and as td for *H6* and *H7*. A singlet attributed to *H11* is also observed around δ 6.0-5.5, except for compounds of **Series i** where this singlet is around δ 4.0. The aromatic hydrogens (*H13-H17*) are observed in the expected region and were attributed to the respective hydrogens of the aryl or pyridyl groups based on coupling constant values (*J*) and ^{1}H-^{1}H COSY experiments.[11] The aliphatic peaks are the most shielded signals at δ 4.0-1.0. The spectra of the **MBs** containing *Boc*-protected amines exhibit a singlet attributed to the three methyl groups, at δ 1.41-1.48. The ^{13}C (APT) NMR spectra show all the expected peaks (see Supplementary data).

(A) *General Scheme*



(B) *Structures of the MB of* ***Series a-i***

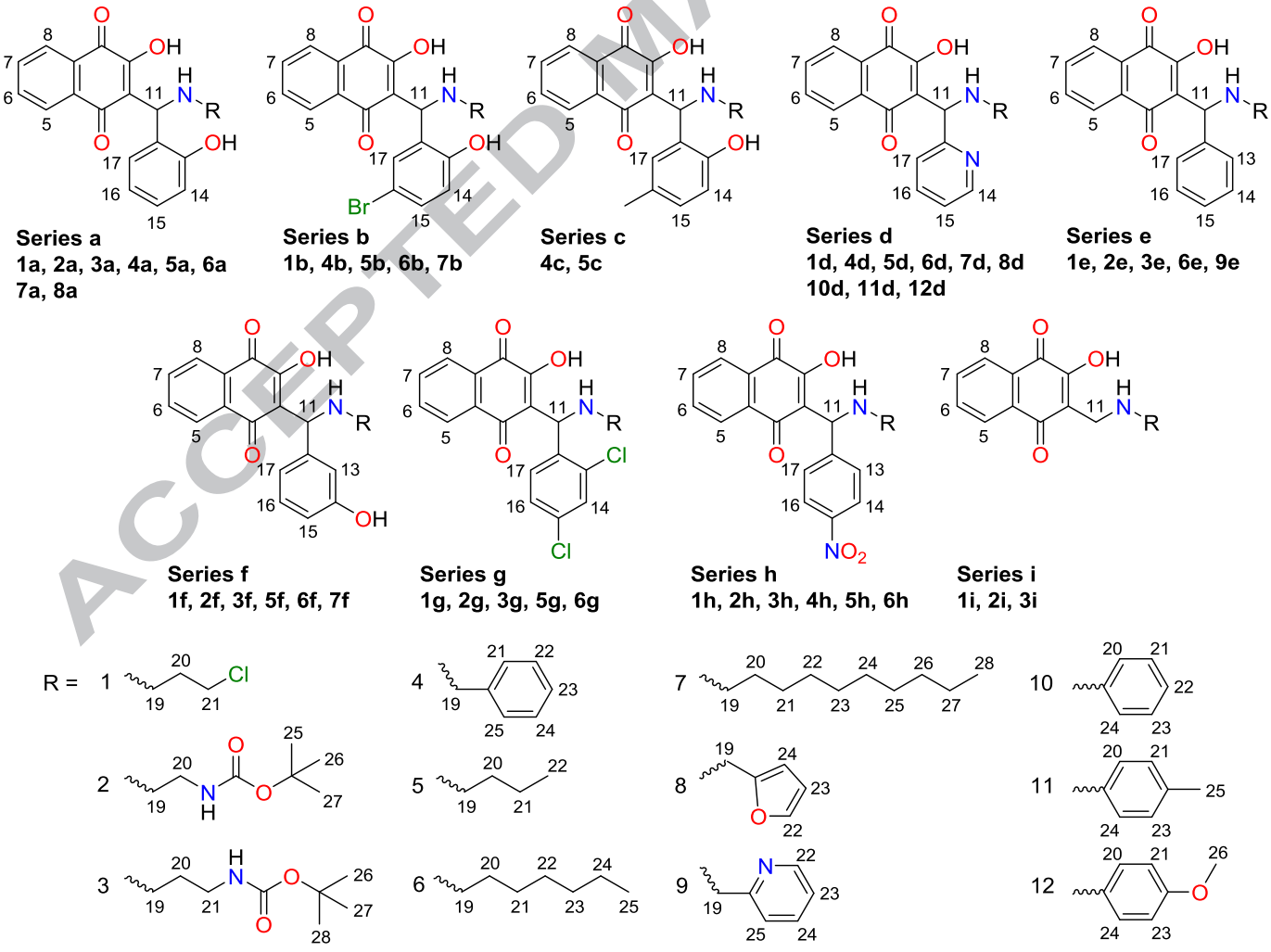


Figure 1. (A) General procedure for the preparation of 3-(R-amino-(R')-methyl)-2-hydroxy-1,4-naphthoquinones and (B) structures of the synthesized **MBs** (**Series a-i**).

Similarly the IR spectra exhibit the expected bands for the synthesized **MBs**[19] (see Supplementary data). The UV-Vis spectra show the same pattern for all compounds, with three bands, λ_1 = 270-280 nm, λ_2 = 330-340 nm and λ_3 = 460-470 nm, attributed, respectively, to π–π* transitions of the benzene and naphthoquinone rings, naphthoquinone π–π* transition and carbonyl groups n–π* transition.[20-22]

The cytotoxicity of the **MBs** was investigated and compared to the data described previously for compounds **4d-8d**.[14] Aiming to improve their solubility in water, some **MBs** (**4a**, **5a**, **8a**, **4b**, **5b**, **4c**, **5c**, **9e**, **5g**, **4h** and **5h**)[11,23] were converted into their hydrochloride forms upon treatment with acetyl chloride. The **MBs.HCl** were obtained in quantitative yields and formulated based on elemental analysis data. Nevertheless they were not soluble enough in water and therefore the cytotoxicity assays of all compounds were undertaken in 1% DMSO (DMSO concentration did not exceed 1% in the highest drug concentration solution, see Experimental, Supplementary data).

All **MBs** described in Fig. 1 have been tested, but only those that have shown cytotoxic activity (IC_{50} < 40 μmol L^{-1}) against the tested tumor cell lines are shown in Table 1, which also contains compounds **4d-8d**[14] for comparison. The results show that the activity of the **MBs** is mostly dependent on the nature of the substituent on C11. Thus, the **MBs** derived from benzaldehyde (**Series e**), 3-hydroxybenzaldehyde (**Series f**), 2,4-dichorobenzaldehyde (**Series g**), 4-nitrobenzaldehyde (**Series h**) and formaldehyde (**Series i**) are inactive.

In general, **MBs** of **Series a** (derived from 2-hydroxybenzaldehyde) and **d** (derived from 2-pyridinecarboxaldehyde) are the most active. Furthermore, the highest activities are associated with aliphatic substituents R on the nitrogen atom, whereas the presence of aromatic substituents (R = C_6H_5, 4-Me-C_6H_4, 4-OMe-C_6H_4) leads to inactive **MBs** (**10d-12d**).

On the whole the compounds in Table 1 are active against all cell lines except for **4d-8d** (**Series d**, derived from 2-pyridinecarboxaldehyde) that are not active against MDA-MB-435. However, these **MBs** present the highest activity against the other cell lines, with IC_{50} ranging from 1.0 to 12.5 μmol L^{-1}.[14] The large majority of **MBs** derived from 2-hydroxybenzaldehyde (**Series a**, **b** and **c**) are active against the four cell lines, and in these cases, with the lowest IC_{50} values against HL-60 (ranging from 1.1 to 19.2 μmol L^{-1}). The presence of a methyl substituent in *para* position with respect to the OH group (**Series c**) had no appreciable effect on the activity compared with analogous compounds of **Series a**. On the other hand, a Br substituent in the same position (**Series b**) leads to marked decrease in the activity (Tables S1-S4). The position of the hydroxyl group on the phenyl ring is crucial, as the **MBs** derived from 3-hydroxybenzaldehyde (**Series f**) are mostly inactive. These results are in accordance with previous studies on the cytotoxicity of some of the compounds of **Series a**, **b**, **c**, **e**, **g** and **h** against MDBK (Madin-Darby Bovine Kidney Epithelial) cells.[12]

Because the **MBs** of **Series a** and **d** present the largest number of active compounds, a possible correlation between activity and nature of the substituent R was examined. In **Series a**, benzyl (**4a**) and furfuryl (**8a**) derivatives were by far the most active ones (IC_{50} from 1.1 to 20.2 μmol L^{-1}) against MDA-MB-435 and HL-60 cell lines (Table 1). Although the benzyl and furfuryl derivatives of **Series d** also present high activity, a noticeable increase in activity with the length of the R chain (*n*-butyl < *n*-heptyl ~ *n*-decyl) is observed against HCT-8, SF-295 and HL-60 cell lines. Previous work suggested a correlation with increased uptake associated with higher lipophilicity.[16] Finally, changing the alkyl chain (*n*-butyl, 3-chloropropyl and 3-(*N*-Boc-amino)-propyl) does not lead to appreciable changes in the activity.

Conversion of the **MBs** into their hydrochlorides (**MBs.HCl**) does not result in increased cytotoxicity in the cases of inactive **MBs** (*e.g.* **Series g** and **h**, Tables S1-S4). Among the **MBs** that present good to moderate activity, *e.g.* those containing R = benzyl (compounds **4a-c**), no improvement is noted for their hydrochlorides. However, in few cases marked enhancement of cytotoxicity is observed upon conversion into the **MBs.HCl**. *E.g.*, **9e** (derived from benzaldehyde and R = pyridin-2-ylmethyl) is inactive against all cell lines except for HCT-8 (IC_{50} = 14.3 ± 0.7 μmol L^{-1}), whereas **9e.HCl** is active against all cell lines with IC_{50} values varying from 3.4 to 13.5 μmol L^{-1}. For **MBs** derived from 2-hydroxybenzaldehydes and R = furfuryl (**8a**) and butyl (**5b-c**), appreciable enhancement of cytotoxicity is noted for **8a.HCl**, **5b.HCl** and **5c.HCl**. As suggested earlier by our group, the cytotoxicity of lawsone derived **MBs** may be associated with their ability to undergo deamination[18,23] in the biological medium (pH 7.4) and form *ortho*-quinone methide species,[16] whose toxic effects are known (*e.g.* through covalent modification of proteins and/or DNA). In phenolic derivatives, the deamination process is known to take place *via* a pre-equilibrium with formation of the *zwitterion*.[24] It is possible that the deamination rate of **MBs.HCl** is altered by the protonation.

As naphthoquinone derivatives are redox active molecules and in principle the mechanism of cytotoxicity could be associated with their redox processes,[1] the electrochemical behavior of the **MBs** was studied by cyclic voltammetry. Measurements were carried out in DMSO, which has been proposed to mimic the cell membrane environment.[17] The cyclic voltammograms (CVs) of compounds **4d-8d** and **9e** were previously reported in MeOH and MeCN.[14,15,23] However, because the nature of the solvent is known to strongly affect the electrochemical behavior of the **MBs,**[25] these compounds were also studied in DMSO.

In general the CVs of the **MBs** in DMSO exhibit three main processes (see Fig. 2A). The first one is assigned to the reduction of 3-(aminomethyl)-2-hydroxy-1,4-naphthoquinone (**NQOH**) into the respective semiquinone – **NQOH**$^{\cdot}$ (irreversible process **Ic/Ia**), which can deprotonate a **NQOH** molecule, affording a protonated semiquinone (**NQOH**$_2^{\cdot}$), which in turn can be reduced to a protonated cathecol – **CATOH**$_2^{\cdot}$ (irreversible process – **Ic*/Ia***). Finally, the *quasi*-reversible process **IIc/IIa** is attributed to the redox processes of the naphthoquinonate (**NQO**$^-$) generated from the autoprotonation (Fig. 3).[26,27] An additional process (**IIIc/IIIa**) is observed in the CVs of the **MBs** derived from 4-nitrobenzaldehyde (**1-6h**) and is attributed to the reduction/oxidation of the nitro group.[28] Differently, the CVs of the **MBs** derived from 2- and 3-hydroxybenzaldehydes (**Series a-c** and **f**, *e.g.*, see Fig. 2B) exhibit an additional redox process (**IIc*/IIa***), which showed dependence on the scan rate and is associated to the presence of the OH group (possibly involving hydrogen bonding or deprotonation processes).[25,29]

Table 1. Selected data of IC_{50} values of **MBs** in μmol L^{-1} after 72 h of incubation.

MB	R	R'	MDA-MB-435	HCT-8	SF-295	HL-60
1a	3-chloropropyl	2-hydroxyphenyl	30.1 ± 8.0	17.7 ± 2.3	17.5 ± 5.3	8.1 ± 2.6
2a	2-(*N*-Boc-amino)ethyl	2-hydroxyphenyl	26.3 ± 4.6	10.8 ± 7.0	15.9 ± 2.0	19.2 ± 1.1
3a	3-(*N*-Boc-amino)propyl	2-hydroxyphenyl	37.8 ± 2.8	22.7 ± 6.2	26.0 ± 3.2	17.2 ± 1.6
4a	benzyl	2-hydroxyphenyl	18.9 ± 0.8	15.6 ± 3.6	23.1 ± 0.2	3.9 ± 1.2
5a	*n*-butyl	2-hydroxyphenyl	25.6 ± 9.6	20.5 ± 3.6	33.3 ± 7.6	6.5 ± 0.4
6a	*n*-heptyl	2-hydroxyphenyl	33.3 ± 2.7	19.7 ± 6.3	18.9 ± 1.9	7.7 ± 3.0
7a	*n*-decyl	2-hydroxyphenyl	30.9 ± 3.5	32.2 ± 2.3	23.1 ± 2.2	12.2 ± 1.5
8a	furfuryl	2-hydroxyphenyl	20.2 ± 2.4	14.4 ± 2.3	23.4 ± 3.7	1.1 ± 0.1
1b	3-chloropropyl	5-bromo-2-hydroxyphenyl	>40	>40	28.8 ± 5.1	8.4 ± 3.3
4b	benzyl	5-bromo-2-hydroxyphenyl	25.2 ± 4.6	14.9 ± 2.3	17.4 ± 0.4	8.4 ± 0.2
4c	benzyl	5-methyl-2-hydroxyphenyl	15.8 ± 2.8	11.3 ± 2.7	8.5 ± 0.6	7.2 ± 1.1
5c	*n*-butyl	5-methyl-2-hydroxyphenyl	26.5 ± 5.7	17.0 ± 4.5	29.6 ± 7.1	6.0 ± 1.1
4d	benzyl	2-pyridyl	>40[a]	2.2 ± 0.1[a]	1.3 ± 0.1[a]	4.8 ± 1.3[a]
5d	*n*-butyl	2-pyridyl	>40[a]	12.5 ± 0.8[a]	9.5 ± 0.2[a]	>40[a]
6d	*n*-heptyl	2-pyridyl	>40[a]	1.6 ± 0.6[a]	1.0 ± 0.4[a]	1.8 ± 0.2[a]
7d	*n*-decyl	2-pyridyl	23.3 ± 3.9[a]	1.7 ± 0.2[a]	1.7 ± 0.6[a]	3.8 ± 1.3[a]
8d	furfuryl	2-pyridyl	>40[a]	4.4 ± 1.2[a]	3.3 ± 2.3[a]	5.8 ± 0.6[a]
9e	pyridin-2-ylmethyl	phenyl	>40	14.3 ± 0.7	>40	>40
7f	*n*-decyl	3-hydroxyphenyl	31.1 ± 4.7	25.8 ± 5.6	>40	11.9 ± 0.7
3g	3-(*N*-Boc-amino)propyl	2,4-dichlorophenyl	16.2 ± 3.4	21.2 ± 5.6	>40	N.D.
6g	*n*-heptyl	2,4-dichlorophenyl	>40	>40	>40	4.5 ± 0.5
4a.HCl	benzyl	2-hydroxyphenyl	18.3 ± 1.1	14.4 ± 2.5	11.6 ± 2.4	4.6 ± 0.8
8a.HCl	furfuryl	2-hydroxyphenyl	2.9 ± 0.2	3.0 ± 0.8	1.8 ± 0.5	1.2 ± 0.4
4b.HCl	benzyl	5-bromo-2-hydroxyphenyl	>40	15.9 ± 10.2	10.7 ± 0.9	4.9 ± 0.5
5b.HCl	*n*-butyl	5-bromo-2-hydroxyphenyl	23.9 ± 15.2	12.5 ± 0.9	21.4 ± 13.9	6.2 ± 1.3
4c.HCl	benzyl	5-methyl-2-hydroxyphenyl	18.7 ± 2.9	12.1 ± 2.0	14.4 ± 2.8	N.D.
5c.HCl	*n*-butyl	5-methyl-2-hydroxyphenyl	11.0 ± 4.2	5.6 ± 1.3	5.5 ± 1.3	1.6 ± 0.4
9e.HCl	pyridin-2-ylmethyl	phenyl	3.6 ± 0.9	6.3 ± 2.2	13.5 ± 2.7	5.4 ± 0.6
Doxorubicin			0.83 ± 0.03	0.05 ± 0.03	0.39 ± 0.05	0.03 ± 0.01

* Experiments were performed in triplicate and data are presented as mean IC_{50} value ± standard deviation of the mean of 3.

[a]These data were previously reported in reference 14.

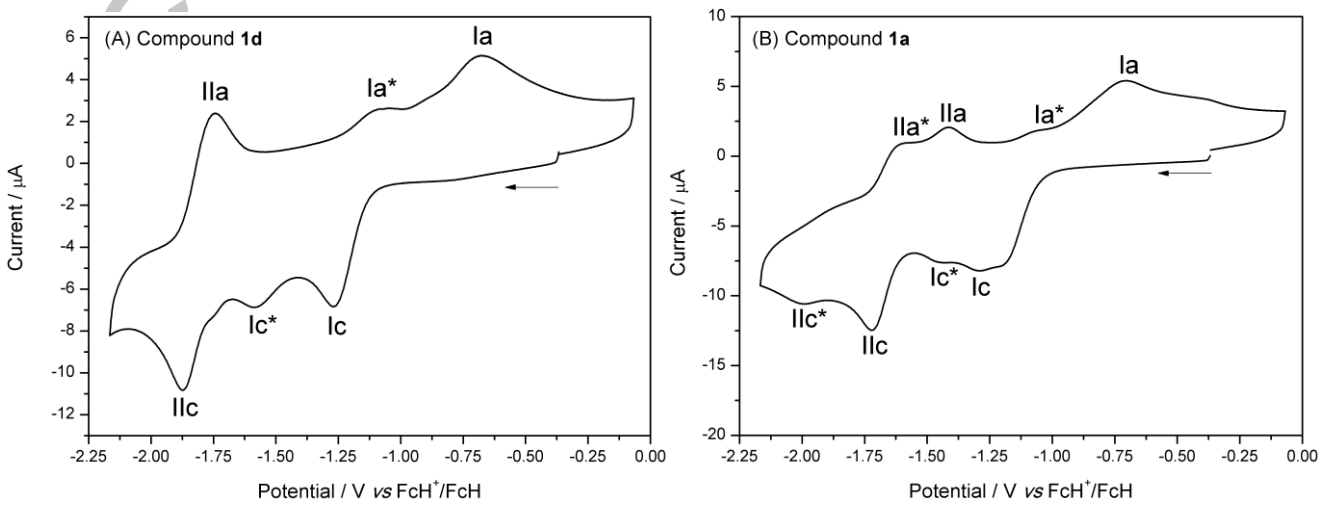


Figure 2. CVs of compounds (A) **1d** and (B) **1a** at scan rate of 0.200 V s^{-1}.

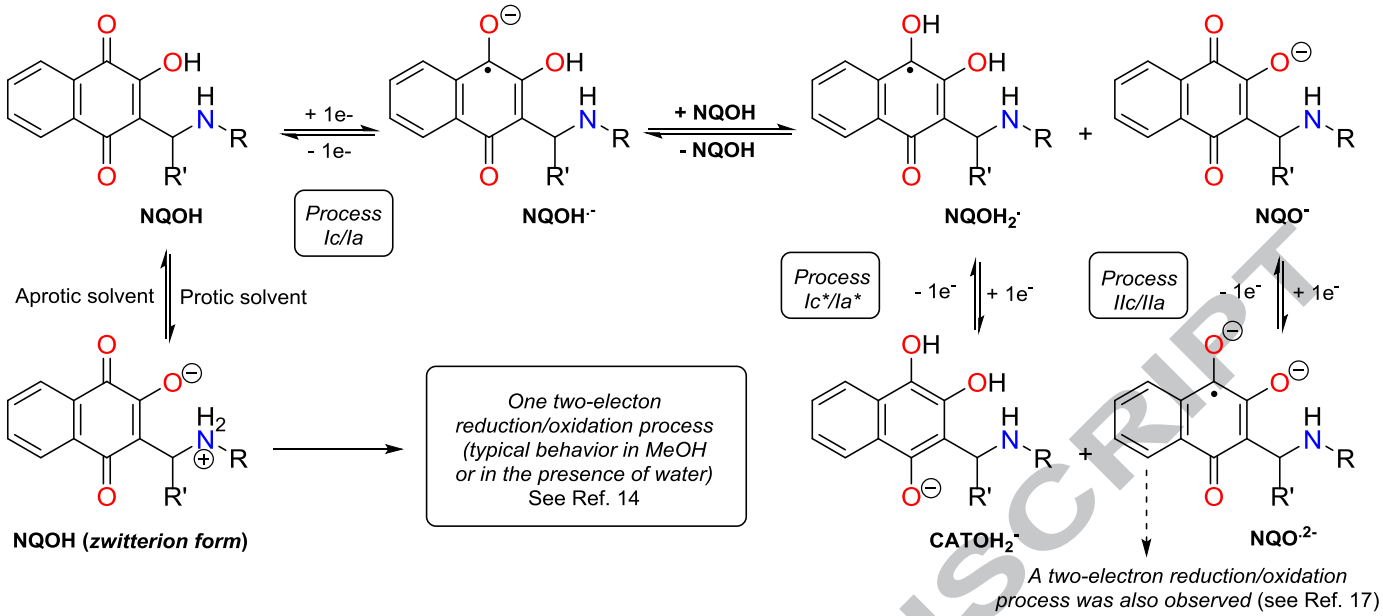


Figure 3. Redox behavior for **MBs** in aprotic solvents.

Attempts have been made to establish a correlation between the first reduction potential (E_{pIc}) and the molluscicidal activity (LD_{50} and LD_{90} values) of 2-hydroxy-naphthoquinones.[17] However, it is known that in these cases, including the **MBs**, autoprotonation strongly affects the kinetics of the first reduction/oxidation process (**Ic**/**Ia**), leading to irreversibility.[30] Thus, in terms of correlation with biological data, reversible or *quasi*-reversible processes, such as **IIc**/**IIa** were shown to be more suitable choices. For example, in another series of 2-hydroxynaphthoquinones, a correlation was found between strong inhibitory effect on the fertilization of sea urchin eggs (LD_{50}) and highest reduction potentials and similarly, low activity was related with the lowest reduction potentials.[30]

According to the data shown in Table S5 the E_{pIIc} values for the **MBs** vary from –1.652 to –1.960 V and depend far more on the nature of the substituent on C11 than that on the nitrogen atom. *E.g.*, E_{pIIc} values for compounds derived from 3-(chloropropyl)amine and containing different substituents on C11 (**1a**,**b**,**d-i**, see Fig. 1) vary as much as 227 mV, whereas in one series, for instance, that of compounds derived from 2-hydroxybenzaldehyde **1a-8a**, the E_{pIIc} values vary only by 66 mV.

Plots of the second reduction process (E_{pIIc}) for all **MBs** *vs* IC_{50} values against the four cell lines were obtained (Fig. 4 and Fig S1). Because the best correlation was observed for the HL-60 cell line, against which the compounds have exhibited the highest activities (Fig. 4), this plot will be further discussed.

Of the **MBs** derived from substituted benzaldehydes, those containing the R' = 2-hydroxyphenyl substituent (**Series a-c**) show the least negative E_{pIIc} values ranging from –1.652 to –1.765 V and are also the most active (IC_{50} from 1.1 to 19.2 μmol L^{-1}), except for the 5-bromo-2-hydroxybenzaldehyde derivatives **5b**-**7b** that are inactive. Furthermore, the presence of other substituents on the phenyl ring (**Series e-h**) leads to the most negative E_{pIIc} values (from –1.812 to –1.960 V) and exhibit low cytotoxicity (IC_{50} > 40 μmol L^{-1}), except for compounds **7f** and **6g** that contain the longest aliphatic carbon chains on the nitrogen atom (*n*-decyl and *n*-heptyl, respectively), previously associated with high uptake.[16] The **MBs** of **Series i** derived from formaldehyde, also present negative E_{pIIc} values (from –1.853 to –1.888 V) and no activity. Thus, in these series a satisfactory correlation was encountered between activity and E_{pIIc} values and follows similar tendency to that previously reported for other 2-hydroxynaphthoquinones.[30]

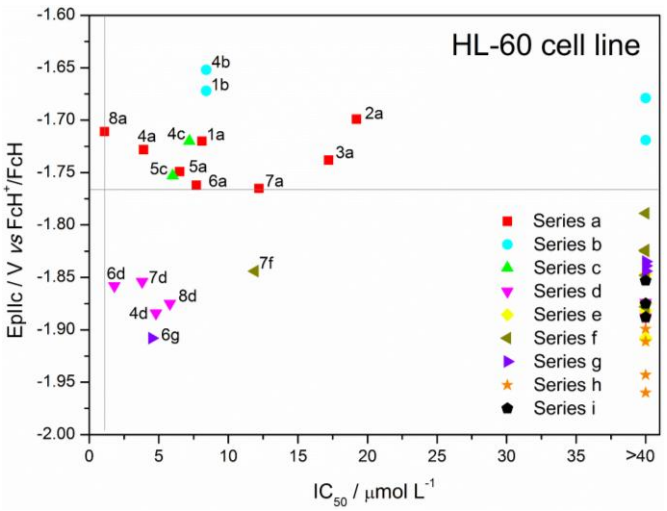


Figure 4. Correlation of the E_{pIIc} (V *vs* FcH^+/FcH) of the **MBs** and the IC_{50} (μmol L^{-1}) against HL-60 cell line.

The **MBs** derived from 2-pyridinecarboxaldehyde, however, appear as a separate group in the plot, with the lowest IC_{50} values (from 1.8 to 5.8 μmol L^{-1}, except for one case) and E_{pIIc} values ranging from –1.854 to –1.888 V). These results indicate that E_{pIIc} values alone do not allow safe prediction of the activity of this class of compounds. Other factors, such as nature of the substituents on C11 and on the nitrogen atom also seem to play important effects on the activity.

In summary, from the structural modifications performed on the naphthoquinone Mannich bases (**MBs**) it was found that their activity depends mostly on the substituent on C11. The ones

containing a 2-pyridyl group have exhibited the highest activities (IC_{50} from 1.0 to 12.5 μmol L^{-1}), except for the melanoma cell line, thus indicating that this group plays an important role in cytotoxicity. The presence of a hydroxyl group in position 2 of the phenyl substituent on C11 (derived from 2-hydroxybenzaldehyde) is also important for the cytotoxicity, leading to compounds with moderate to high activities. Differently, other substituents on C11, such as C_6H_5 (**Series e**), 3-OH-C_6H_4 (**Series f**), 2,4-Cl-C_6H_4 (**Series g**), 4-NO_2-C_6H_4 (**Series h**) and H (**Series i**) mostly resulted in inactive compounds. The nature of the R substituent on the nitrogen atom also has important consequences in the activity of the **MBs**. Aromatic substituents (R = C_6H_5, 4-Me-C_6H_4 or 4-OMe-C_6H_4) gave completely inactive **MBs**, thus indicating that cytotoxicity is associated with aliphatic substituents R, especially the benzyl and furfuryl groups that led to the most active **MBs**. Besides, increasing the length of the alkyl chain on the nitrogen atom also resulted in increased activities (*n*-butyl << *n*-heptyl ~ *n*-decyl), which may be attributed to the greater cellular accumulation of these derivatives. Conversion of the **MBs** into their hydrochlorides (**MBs.HCl**) may result in improvement of cytotoxicity, especially of the **MBs** with good to moderate activities. Finally, a satisfactory correlation was encountered between cytotoxicity (IC_{50} values for HL-60 cell line) and the second reduction potential (E_{pIIc} values) of the naphthoquinone **MBs**, except for **Series d** (derived from 2-pyridinecarboxaldehyde). Those with the least negative E_{pIIc} values (**Series a-c** derived from 2-hydroxybenzaldehyde) are also the most active, which could suggest that the ease of reduction of the **MBs** may play a role in their activities. However, **MBs** of **Series d** exhibited the lowest IC_{50} values and yet also the most negative E_{pIIc} values, thus indicating that other factors also influence the cytotoxicity of the **MBs**, amongst which lipophilicity, electronic distribution and hydrogen bonding as a result of the nature of the substituents on C11 and on the nitrogen atom.

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Graphical Abstract

New insights into 3-(aminomethyl)-naphthoquinones: evaluation of cytotoxicity, electrochemical behavior and search for structure-activity correlation

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