Chapter 7

Structure-activity relationship of naphthoquinones

Chapter 7
Structure-activity relationship of naphthoquinones

7.1 Introduction

The aim of any structure-activity relationship study should be to find the most potent and least toxic compound. To be able to find such a compound a structure-activity relationship (SAR) study must be carried out. Crum-Brown and Fraser suspected in 1868 that the ammonium character of the arrowhead poison, curare, was responsible for its paralytic properties. They tested various ammonium salts and quaternized alkaloids in animals and from this data concluded that the physiological action of a compound was a function of its chemical constitution. These observations were the basis for the study area of structure-activity relationships (Silverman, 2004).

Compounds (drugs) can be classified into structurally specific and structurally non-specific drugs. The specific drugs act at a specific site such as a receptor or enzyme. Small changes in their molecular structure have a large influence on their potency. Furthermore, molecules with similar biological activities tend to have common structural features. Non-specific drugs have no specific target and they tend to have lower potency. Similar biological activities might be caused by a variety of structures. The aim of SAR studies is therefore to synthesise as many analogs as possible from the lead compound and testing the effect the structure has on the potency as well as the toxicity.

Several structurally related compounds (isolated, synthesised or commercially obtained) have been tested for potency against TB to determine the active site (pharmacopore) of the lead compound. In total thirteen compounds have been tested for activity (with the use of the radiometric BACTEC bioassay) and for toxicity with the use of vero cells.

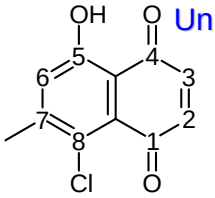
7.2 Materials and methods

7.2.1 MIC determination

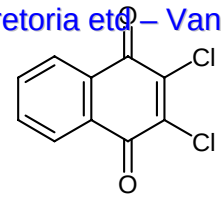
The naphthoquinones used during these experiments included the isolated compounds: diospyrin **(4),** isodiospyrin **(6),** neodiospyrin **(11),** shinanolone **(13),** mamegakinone (isolated from *E. natalensis*) and plumbagin **(12)** (isolated from *Plumbago auriculata*). The synthetic compounds were 8-chloro-7-methyljuglone **(1)** and 7-methyljuglone **(10).** The compounds obtained from Sigma-Aldrich includes: dichlon **(2),** 2,3-dimethoxynaphthoquinone **(3),** 2-hydroxynaphthoquinone **(5),** juglone **(7),** lapachol **(8)** and menadione **(9)** (Fig. 7.1).

A susceptible strain of *Mycobacterium tuberculosis* H37Rv, obtained from the American Type Culture Collection (Rockville, MD, USA), was used to investigate the activity of the naphthoquinones. The 7H12 Middlebrook TB medium (Middlebrook *et al.,* 1977) used during these studies, consisted of an enriched Middlebrook 7H9 broth base supplemented with bovine serum albumin, catalase, casein hydrolysate and ^{14}C-labelled substrate (palmitic acid) as a source of carbon. Growth of the mycobacterium leads to the consumption of the carbon source, with subsequent release of labelled $^{14}CO_2$. This labelled CO_2 moves into the atmosphere above the medium in the sealed vial and the BACTEC TB 460 instrument detects the amount of $^{14}CO_2$ and records it as a growth index (GI) on a scale of 0-999 (Heifets and Good, 1994).

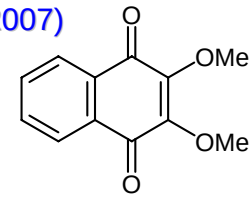
For the MIC determination, a vial containing 7H12 Middlebrook TB medium was inoculated with homogenised cultures in a special diluting fluid (Middlebrook-Dubos 7H9 broth having the no.1 McFarland standard optical density). When growth in this vial reached a GI reading of 400-500, the 7H12 broth culture was used undiluted to inoculate a set of vials. The isolated compounds, dissolved in dimethylsulfoxide (DMSO), were added into the vials to give a final concentration of 1 % DMSO. The positive drug controls ethambutol, izoniazid and rifampicin and a 1 % DMSO control were used.



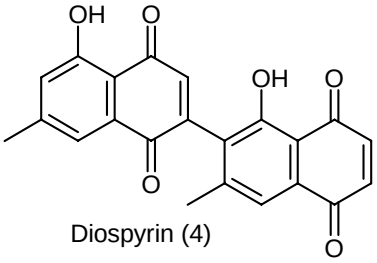
Chloro-7-methyljuglone (1)



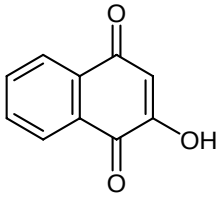
Dichlon (2)



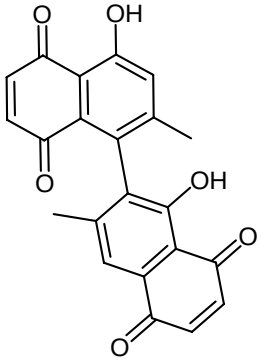
2,3-dimethoxynaphthoquinone (3)



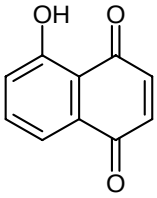
Diospyrin (4)



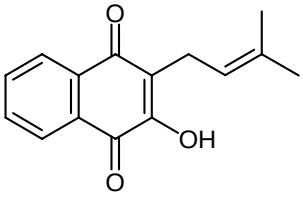
2-hydroxynaphthoquinone (5)



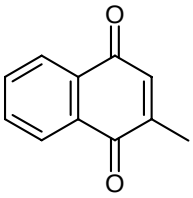
Isodiospyrin (6)



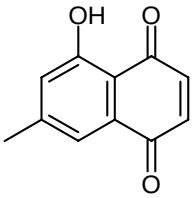
Juglone (7)



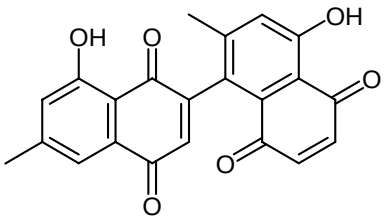
Lapachol (8)



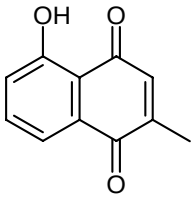
Menadione (9)



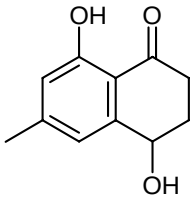
7-methyljuglone (10)



Neodiospyrin (11)



Plumbagin (12)



Shinanolone (13)

Fig. 7.1: The chemical structures of the compounds used for SAR studies. The numbering system is indicated for chloro-7-methyljuglone.

Inoculated vials were incubated at 38 ºC and each vial was assayed daily at the same hour until cumulative results were interpretable. The GI value of the control vial was compared with the readings from the vials containing the compounds. The control vial contains a 1:100 dilution of the inoculum and when it reached a reading of 30 the readings were stopped. The difference in GI readings for the last two days (ΔGI) was used. If ΔGI readings of any of the compounds were less than the control vial, that compound was considered to be active. All compounds were tested in triplicate at concentrations of 0.5, 1.0, 5.0, 10.0, 25.0, 50.0, 100.0µg/ml.

7.2.2 Toxicity bioassay

The toxicity bioassay on vero cells was done as described in Chapter 5.

7.3 Results

7.3.1 MIC and toxicity determination

The results of the anti-TB and toxicity of the naphthoquinones are given in Table 7.1 and are expressed in nM/ml. The MIC's of the positive controls: isoniazid, rifampicin and ethambutol were 0.45, 0.151 and 61.27 nM/ml respectively.

Table 7.1: The MIC and toxicity of naphthoquinones used during this study in nM/ml

Compound	MIC	IC50	Selectivity index
(1)	17.90	11.01	0.62
(2)	440.23	128.20	0.29
(3)	458.30	257.73	0.56
(4)	21.40	41.44	1.93
(5)	574.18	287.36	0.50
(6)	26.73	80.21	3.00
(7)	5.75	6.90	1.20
(8)	41.30	413.20	10.00
(9)	29.07	37.97	1.30
(10)	2.66	6.54	2.46
(11)	26.74	86.04	3.20
(12)	5.32	16.49	0.59

(13)	335.00	173.30	0.52

7.3.2 Structure - activity relationship

Fig. 7.1 illustrates the chemical structures of the naphthoquinones. The structure of the naphthoquinones shows that the double ketone motif on carbon 1 and 4 is needed for potency. The activity declines dramatically when the ketone is reduced to the corresponding hydroxyl group (e.g. MIC of **(13)** = 335 nM/ml). The aromaticity of the naphthalene ring also influences the potency, with the reduction of the aromatic ring the activity declines sharply (shinanolone). The presence of the hydroxyl group confirs activity. By blocking carbon 2 and 3 the possibility that a Michaels adduct can form is reduced. Fig. 7.2 indicates the mechanism of a Michaels adduct.

The conjugate nucleophilic addition to α-β-unsaturated carbonyl groups play an important role, and it might be due to interaction via this mechanism with thiol groups (nucleophilic groups) on proteins, that cause the toxicity of these compounds (McMurray, 1996; Lipinsky, personal communication). All the compounds with functional groups on both carbons (C2 and C3) have IC50 values of 128.20 nM/ml and higher. The only compound that showed good activity with these two carbons blocked is lapachol. The other two compounds **((2)** and **(3))** lost their activity as well as their toxicity.

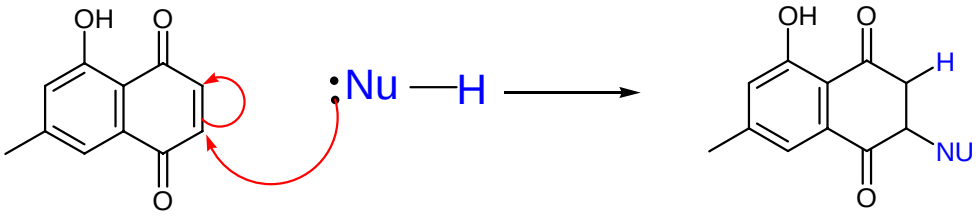


Fig. 7.2. The mechanism of a Michael's reaction.

7.4 Discusion and conclusions

In general the toxicity increases when the activity increases, which would indicate that the pharmacophore and the toxic site are similar. The active site may however be the 1,4 ketone motif with the toxic site the unsaturated unblocked carbon 2 and 3. Due to the above results we are currently in the process of synthesising more compounds with positions C2 and C3 blocked. This will decrease the probability that a Michaels adduct can form while the redox potential of the ketone groups remain the same.

Lapachol is one of the compounds that showed the most promise. The compound will be derivatised further with the addition of hydroxyl functional groups at different positions which might increase its activity further. The instability of the compounds in the toxicity tests and the potency bioassays are a cause for concern. This might yield inaccurate results. The high hydrophobicity of the NQ's increases the problem even further. In the next chapter the possible mode of action is discussed with the redox potential of the ketone groups as the main active mechanism.

The main aim of the SAR studies in future will be to find a compound that will only interact with menaquinone (occurring in mycobacterium) and not with ubiquinone (occurring in mammals). Therefore the structure would have to be modified further in order to enhance the specific activity and to decrease the toxicity.

7.5 References

Heifets, L.B. & Good, R.C. (1994). Current laboratory methods for the diagnoses of tuberculosis. In: Tuberculosis: pathogenesis, protection and control, ed. B.R. Bloom, pp 85-108. ASM Press, Washington, DC.

McMurry, J. (1996). Organic chemistry. 4th ed. pp 914-917, Brookes/Cole Publishing, USA.

Middlebrook, G., Reggiards, Z. & Tigertt, W.D. (1977). Automable radiometric detection of growth of *Mycobacterium tuberculosis* in selective media. *American Review of Respiratory Diseases*. 115: 1067-1069.

Silverman, R.B. (2004). The organic chemistry of drug design and drug action. pp 21-22, Elsevier Academic Press, USA.

University of Pretoria etd – Van der Kooy, F (2007)

Chapter 8

The mode of action of naphthoquinones in *Mycobacterium smegmatis*

University of Pretoria etd – Van der Kooy, F (2007)

Chapter 8

The mode of action of naphthoquinones in *Mycobacterium smegmatis*

8.1 Introduction

The focus of this chapter was on the mechanism of action of our lead compound, 7-methyljuglone in *Mycobacterium smegmatis*. The non-pathogenic *M. smegmatis* is usually used as an indicator species in laboratories that are not equipped to culture the pathogenic *M. tuberculosis*. Another species that are sometimes used as a substitute is *M. aureum*. The possible implication of using indicator species, is that the lead compound might not show any activity, as is the case with the known TB drug Izoniazid. Izoniazid shows very good activity against *M. tuberculosis,* but is not active against *M. smegmatis*. The compounds should therefore first be tested for activity against *M. smegmatis,* before continuing with the mode of action experiments.

No literature could be found on the antimycobacterial mode of action of 7-methyljuglone**.** There are however various reports on the biological activity in other organisms e.g. termicidal activity (Carter *et al.,* 1978) and antifungal activity (Steffen & Peschel, 1975). It appears from the literature that the biological activity of this compound is very broad, which will probably classify it as a non-specific binding site "drug". Published reports on the other NQ's showed that diospyrin and isodiospyrin bind or inhibit topoisomerase I and therefore might be useful as anticancer drugs. It is also speculated by Cushion *et al*. (2000) that diospyrin can bind to the mithochondrial electron transport chain. The literature review (Chapter 1) gives a complete list of activities associated with the naphthoquinones while Table 8.1 recaps the most important mode of action publications that could be found for the naphthoquinones. It can be concluded from the literature reviews that the mode of action of the lead compound remains unknown. Structurally similar naphthoquinones do however have speculative mode of actions including respiration inhibition (juglone).

University of Pretoria etd – Van der Kooy, F (2007)

Table 8.1: Mechanism of action associated with naphthoquinones as found in literature

Compound	Mode of Action	Reference
diospyrin	Binds electron transport chain Prevent or reverse Topoisomerase I and DNA complex from forming	Cushion *et al*. (2000) Bailly (2000)
isodiospyrin	Binds topoisomerase I - preventing it from binding to DNA	Ting *et al.* (2003)
plumbagin	Superoxide generator	Wang *et al*. (1998)
juglone	Inhibited respiration in bean and lettuce plants and binds to SH groups of peptides	Li *et al.* (1993)

According to Weinstein et al. (2005), M. tuberculosis is an obligate aerobe but can survive for long periods under low oxygen conditions. Fig. 8.1 illustrates an electron transport chain system. The mycobacterial electron transport chain occurs in the cell wall of M. tuberculosis and consists of 4 complexes. In complex 1, NADH dehydrogenase, receives electrons from FAD and NADH. The redox potential of this complex is lower than that of complex 2. The electrons are transported to the second complex by the mobile menaquinone. Menaquinone becomes reduced to menaquinol and shuttles the electrons to complex 3, the cytochrome bc_1 complex (under anaerobic conditions the electrons are shuttled to the cytochrome bd complex). During the transfer of electrons the menaquinol becomes oxidised back into menaquinone. From the cytochrome bc_1 complex the electrons flow to cytochrome aa_3 and convert O_2 into water. The resulting H^+ gradient phosphorolates ADP to form ATP in the ATP synthase complex (Matsoso et al. 2005).

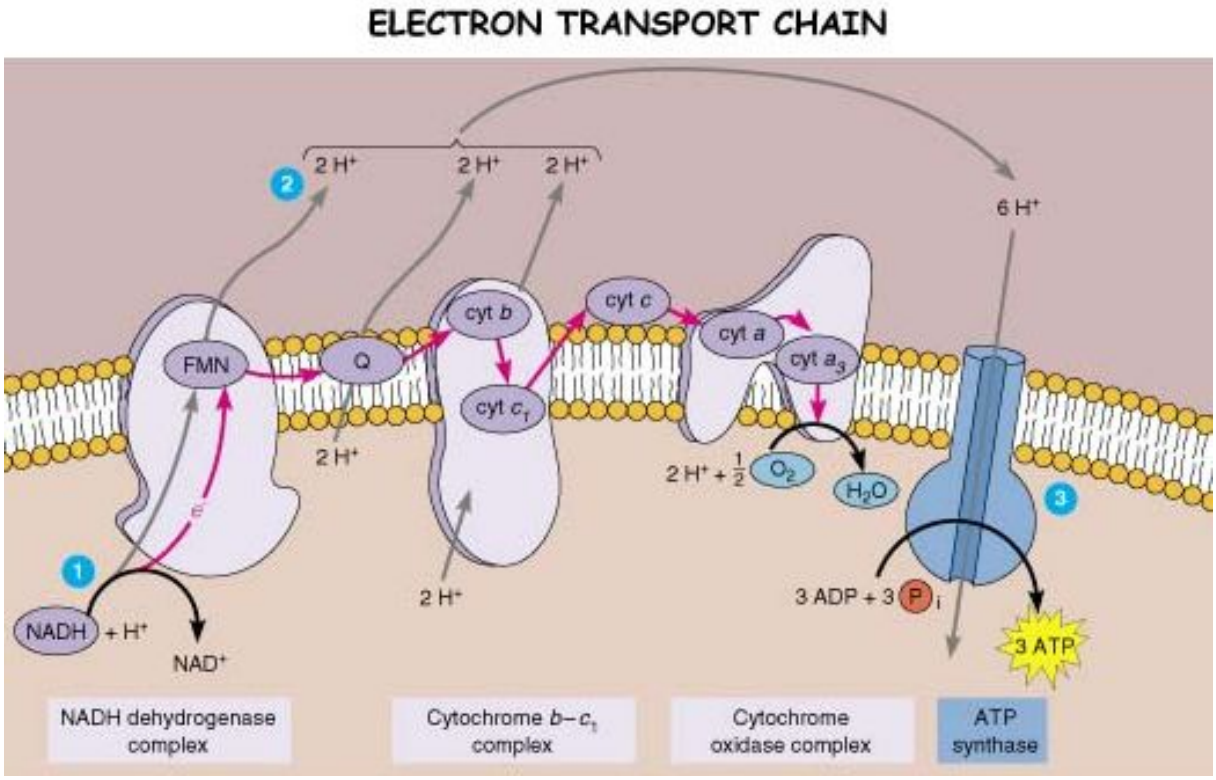


Fig 8.1: The electron transport chain with its different complexes. Q represents ubiquinone. Mycobacterium tuberculosis has menaquinone in this position (http://www.columbia.edu/cu/biology/courses/c2005/handouts/etccomplexes.jpg).

The similarities between 7-methyljuglone and menaquinone shows a possible interference of the electron transport system in mycobacteria (Fig. 8.2). Menaquinone consists of a 1,4-napthoquinone skeleton with two side chains attached on carbon 2 and 3. The lengh of the isoprene unit on carbon two differs between species but consists of 9 units in *M. tuberculosis* (45 carbons). The role of the side chain is to make the compound hydrophobic enough so that it can move inside the cell wall. Ubiquinone (found in humans) consists of a phenolic skeleton with similar side chains on carbon 2 and 3. This might indicate that menaquinone is an attractive drug target because it lacks a human equivalent.

The mode of action might occur in one of three ways. 7-Methyljuglone can enzymatically be incorporated into a menaquinone-like compound with the subsequent interferences in the chain due to an altered redox potential. This can be achieved by competitive binding of 7-methyljuglone to the enzymes producing menaquinone. Fig 8.3 illustrates the formation of menaquinone and where 7-methyljuglone might interfere (indicated with arrows).

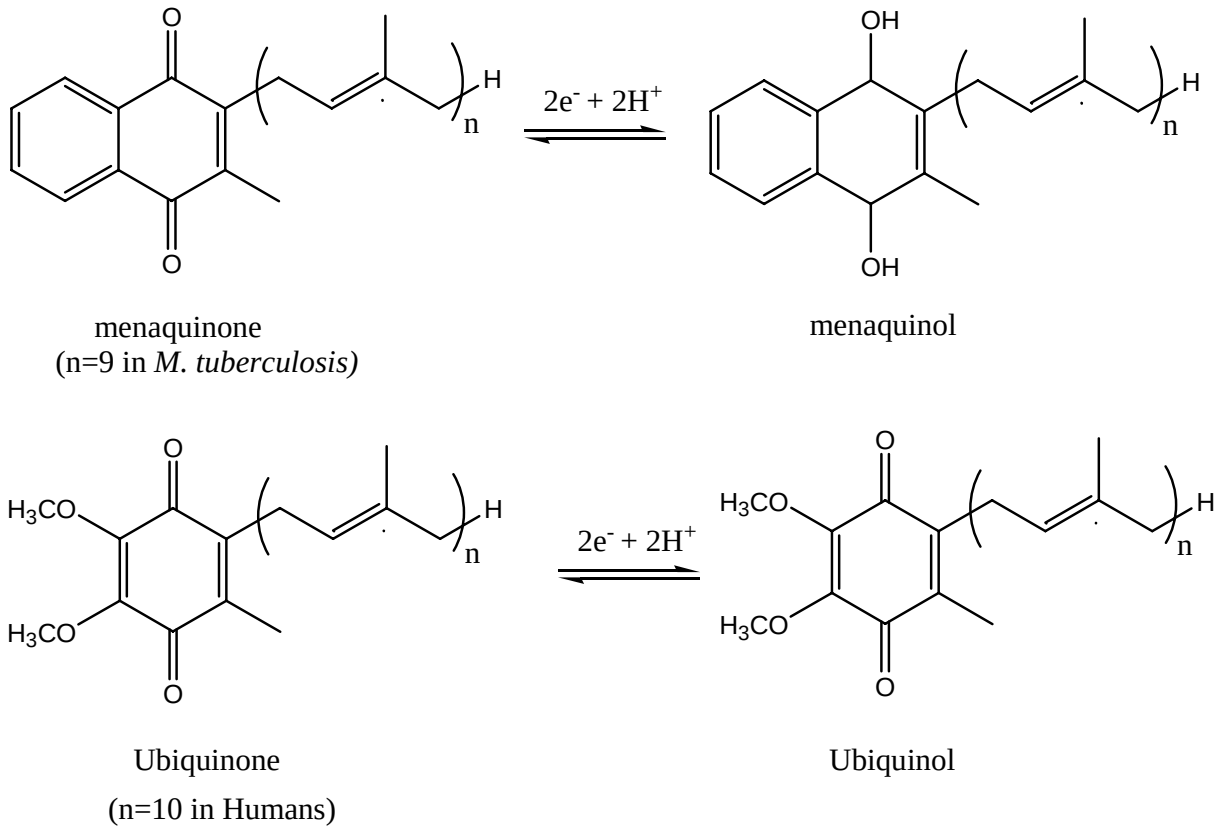


Fig. 8.2: The structural difference between the redox reactions of the mycobacterial menaquinone and ubiquinone found in humans (Truglio *et al.*, 2003)

7-Methyljuglone might compete with *men* A or the *men* G enzyme and thereby either stops the production of menaquinone or it might be enzymatically incorporated into a menaquinone-like compound with the addition of a long carbon sidechain. It might therefore also be possible to stop the addition of the methyl group on carbon three. The result of this interference would be two-fold. Firstly menaquinone production can be stopped or the tempo of production might be influenced. Secondly, 7-methyljuglone can be incorporated into the role of menaquinone. This will have an influence on the rate of electron transport due to a different redox potential that this new compound will have. This will effectively either increase respiration or decrease respiration.

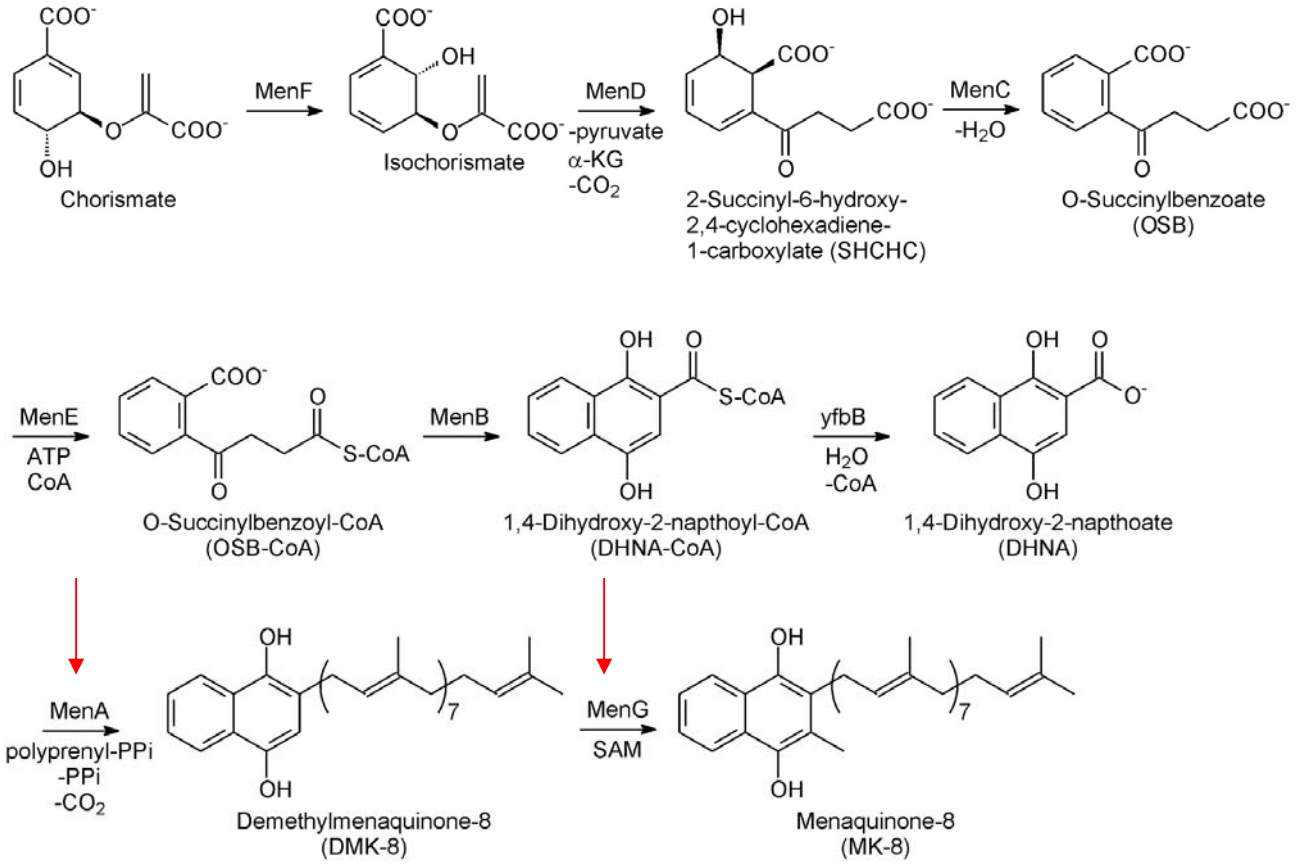


Fig. 8.3 Menaquinone production with the two possible sites of interference indicated with arrows (Truglio *et al*., 2003)

7-Methyljuglone might competitively bind to the menaquinone enzyme, thereby inhibiting menaquinone from forming. This will effectively stop the electron transport chain. 7-Methyljulone might also bind to one of the complexes in the chain and thereby prohibit electrons from flowing down the chain. Either by scavenging electrons from complex 1 or binding to the cytochrome complexes.

During this chapter the influence of some of the NQ's on the mycobacterial electron transport chain was studied in *M. smegmatis.* The mycobacterium was cultured and treated with naphthoquinones. The treated cells as well as a control group was quantitatively extracted and subjected to HPLC analysis.

University of Pretoria etd – Van der Kooy, F (2007)

8.2 Materials and methods

8.2.1 Activity against *M. smegmatis*

The fluorinated 7-methyljuglone, juglone, 7-methyljuglone and plumbagin and the known TB drug izoniazid were chromatographed on normal phase silica gel TLC plates using hexane:ethyl acetate (5:2) as mobile phase. In addition a phase three clinical trial anti-TB drug, moxifloxacin, was also used as a positive control. The volumes of the compounds spotted were 50, 20 and 5 µl of 1 mg/ml solutions. The plates were dried and sprayed with freshly subcultered *M. smegmatis* in 7H9 Middlebrook broth with OADC growth supplement (Begue & Kline, 1972; Eloff, 1998). The plates were incubated at 37 °C for 24 hours after which they were sprayed with INT (2 mg/ml). The plates were incubated for an additional 4 hours before being inspected for activity. The zones in which INT did not give a purplish colour were indicative of activity.

8.2.2 Mechanism of action bioassay

The mycobacterial cultures were obtained from the Medical Research Council in Pretoria. The cultures were sub-cultured in 7H9 Broth (4.5 g / 450 ml) with 2 ml glycerol. The broth was autoclaved and ADC growth supplement added when the broth cooled down to 45 °C. The mycobacteria were placed in a shaker in an incubator at 37 °C in the dark (Weinstein *et al.*, 2005). After 24 hours the mycobacteria were centrifuged at 4000 rpm and the supernatant discarded. Fresh broth was added to the cultures. The sub-culturing was repeated daily for four days in order to obtain a large amount of cells. On day four the medium was removed by centrifugation and the subcultures with fresh medium subdivided into five equal groups. They consisted of a control group and four NQ treated groups. Fresh broth was added while the four NQ's were dissolved in DMSO. The compounds were added to the cultured mycobacteria to achieve a final concentration of 100 µg/ml. The DMSO had a final concentration of 1 %. The control group also contained DMSO.

University of Pretoria etd – Van der Kooy, F (2007)

8.2.3 Extraction of *M. smegmatis* cells

The five cultures were placed on a shaker inside an incubator and after 12 hours they were centrifuged and the amount of cells present in each group weighed, after which it was dried at 100 °C and again weighed (Lester and Crane, 1959). The dried cells were crushed with a mortar and pestle after which they were extracted with dichloromethane and hexane three times each. The extracts were dried on a rotary evaporator and subjected to HPLC analysis.

8.2.4 HPLC analysis

The HPLC system consisted of a Phenomenex RP18 (150 X 4.6mm) column with 100 % acetonitrile as mobile phase (Kroppenstedt, 1982). The column temperature was set to 40 °C with a flow rate of 2 ml/min. The retention time of menaquinone was 25 min. A blank broth sample was injected to see if the broth would cause peak overlap.

8.3 Results

8.3.1 Activity against *M. smegmatis*

The zones of inhibition could clearly be seen on the TLC plates (Fig. 8.4-8.6). INH did not inhibit the growth as was expected.

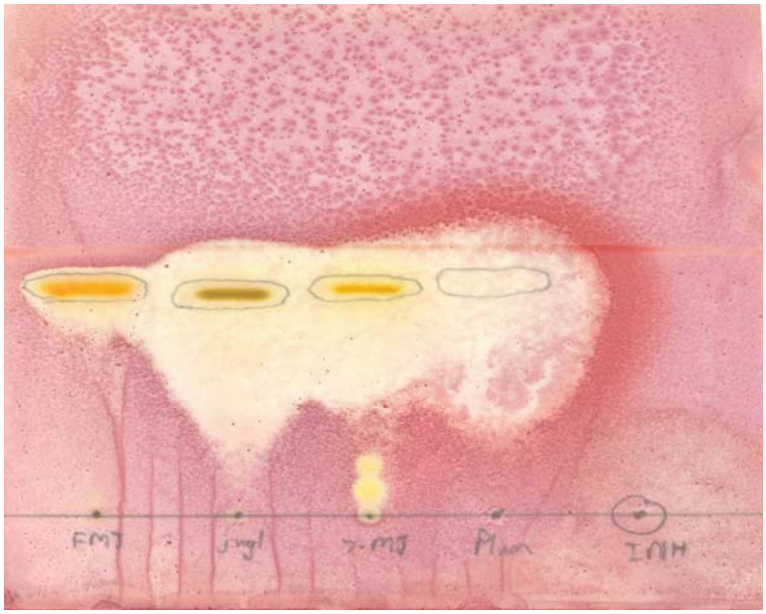


Fig. 8.4: TLC plate with NQ's after spraying with *M. smegmatis*. Lanes 1-5 contain the fluorinated 7-methyljuglone, juglone, 7-methyljuglone, plumbagin and INH at amounts of 50 μg. The compounds are circled in pencil.

University of Pretoria etd – Van der Kooy, F (2007)

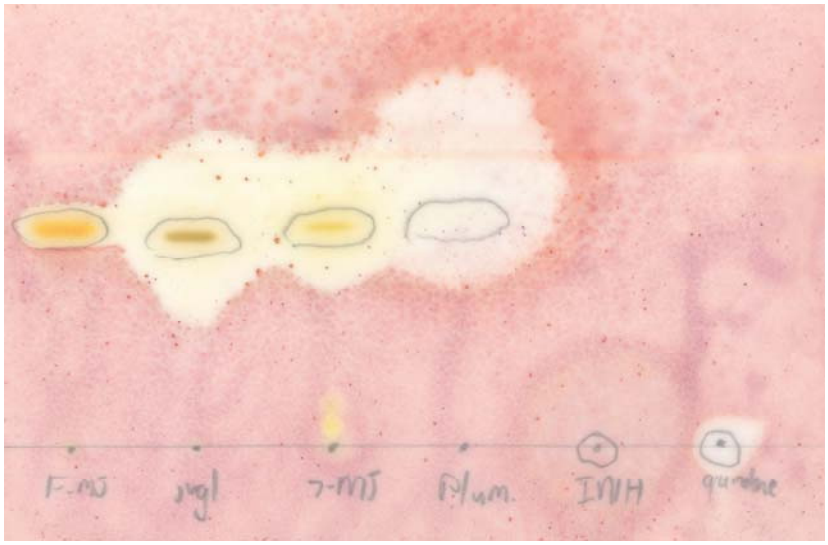


Fig. 8.5: Naphthoquinones developed on a TLC plate sprayed with *M. smegmatis*. Lanes 1-6 contain the fluorinated 7-methyljuglone, juglone, 7-methyljuglone, plumbagin, INH and moxifloxacin at amounts of 20 μg.

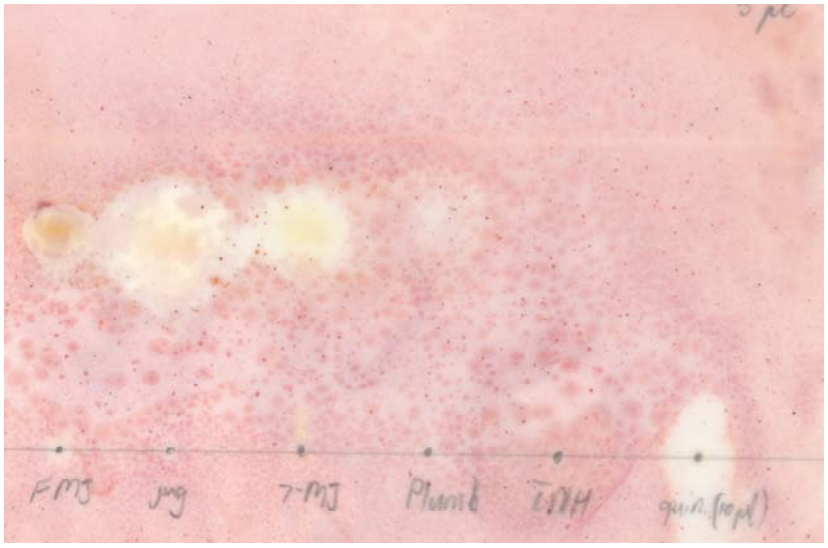


Fig. 8.6: Naphthoquinones developed on a TLC plate sprayed with *M. smegmatis*. Lanes 1-6 contain the fluorinated 7-methyljuglone, juglone, 7-methyljuglone, plumbagin, INH and moxifloxacin at amounts of 5 μg. The compounds are circled in pencil.

The fluorinated 7-methyljuglone has the highest log P value which would indicate that less diffusion through the 7H9 Middlebrook broth occurred. This might be the reason why it has a smaller zone of inhibition on the TLC plate. The difference between the polarity of INH and the naphthoquinones can also clearly be seen. The known drugs INH and moxifloxacin are more hydrophilic and stayed on the base line while the naphthoquinones are more hydrophobic.

8.3.2 *M. smegmatis* cultures

The compounds are stable for the first day after which they gradually break down or precipitate from solution. Therefore it was decided to stop the experiments 12 hours after the addition of the NQ's. The mass of the cells (wet and dry mass) are given in Table 8.2

Table 8.2: The mass of the *M. smegmatis* cells after 12h treatment with NQ's.

NQ	Mass (wet) mg	Mass (dry) mg
plumbagin	384	109
juglone	105	79
7-methyljuglone	181	74
fluorinated 7-methyljuglone	178	52
control	503	116

8.3.3 HPLC analysis

The HPLC chromatograms indicated that menaquinone was present in all the extracts except in the fluorinated 7- methyljuglone. This was clearly visible with the complete absence of the menaquinone peak. Figures 8.7-8.9 show the control, 7-methyljuglone and the fluorinated 7 -methyljuglone chromatograms. It is quite easy to recognise the apparent similarities between the chromatograms. There are four peaks with retention times between 3 and 7 minutes. These peaks are probably the shorter chained menaquinones. The ratio's and intensities between them are very similar and could therefore be used as indicator peaks.

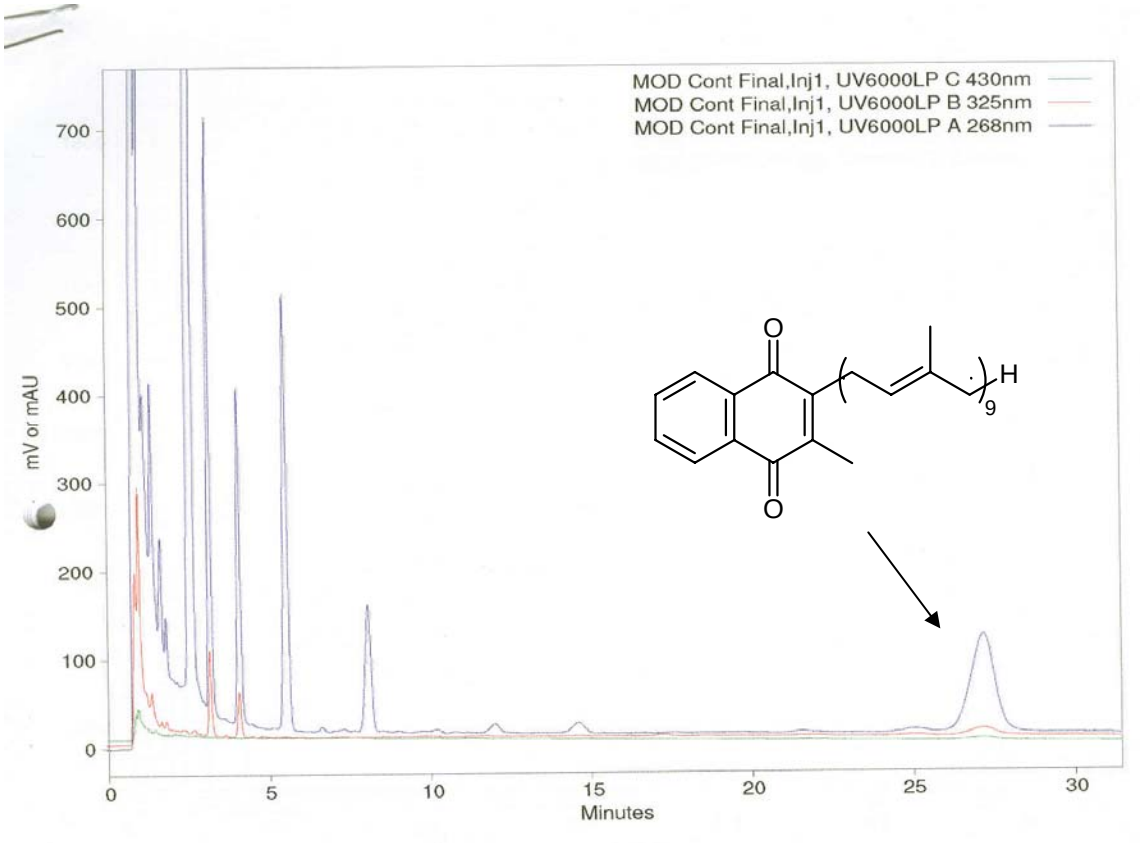


Fig. 8.7: HPLC chromatogram of the control sample indicating the presence of menaquinone at RT 27 min.

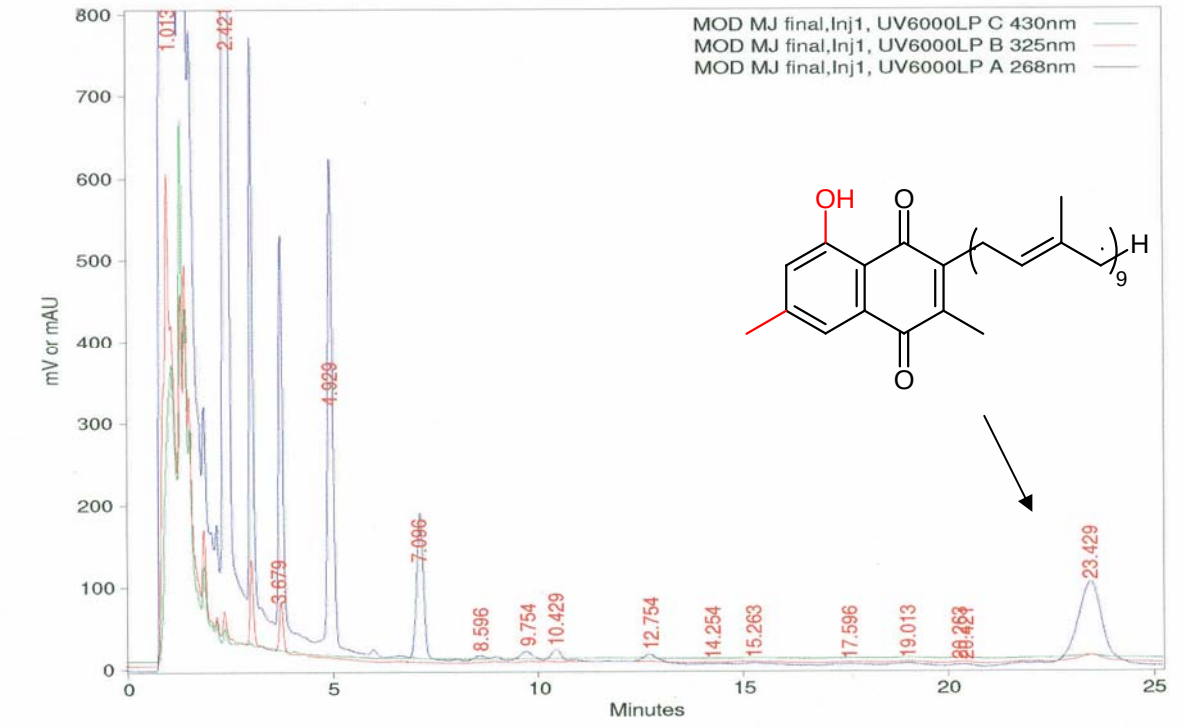


Fig. 8.8: Chromatogram indicating the presence of a menaquinone-like compound in the 7-methyljuglone sample. The retention time might indicate that this is a 7-methyljuglone with a terpenoid side chain.

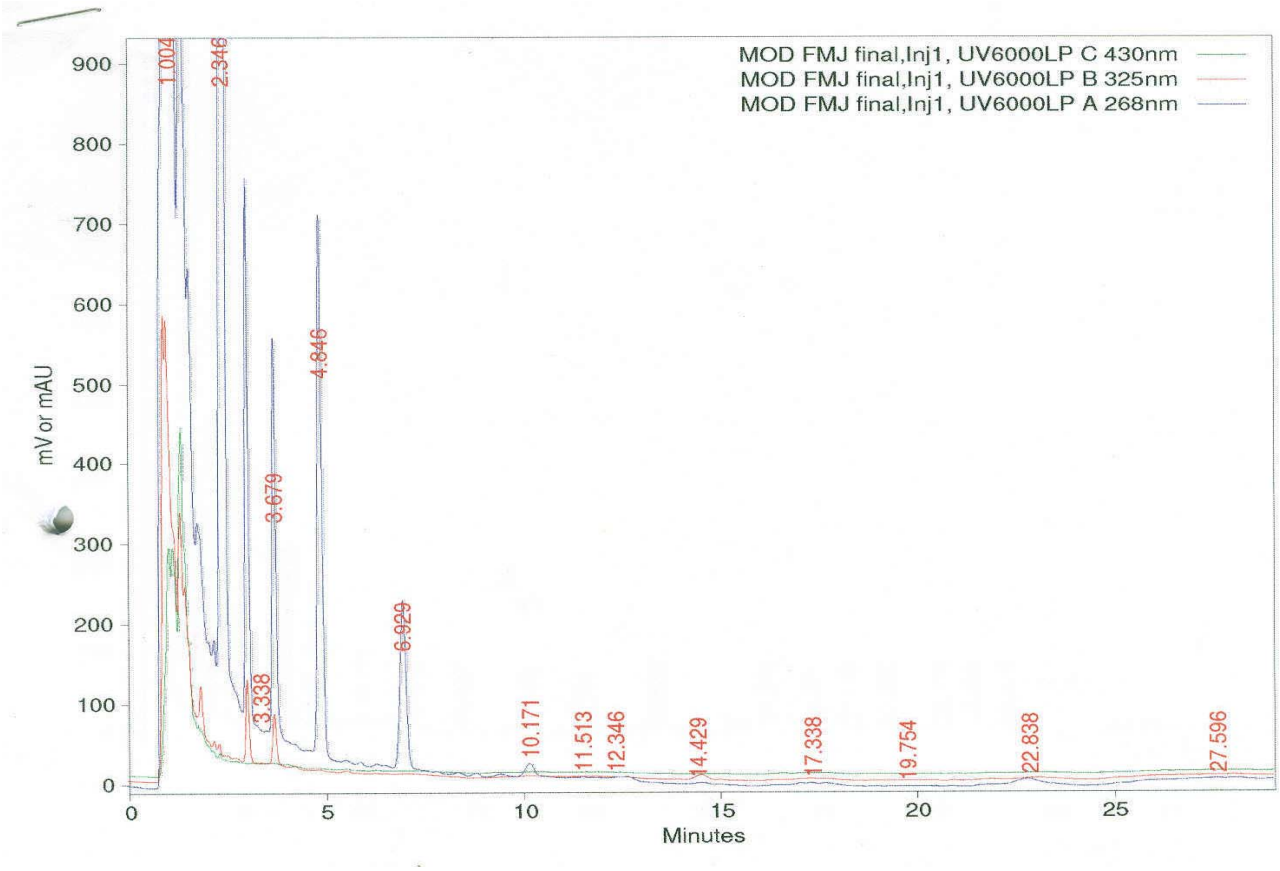


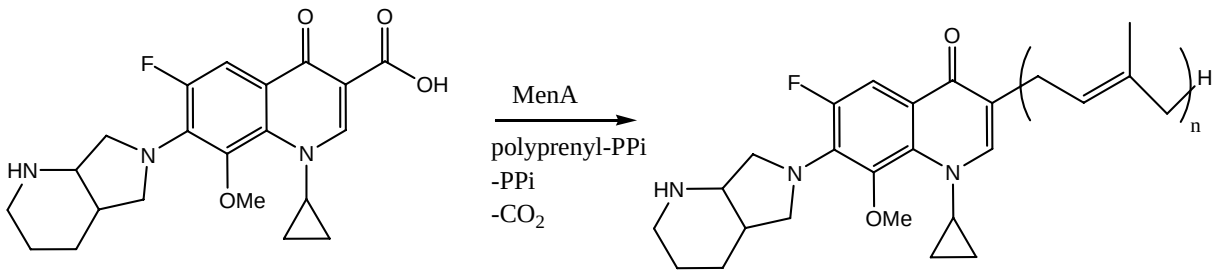
Fig. 8.9: Chromatogram of the fluorinated 7-methyljuglone sample indicating the absence of menaquinone.

8.4 Discussion and conclusions

The naphthoquinones tested on the TLC bioassay showed that the compounds are active against *M. smegmatis* and therefore it can be assumed that the mechanism of action could be the same as in *M. tuberculosis.* The yield of cells after sub-culturing for 5 days appears to be sufficient for one experiment. This must however be increased so that the experiments can be repeated in duplicate or triplicate. The mass of the cells give a good indication of the efficiency or potency of the compounds. Juglone stopped the growth of the cells the fastest while the fluorinated 7-methyljuglone and 7-methyljuglone**,** took slightly longer. The plumbagin sample appeared to be the most inefficient of the four NQ's tested. The HPLC results showed that menaquinone was completely inhibited by the fluorinated 7-methyljuglone. This will most probably be by binding to the men A or men G enzymes in the menaquinone biosynthetic pathway.

In the case of juglone and 7-methyljuglone**,** menaquinone still appeared to be present in the chromatogram. The altered retention time (2-3 minutes shorter RT) of the 7-methyljuglone treated sample might indicate that 7-methyljuglone could be incorporated into a menaquinone-like compound (which will have a reduced retention time due to its slightly more polar nature). This can also indicate that these compounds might bind to cytochrome bc complex or it can interfere with the electrons shuttled from the NADH dehydrogenase complex. This in fact will disrupt respiration with detrimental effect on the organism. This effect was also noticed with juglone and its influence on lettuce (Li *et al.,* 1993). This will influence the rate of respiration due to the altered redox potential that this new compound might have. The rate of menaquinone production and its half-life is however the key in understanding this system.

Currently a similar compound, moxifloxacin, in phase 3 clinical trials exhibits characteristics for this mechanism of action. This compound could interfere with the production of menaquinone at the *men* A biosynthetic step (Fig. 8.10). At the right pH the polyprenyl side chain can be attached to moxifloxacin, catalysed by men A. This hypothesis will be tested in future experiments which will also include 7-methyljuglone and the fluorinated 7-methyljuglone.

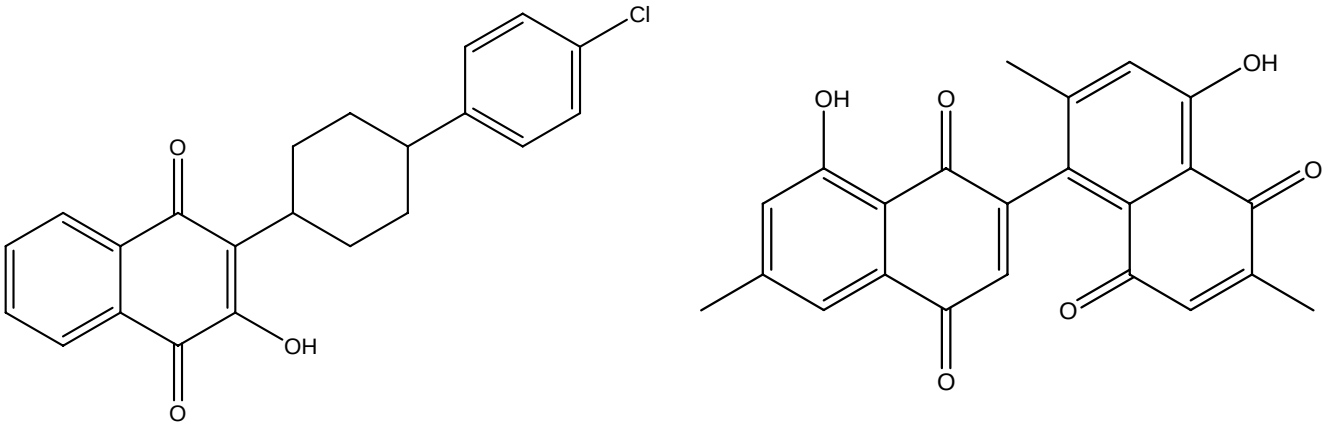


Moxifloxacin prenylated moxifloxacin

Fig. 8.10: Possible mode of action of moxifloxacin if it becomes prenylated through the enzymatic action of MenA, it might cause disruption of the electron transport chain

The structurally similar compound atovaquone (prescribed drug – mepron) is known to bind to the cytochrome *bc* complex in *Plasmodium falcipirum* (Fig. 8.11) (Yeo *et al.*, 1997;

Srivastava *et al*., 1999; Syafruddin *et al.,* 1999). During initial screening in our laboratory (unpublished results) of the structurally similar compound, neodiospyrin, the activity was found to be 60 ng/ml against *P. falcipirum* compared to 63 ng/ml for chloroquine (positive control). This would indicate that these compounds do indeed interfere with the electron transport chain of various organisms. The main challenge is therefore to change the structure of the compounds to such a degree as to make it species specific (and less toxic to people). In order to achieve this goal a large database of structurally similar compounds must be screened against various organisms.



(a) **(b)**

Fig. 8.11: The structural similarities between atovaquone (a) and neodiospyrin (b) indicates that the mode of action might be the same.

8.5 References

Bailly, C. (2000). Topoisomerase I poisons and suppressors as anticancer drugs. *Current Medicinal Chemistry*. 7(1), 39-58.

Begue, W. J. & Kline, R. M. (1972). Use of tetrazolium salts in bioautographic procedures. *Journal of Chromatography*. 64(1), 182-4.

Carter, F.L., Garlo, A.M., Stanley, J.B. (1978). Termicidal components of wood extracts: 7-methyljuglone from *Diospyros virginiana*. *Journal of Agriculture and food chemistry*. 26(4), 869-73.

Cushion, M. T., Collins, M., Hazra, B. & Kaneshiro, E. S. (2000). Effects of atovaquone and diospyrin-based drugs on the cellular ATP of *Pneumocystis carinii* f. sp. *carinii*. *Antimicrobial Agents and Chemotherapy*. 44(3), 713-719.

Eloff, J. N. (1998). Which extractant should be used for the screening and isolation of antimicrobial components from plants? *Journal of Ethnopharmacology*. 60(1), 1-8.

Kroppenstedt, R. M. (1982). Separation of bacterial menaquinones by HPLC using reverse phase (RS18) and a silver loaded ion exchanger as stationary phases. *Journal of Liquid Chromatography*. 5(12), 2359-67.

Lester, R. L. & Crane, F. L. (1959). Natural occurrence of coenzyme Q and related compounds. *Journal of Biological Chemistry*. 234, 2169-75.

Li, H. H., Nishimura, H., Koji, H. & Mizutani, J. (1993). Some physiological effects and the possible mechanism of action of juglone in plants. *Zasso Kenkyu*. 38(3), 214-22.

Srivastava, I. K., Morrisey, J. M., Darrouzet, E., Daldal, F. & Vaidya, A. B. (1999). Resistance mutations reveal the atovaquone-binding domain of cytochrome b in malaria parasites. *Molecular Microbiology*. 33(4), 704-711.

Steffen, K. & Peschel, H. (1975). Chemical constitution and antifungal activity of 1,4-naphthoquinones, their biosynthetic intermediates, and chemically related compounds. *Planta Medica.* 27(3), 201-12.

Syafruddin, D., Siregar, J. E. & Marzuki, S. (1999). Mutations in the cytochrome b gene of *Plasmodium berghei* conferring resistance to atovaquone. *Molecular and Biochemical Parasitology*. 104(2), 185-194.

Ting, C., Hsu, C., Hsu, H., Su, J., Chen, T., Tarn, W., Kuo, Y., Whang-Peng, J., Liu, L. F. & Hwang, J. (2003). Isodiospyrin as a novel human DNA topoisomerase I inhibitor. *Biochemical Pharmacology*. 66(10), 1981-1991.

Truglio, J. J., Theis, K., Feng, Y., Gajda, R., Machutta, C., Tonge, P. J. & Kisker, C. (2003). Crystal Structure of Mycobacterium tuberculosis MenB, a Key Enzyme in Vitamin K2 Biosynthesis. *Journal of Biological Chemistry*. 278(43), 42352-42360.

Wang, J., Burger, R. M. & Drlica, K. (1998). Role of superoxide in catalase-peroxidase-mediated isoniazid action against mycobacteria. *Antimicrobial Agents and Chemotherapy.* 42(3), 709-711.

Weinstein, E. A., Yano, T., Li, L., Avarbock, D., Avarbock, A., Helm, D., McColm, A. A., Duncan, K., Lonsdale, J.T. & Rubin, H. (2005). Inhibitors of type II NADH:menaquinone oxidoreductase represent a class of antitubercular drugs. *Proceedings of the National Academy of Sciences of the United States of America*. 102(12), 4548-4553.

Matsoso, L.G., Kana, B. D., Crellin, P. K., Lea-Smith, D. J., Pelosi, A., Powell, D., Dawes S. S., Rubin, H.., Coppel, R.L. & Mizrahi, V. (2005). Function of the cytochrome bc1-aa3 branch of the respiratory network in mycobacteria and network adaptation occurring in response to its disruption. *Journal of Bacteriology*. 187(18), 6300-8.

Yeo, A. E., Edstein, M. D., Shanks, G. D. & Rieckmann, K. H. (1997). Potentiation of the antimalarial activity of atovaquone by doxycycline against *Plasmodium falciparum in vitro*. *Parasitology research*. 83(5), 489-91.

Chapter 9

General discussion

It has been reported that the roots of *Euclea natalensis* A.DC. are used by indigenous people of southern Africa for various bacterial infections. The Zulu people used the roots for a purgative and for abdominal complaints and the root bark to treat tuberculosis (TB) related symptoms such as chest diseases, bronchitis, pleurisy and asthma. The Shangaan people apply the powdered root bark to skin lesions in leprosy and take it internally for ancylotomiasis while the Tonga people use the root for the relief of toothache and headache (Watt & Breyer-Brandwijk, 1962).

According to the Global TB Alliance's annual report, over 2 billion people carry the *Mycobacterium tuberculosis* bacterium. Millions of these infected people die each year. Most TB patients must complete 130 doses – up to eight tablets a day over a period of 6 months, while multi-drug resistant TB takes 2 years to treat. TB is also the leading killer of people with HIV-Aids, as current therapy cannot be combined easily with most HIV therapies. The incidence of TB is also on the increase with the last TB drug being commercialised over 30 years ago. In order to reverse the trend new anti-TB compounds must be found.

In total eleven naphthoquinones have been isolated from *E. natalensis,* while two of these compounds were isolated for the first time, during the authors M.Sc. studies (Van der Kooy, 2003). After finding a class of compounds that is active against a specific disease, the biochemical, chemical and biological aspects should be studied further. The aim of such a study is to better understand the biological role of these compounds in plants, humans (as medicine) and animals. During this thesis the chemical investigation included the organic synthesis of some of these naphthoquinones, as well as their stability and toxicity. In order to improve the potency of these compounds a structure–activity relationship has been conducted. From these preliminary findings the active and toxic sites could be identified. Future studies could focus on improving the potency with the addition of different side chains on carbon two and three. Due to the costs and time involved to synthesise these compounds additional funding and equipment need to be acquired. The semi-large scale production of the active compounds is needed for the various biological tests that have to be performed. Although the toxicity and stability tests made use of quantities in milligram, the *in vivo* mice work used

gram quantities. It is therefore essential to either isolate or synthesise enough material so that the various tests can be performed.

The compound, 7-methyljuglone**,** was synthesized by Musgrave & Skoyles (2001), in a two-step procedure. The overall yield of the two-step procedure (3 % calculated from the publication) is not satisfactory. During this thesis the synthesis was reinvestigated and improved to a one-step procedure with yields of 16 %. The synthesis of diospyrin**,** a dimer of 7-methyljuglone was published by Yoshida & Mori, *et al*. (2000). This synthetic route entails a 14 step sequence with yields of less than 4 %. Keeping in mind the costs, time and the experience needed for such a synthesis, it was decided that an alternative pathway was needed. During this thesis a new 3-step synthetic route with 7-methyljuglone epoxide as intermediate was investigated, to yield small quantities of diospyrin**.**

The stability of organic compounds in biological systems and bioassays was investigated in Chapter 5. This is a readily overlooked chemical test that bioactive compounds should be subjected to. It was found that the stability in almost all the bioassays used was unsatisfactory. This would indicate that the organic solvents used to dissolve hydrophobic compounds must be improved or the lead compounds need to be derivatised. The almost universal use of DMSO to dissolve hydrophobic compounds should be re-investigated.

The toxicity of the naphthoquinones was investigated in Chapter 6. The compounds proved to be toxic to vero cells, but these inflated results might be due to the high log P values of the compounds. A clear correlation could be seen between the log P values and the rate of precipitation in MEM. The toxicity of 7-methyljuglone was in addition also tested on *Musca domestica* to establish a possible mode of action of these compounds in diverse organisms. It proved to be non-toxic to the flies but did show some repellent properties.

Chapter 7 discussed the structure-activity relationship. This study was conducted in order to find the most potent and least toxic compound. Several structurally related compounds (isolated, synthesised or commercially obtained) have been tested for potency against TB to determine the active site (pharmacopore). In total thirteen compounds were tested for potency (with the use of the radiometric BACTEC bioassay) and for toxicity with the use of vero cells. In the pharmaceutical industry thousands of compounds are usually tested for potency with

the accuracy of the tests (structure-activity analysis) increasing with the increasing size of the data set.

It was found that the conjugate nucleophilic addition to α-β unsaturated carbonyl groups plays an important role, and it might be due to interaction via this mechanism with thiol groups (nucleophilic groups) on proteins that causes the toxicity of these compounds (McMurray, 1996: Lipinsky, personal communication). All the compounds with functional groups on both carbons (C2 and C3) have IC50 values of 128.5 μg/ml and higher. Only one of the three compounds, lapachol, showed good activity as well. This compound will be further derivatised in order to enhance its activity.

The mechanism of action of our lead compound and the biological activity in *M. smegmatis* indicated that there is a possible interference of the mycobacterial electron transport chain. These initial tests showed that 7-methyljuglone might be incorporated into a menaquinone-like compound, thereby inhibiting the flow of electrons or the rate of electron flow. The fluorinated 7-methyljuglone inhibited the production of menaquinone. The obtained results could be verified with the use of an LC-MS. This should give an indication if 7-methyljuglone was incorporated into a menaquinone-like compound (addition of 31 AMU). The use of *M. smegmatis* and *M. aureum* and the phase of bacterial growth (log phase etc.) and the influence this will have on potency should also be investigated. This can be done by administering the compounds during the different stages of growth. The bacteria could be cultured in different conditions especially aerobic and anaerobic conditions.

Future research could focus on finding additional structurally similar compounds from different sources in order to study the potency of these compounds on the electron transport chain. The structurally similar compound, atovaquone, is known to bind to the cytochrome bc complex in *Plasmodium falcipirum* (Srivastava *et al*., 1999). During initial screening of neodiospyrin the activity was in the region of 60 ng/ml against *P. falcipirum*, which is comparable to chloroquine. This would indicate that these types of compounds do indeed interfere with the electron transport chain of various organisms. The main challenge is therefore to change the structure of the compounds to such a degree as to make it species specific. In order to achieve this goal a large database of structurally similar compounds must be screened against various organisms.

References

Global Alliance for TB Drug Development. (2005). pp1-3. Broad Street, 31st floor,New York, US.

McMurry, J. (1996). Organic chemistry. 4th ed. pp 1-3, 583, 882. Brookes/Cole Publishing, USA.

Musgrave, O.C. & Skoyles, D. (2001). Ebenaceae extractives. Part11. The synthesis of 7-methyljuglone. A re-examination. *Journal of the Chemical Society. Perkin Transactions*. 1 : 1318-1320.

Srivastava, I. K., Morrisey, J. M., Darrouzet, E., Daldal, F. & Vaidya, A. B. (1999). Resistance mutations reveal the atovaquone-binding domain of cytochrome b in malaria parasites. *Molecular Microbiology*. 33(4), 704-711.

Van Wyk, B. & Van Wyk, P. (1997). Field guide to trees of Southern Africa, pp 184-185. Struik, McKenzie street, Cape Town.

Van der Kooy, F. (2003). Characterisation, synthesis and antimycobacterial activity of naphthoquinones isolated from *Euclea natalensis.* Unpublished. M.Sc. dissertation. University of Pretoria. South Africa.

Watt, J.M. & Breyer-Brandwijk, M.G. (1962). The medicinal and poisonous plants of southern and eastern Africa. 2nd ed. p 390. Livingstone, Londen.

Yoshida, M. & Mori, K. (2000). Synthesis of diospyrin, a potential agent against Leishmaniasis and related parasitic protozoan diseases. *European Journal of Organic Chemistry.* 1313-1317.