ROLE OF LIQUID MEMBRANE PHENOMENA IN THE BIOLOGICAL ACTIONS OF TINIDAZOLE

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Summary

The role of surface activity has been studied in the mechanism of action of nitroimidazole like Tinidazole. Tinidazole has been shown to generate liquid membrane. In the present study the transport of relevant permeants (glucose, p-amino benzoic acid, and various ions such as ammonium, phosphate, calcium, sodium, potassium and chloride) through liquid membrane generated by Tinidazole alone, and in association with lecithin and lecithin-cholesterol mixture in series with supporting membrane has been studied. The results indicate that the liquid membrane generated by Tinidazole inhibit the transport of various essential bio-molecules and permeants in to the cell. The data indicate that modification in permeability of different permeants in the presence of the liquid membrane is likely to play significant role in the biological actions of Tinidazole along with the conventional mechanism of antibacterial action.

Key words: Liquid membrane hypothesis, Tinidazole, Altered permeability, Kesting's hypothesis

Introduction

Many pharmacologically active compounds are amphiphillic in nature, which may undergo different types of association, and whose site of action is frequently, the plasma membrane. Amphiphillic compounds bear an ionic (zwitter ionic, anionic or cationic) or non-ionic polar head groups and a hydrophobic head portion. In aqueous medium, they are able to organize themselves as micelles, bilayer, monolayer and hexagonal or cubic phases. A wide variety of drugs are in fact, are known to be surface active in nature. In many cases excellent correlation between surface activity of drugs and their biological actions was demonstrated[1-4].The literature survey reveals that, wide variety of surface active drugs might exert their biological effects due to a common mechanism i.e. surface activity[5-16]. It has been shown that the surface-active drugs at the site of their action generate liquid membranes, which acts as barrier to the transport of relevant permeants. These investigations have led to phenomenon called as "liquid membrane hypothesis of drug action". Thus, the generation of the liquid membrane by the surface active drugs is aimed to add a new dimension towards the mechanism of pharmacological actions of drugs. According to liquid membrane hypothesis [5-16], propounded in the context of water desalination by reverse osmosis, a surface active substance when added to an aqueous phase generates a surfactant layer of liquid membrane at the interface. As concentration of the surfactant is increased, the interface gets progressively covered with the more and more surfactant molecules to form liquid membrane and at the critical micellar concentration (CMC) it is completely covered. In view of the liquid membrane hypothesis, it was supposed that the liquid membranes generated at the site acts as a barrier to transport of relevant permeants, which might be an important step, common to the mechanism action of all surface active drugs.

Nitroimidazoles are very popularly adopted not only as anti-amoebic but also used extensively in treating anaerobic bacterial infections. The mechanism of action of this group of drugs is generally agreed to be a pro-drug and that anaerobic

organisms reduce the nitro group of these drug into a hydroxylamine, during which a reactive derivative or reactive oxygen species are produced, and destroy the cellular components (DNA, proteins and membranes). It has been reported that nitro aryl compounds (nitroimidazoles, nitro furans) are reduced to nitro radical anions which in turn reacts with oxygen to regenerate the nitro aryl and the super oxide radical anion. Further reduction of super oxide radical anion leads to hydrogen peroxide and homolytic cleavage of the latter leads to hydroxyl radical formation. Super oxide radical anion, hydrogen peroxide and hydroxyl radicals are referred to as reactive oxygen species (ROS) and are the reactive substances which are implicated in damage to critical cellular components of the parasite. However, the understanding of mechanism action of these agents is incomplete [17].

Nitroimidazoles are a group of drug entities, used to treat various infections. There are reports that many antimicrobial agents like norfloxacin, ciproflaxacin, cefuroxime sodium etc which are amphiphillic in nature and generate the liquid membrane at the site of their action may also contribute for their antimicrobial action [18-19].

In the present study Tinidazole, (Fig. 1) a 5-nitro imidazole was used, having both hydrophilic and hydrophobic domains in its structure. Tinidazole is therefore expected to generate a liquid membrane at the interface. The cytoplasmic membrane consists of Phospholipids and Proteins. The phospholipid molecules are arranged in a bimolecular layer with polar groups directed towards both sides. At critical micellar concentration (CMC) of Tinidazole, it may form the liquid membrane over the cytoplasmic membrane. Because of the liquid membrane formed by Tinidazole, the transport of essential ions, glucose, glycine and p-amino benzoic acid may alter. The present study was designed so as to assess this hypothesis and its role in the anti-microbial activity of the study drug. Cellulose acetate microfiltration membrane has been specifically chosen as the site for formation of liquid membrane.

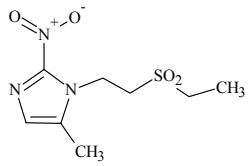


Figure1. Tinidazole.

Material and methods

Materials

Tinidazole (KAPL. Bangalore), glucose (NICE chemicals Pvt. Ltd.), Glycine (Loba chemicals, Mumbai), calcium chloride (NICE chemicals Pvt.Ltd.), sodium chloride(NICE chemicals Pvt. Ltd.), ammonium chloride (NICE chemicals Pvt. Ltd.), Potassium di hydrogen o-phosphate (Loba Chemicals.), Para amino Benzoic Acid (SD Fine Ltd.), Lecithin(Genuine Chemicals.), Cholesterol(Qualigens Ltd.), de-ionized water, Calcium diagnostic kit, Sodium, Potassium and chloride diagnostic kit(ERBA diagnostics.), glucose diagnostic kit (Agappe Diagnostics.). All chemicals used were analytical grade.

Methods

The critical micellar concentration of aqueous Tinidazole was determined from variation of surface activity with concentration. The surface tension was measured using Du Nouy surface tensiometer (model-144 Komal scientific Co., Bombay–47) at 37 ± 0.1^0 C and was found to be 10 X 10^{-5}M. The all glass transport cell as shown in fig–2[1-10] was used to obtain hydraulic permeability and solute permeability data. It essentially consists of two components, C and D separated by Sartorius cellulose acetate micro filtration membrane (Cat.No.11107, pore size 0.2µm, thickness $1X10^{-4}$m, area $2.55X10^{-5}m^2$), which acts as a supporting membrane for the liquid membrane. The cellulose acetate membrane was coated with lecithin (1.919 $X10^{-5}$M) and lecithin-cholesterol (1.919 $X10^{-5}$M lecithin and $1.175X10^{-6}$M cholesterol) mixture so as

to simulate the bacterial and human cell membranes respectively[11]. For the measurement of hydraulic permeability data, aqueous solution of Tinidazole at various concentrations (0-3 CMC) was placed in compartment C and compartment D was filled with de-ionized water. The hydraulic permeability was determined separately for Sartorius cellulose acetate micro filtration membrane coated with lecithin alone and lecithin-cholesterol mixture. The procedure as described by Srivastava et al [7] was adopted for obtaining the hydraulic permeability data.

To measure the solute permeability (ω) of the relevant permeants, the following equation was used [20-22].

$$\omega = [Js / \Delta\pi]_{Jv=0} \quad (1)$$

Where, *Js* and *Jv* are the solute flux and volume flux per unit area of the membrane, respectively. $\Delta\pi$ Is the osmotic pressure difference across the membrane. For the measurement of ω, compartment C of the transport cell was filled with aqueous solution of Tinidazole (2CMC) along with permeant. Compartment D was filled with de-ionized water. In control experiments, no drug was used; concentration of the drug in these experiments was always kept higher than its CMC so as to ensure complete coverage of the supporting membrane with the liquid membrane generated by Tinidazole[12-13]. All measurements were made at constant temperature ($37^0 \pm 0.1\ ^0$C) using a thermostat.

Estimation of permeants transported through liquid membrane generated by Tinidazole[19].

The amounts of sodium, potassium, chloride, calcium and D-glucose were estimated using semi-auto analyzer (Star-21). Amount of p-amino benzoic acid, glycine, ammonium and phosphate ions were estimated using UV-VIS Spectrophotometer (shimadzu-1700). Amount of magnesium was estimated using atomic absorption spectrophotometer (Chemito make).

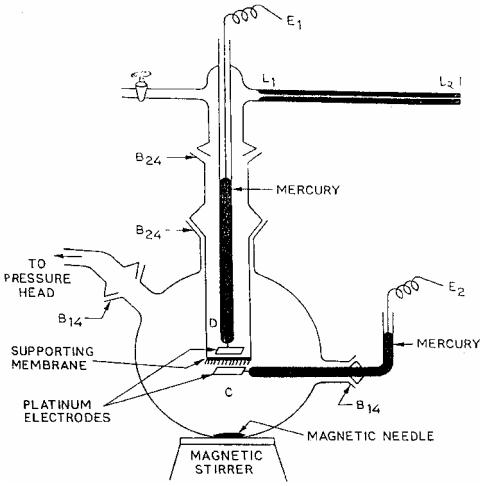


Figure2. All glass Transport Cell.

Results and discussion

Hydraulic permeability data at various concentrations of Tinidazole was found to obey the linear relationship i.e.

$$Jv = Lp.\Delta p \quad \text{(2)}$$

Where *Jv* represents the volume flux per unit area of the membrane, Δp is the applied pressure difference and *Lp* is the hydraulic conductivity coefficient. The values of *Lp* recorded at varying concentrations of Tinidazole were obtained from the slopes of *Jv* Vs Δp plots (fig-3). The *Lp* values are compiled in table–1. The value of *Lp* shows decreasing trend with increasing concentration of Tinidazole up to CMC. Beyond which it becomes more or less constant. This is indicative of progressive coverage of the supporting membrane with liquid membrane generated by the drug in accordance with the kesting's hypothesis [23].

Analysis of hydraulic permeability data in the light of mosaic membrane model [24-25] further supports the existence of the liquid membrane in series with supporting membrane. The

argument given earlier [3] it can be show that concentration of the surfactant is n times its CMC≤1. The value of Lp would be equal to [(1-n) L^s p + n L^c p], where L^s p and L^cp represents the value of Lp at 0 and the CMC of the surfactant respectively. The values of Lp thus computed for 0.25 CMC, 0.5 CMC and 0.75 CMC of Tinidazole are in good agreement with the experimentally determined values.

It was shown by Srivatsava et al [7-8] that lecithin aqueous solution ($1.919X10^{-5}$M), form liquid membrane, which completely covers the supporting membrane indicate the fall in Lp values when 1 CMC solution was added to the compartment C, which provides additional evidence regarding incorporation of Tinidazole in lecithin membrane [11].

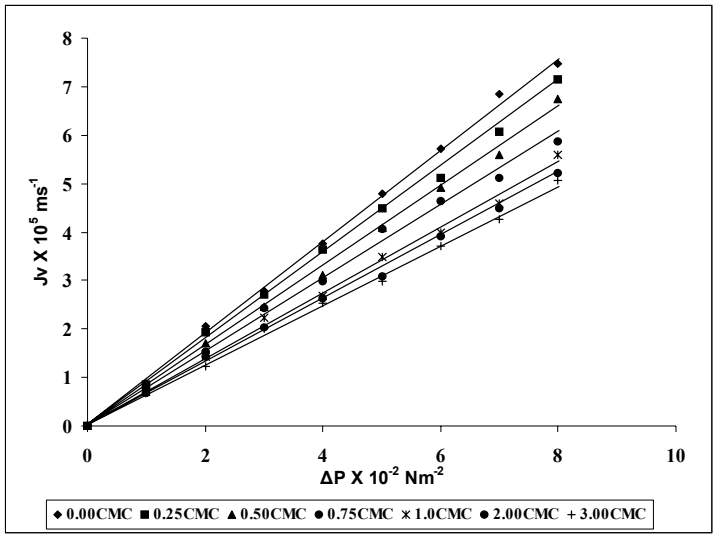
Incorporation of Tinidazole in the liquid membrane generated at the interface by lecithin-cholesterol (lecithin $1.919X10^{-5}$ M and cholesterol $1.175X10^{-6}$ M concentrations) mixture of fixed composition obtained on hydraulic permeability at varying concentrations of Tinidazole [11]. The hydraulic permeability in this case was found to be represented by Eq. (2). The values of L p decreases with increasing concentration of Tinidazole up to certain concentration and then become nearly constant.

Table1. Values of Lp at various concentrations of Tinidazole.

Critical Micellar Concentration CMC	Experimental Lp X 10^5 m^3 $S^{-1}N^{-1}$	Calculated Lp$X10^5$, m^3 $S^{-1}N^{-1}$
0.00	0.935 ± 0.188	
0.25	0.893 ± 0.150	0.875 ± 0.118
0.50	0.843 ± 0.167	0.818 ± 0.082
0.75	0.732 ± 0.195	0.757 ± 0.117
1.00	0.698 ± 0.186	
2.00	0.653 ± 0.114	
3.00	0.632 ± 0.128	

The values are presented as arithmetic mean ±standard deviation of 10 determinations.$m^3S^{-1}N^{-1}$-Meter cube per second per newton

Figure3. Hydraulic permeability data at various concentrations of Tinidazole



The value of solute permeability for various permeants (D-glucose, p-amino benzoic acid, glycine and ions like magnesium, phosphate, and ammonium, sodium, potassium, calcium and chloride) are recorded in table no-2 in presence of lecithin. Lecithin was chosen to simulate membrane components of bacterial cell membrane. From the solute permeability (ω) data, it was observed that Tinidazole reduce the permeation of these permeants. It seems Tinidazole forms the liquid membrane over the cell membrane and inhibits the transport of the essential ions and bio-molecules and thereby inhibits the normal functioning of cell. This may also contribute for the bactericidal effect of the Tinidazole.

In addition to this the permeability of various solutes through lecithin-cholesterol membrane (table No.3) was also reduced significantly. Lecithin-cholesterol membrane simulates the human cell membrane. The reduced permeability of various essential permeants indicates that some of the side-effects/adverse reactions may be due to the formation liquid membrane over human cell membrane. However the quantity

of the drug may be very less so as to cover whole of the human tissue; hence, the human cell toxicity is minimum. Probably liquid membrane phenomenon may contribute for the effects associated with toxic doses. The membrane permeability data shows that the reduction in the permeation of Ca, Na, K, Glucose, PO_4, NH_4, p-amino benzoic acid, Cl ions are reduced to a higher extent through lecithin + cholesterol membrane than lecithin membrane.

The results are indicating that probably these ions play vital role in bacterial cell and the drug concentration is insufficient to cover whole of the human cell and hence, the contribution of liquid membrane on the human cell is minimum. However, further studies are needed to confirm this. From the present study it may be concluded that the proposed hypothesis (i.e. the capability of formation of liquid membrane over the bacterial cell wall by Tinidazole may also contribute for the bactericidal effect of the drug in addition to its conventional mechanism, which involves the inhibition of cell wall synthesis) is justifiable.

The studies on nutrient (D-Glucose), metabolite (p-amino benzoic acid), and amino acid (glycine), ions like magnesium, phosphate, and ions like ammonium, sodium, potassium, calcium and chloride were done as these permeants play an important role in normal functioning of the cell. The role of different permeants is as follows

Glucose is essential for bacterial cell as a source of energy and carbon. It also helps in the synthesis of pentoses like ribose which is the constituents of nucleic acid and coenzyme. It serves as key precursor for the synthesis of non-essential amino acid and peptidoglycan[26].

PABA is essential intermediate and growth factor of micro organisms. The micro organisms have the capacity to the synthesis their own folic acid by using PABA[27].

The amino acids are serving as the source for carbon and nitrogen. They also serve as the building blocks for the synthesis of various proteins, co-enzymes, hormones, immuno

globulins [28] etc. These macro molecules are involved in the various physiochemical reactions, formation of cellular structure and maintenance of osmotic pressure and also serve as suppliers of energy.

Ammonium salts are not only as nitrogen source for the organism but also as the electron donor in some kind of organism. These organisms obtain energy by oxidizing ammonium ion to nitrite ion [29]. Ammonium salts are also require for the formation of class – II reaction products like nucleotides, phospholipids, amino sugars and some growth factors.

All the living organisms requires metal ions like K^+, Ca^{++}, Mg^{++}, Na^+ , $PO_4^-$and Cl^-, for normal growth and several other metallic ions are also needed in very low concentration and these are termed as trace elements. Many of these metallic ions are known to act as co-factors for various enzymes [30]. Even these ions are also involved in the maintenance of osmotic pressure, acid base balance, membrane integrity, activation of enzymes etc.

Due to the generation of liquid membrane by Tinidazole probably on the cytoplasmic membrane of bacterial cell seems to impede the transport of all the essential ions like thereby affect the physiological activities associated with these permeants. Therefore, the altered transport of various above mentioned permeants may also contribute to the antibacterial activity of the selected drugs in addition to the established molecular mechanisms involved.

It seems the molecular specificity for the receptor majorly contributes for the antibacterial activity of the Tinidazole. It is evident from the result that the hydraulic permeability and solute permeability are decreased significantly but not completely blocked due to liquid membrane granules. Therefore, it may be impeded that, there exist some other transport mechanism other than passive diffusion. Hence the involvement of liquid membrane in antibacterial activity may be a complementary to molecular mechanism.

Table 2. **Solute permeability (ω) of various permeants in presence of liquid membrane generated by Tinidazole in presence of lecithin.**

Permeant	Initial concentration	ω_0 ($X10^6$) (mole $s^{-1}N^{-1}$)	ω_1 ($X10^6$) (mole $s^{-1}N^{-1}$)	ω_2 ($X10^6$) (mole $s^{-1}N^{-1}$)	ω_3 ($X10^6$) (mole $s^{-1}N^{-1}$)
Chloride	10mg/ml	03.948±0.21	03.529±0.16	02.937±0.10	02.459±0.19
Glucose	500mg/lt	00.702±0.02	00.502±0.01	00.484±0.01	00.437±0.02
PABA	20mg/ml	53.630±0.83	47.021±0.99	46.821±0.64	35.466±1.04
Sodium	01mg/ml	00.227±0.01	00.200±0.00	00.150±0.00	00.099±0.00
Potassium	5.382mg/ml	00.079±0.00	00.061±0.00	00.060±0.00	00.033±0.00
Calcium	10.43mg/ml	39.850±1.72	24.052±1.50	25.801±2.10	16.011±1.37
Phosphate	10mg/ml	14.961±0.81	08.980±0.77	09.989±1.20	06.281±0.31
Ammonium	10mg/ml	19.793±0.06	17.153±0.12	16.922±0.28	14.089±0.18
Glycine	10mg/ml	03.852±0.14	03.201±0.12	03.114±0.07	01.701±0.18
Magnesium	01mg/ml	20.040±0.10	08.606±0.26	10.282±0.36	05.312±0.40

The values of ω are reported as ± S.D of 10 repeats: ω_0 : values of ω when no drug was used, ω_1 : values of ω in presence of lecithin, ω_2 : values of ω in presence of Tinidazole, ω_3 : values of ω in presence of Tinidazole and lecithin.

***Table 3*. Solute permeability (ω) of various permeants in presence of liquid membrane generated by Tinidazole in presence of lecithin – cholesterol mixtures.**

Permeant	Initial concentration	ω_0 ($X10^6$) (mole $s^{-1}N^{-1}$)	ω_1 ($X10^6$) (mole $s^{-1}N^{-1}$)	ω_2 ($X10^6$) (mole $s^{-1}N^{-1}$)	ω_3 ($X10^6$) (mole $s^{-1}N^{-1}$)
Chloride	500mg/lt	03.948±0.21	03.538±0.08	02.937±0.10	01.137±0.08
Glucose	20mg/ml	00.702±0.02	00.574±0.01	00.484±0.01	00.321±0.01
PABA	01mg/ml	53.631±0.83	45.922±1.99	46.823±0.64	31.978±1.32
Sodium	5.382mg/ml	00.227±0.01	00.190±0.00	00.150±0.00	00.085±0.00
Potassium	10.43mg/ml	00.079±0.00	00.061±0.00	00.060±0.00	00.031±0.00
Calcium	10mg/ml	39.856±1.72	26.785±1.82	25.808±2.10	15.976±1.95
Phosphate	10mg/ml	14.962±0.81	10.424±0.96	09.989±1.20	05.138±0.62
Ammonium	10mg/ml	19.798±0.06	17.782±0.23	16.920±0.28	13.771±0.27
Glycine	01mg/ml	03.852±0.14	03.190±0.03	03.114±0.07	01.429±0.07
Magnesium	10mg/ml	20.045±0.10	11.206±0.43	10.282±0.36	06.873±0.65

The values of ω are reported as ± S.D of 10 repeats: ω_0 : values of ω when no drug was used, ω_1 : values of ω in presence of lecithin-cholesterol, ω_2 : values of ω in presence of Tinidazole, ω_3 : values of ω in presence of Tinidazole & lecithin-cholesterol.

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