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REDUCTIVE ACTIVATION OF NITROIMIDAZOLES IN ANAEROBIC MICROORGANISMS

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The recognition of the antimicrobial and later antiparasitic potential of nitroheterocyclic compounds opened up the possibility of major advances in chemotheraphy in the 1940s and 1950s. Of the innumerable derivatives synthesized and tested, a number found clinical application, among others the antibacterial nitrofurans, the antianaerobic 5-nitroimidazoles and the schistosomicidal nitrofurans and nitrothiazoles.

Although the mode of action on microorganisms of these compounds is not fully understood, it was noted early that derivatives without a nitro-group are not active, showing that the presence of a nitro-group is essential for activity. It was also recognized already in the 1940s that the process of antimicrobial activity of nitrofurans is connected with the metabolic reduction of the nitro-group [1]. When the prototype 5-nitroimidazole derivative, metronidazole began its unparalleled success story in 1959, the analogy with other nitroderivatives surprisingly was not recognized. The mode of action remained an enigma [2] until the pioneering work of Edwards and co-workers [3, 4] about a decade later pointed to the role of reductive activation, a notion that has received extensive experimental support in recent years. This paper focuses first on the action of metronidazole and 5-nitroimidazoles and then compares them with other nitroderivatives, including imidazole derivates having a nitrosubstituent on position other than 5-C. This topic has been reviewed repeatedly in the past [5, 6] but recent progress warrants a renewed evaluation of the data. Although the major conclusions made earlier are still valid, several details of our earlier interpretation require modification. Only selected references will be given to illustrate the more important points and no attempt will be given to provide a comprehensive bibliography.

REDUCTIVE ACTIVATION OF 5-NITROIMIDAZOLES

The action of metronidazole and related 5-nitroimidazole derivatives on microorganisms is characterized by selective killing of anaerobic prokaryotes and eukaryotes. Inhibitory concentrations for facultative anaerobes and aerobes are at least two orders of magnitude higher than those for anaerobes. Mutagenicity is often observed in microorganisms that are not highly susceptible to 5-nitroimidazoles.

In vitro studies with *Trichomonas vaginalis*, against which metronidazole was developed, show that 5-nitroimidazoles exert a high antitrichomonad activity under both anaerobic and aerobic conditions [7]. In most experiments, however, inhibitory concentrations are higher in aerobic tests than in anaerobic ones [7] (M. Müller *et al.*, submitted), showing that the presence of oxygen interferes with the antitrichomonad activity of the compounds. This effect of aerobiosis has been observed in other microorganisms as well [8, 9].

According to current views, the process whereby metronidazole exerts its action on anaerobic microorganisms can be divided into four stages: entry of the compound into the target cell, its reductive activation, interaction of the "active" species with intracellular targets and breakdown, i.e. "inactivation" of the "active" species. Of these stages the present review will concentrate only on the reductive activation and will not deal with the rest for several reasons. Activation, as mentioned above, is a crucial step of the process but more importantly, the metabolic basis of activation is most critical in determining the specificity and selectivity of various nitroimidazole derivatives. Our research interest concerns largely this aspect of the process. Other stages have been reviewed in detail at this symposium, notably the interaction of the active species with intracellular targets [10, 11] and the fragmentation of the active species [12].

In the process of reductive activation an intracellular electron donor provides electrons to the nitro-group of the nitroheterocyclic compound:

$$e^- + R - NO_2 \rightarrow R - NO_2^- .$$

Extensive evidence shows that the first product is the nitro-free radical derivative [13, 14], indicating that the transfer of electrons occurs in one-electron steps. Even in a simplified model the rate of formation and disappearance of the free radical will depend on several factors. These include the supply of electrons which depends on the rate of their metabolic production and transfer to physiological or artificial acceptors other than the nitroderivative, the electron affinity and concentration of the nitroderivative, and the rates of dismutation of the nitro-free radical,

$$2R - NO_2^- \rightarrow R - NO_2 + R - NO,$$

and of its reactions with other compounds. One of the major factors in these processes is molecular oxygen which can compete for the electrons needed for nitroreduction or can oxidize the nitro-free radical to the original compound and be reduced to superoxide anion:

$$R - NO_2^- + O_2 \rightarrow R - NO_2 + O_2^- .$$

This establishes a futile cycle in which electrons from

metabolism are consumed without net reduction of the nitroderivative.

The question of the nature of the active species has to be raised, even if its interaction with intracellular targets will not be discussed. Unreduced nitroimidazoles or the final products of reduction have no antimicrobial activity [15] thus the active species most likely represents a product (or products) intermediate between these two end points. Candidate compounds include the nitro-free radical and the superoxide anion formed by its reaction with O_2 as well as products of further reduction of the nitro-group [i.e. nitroso ($R-NO$), nitroso-free radical ($R-NO^-$), or hydroxylamine ($R-NHOH$) derivatives] arising by dismutation of the free radical or through direct transfer of additional electrons. It is possible that under different conditions, for different compounds and different organisms different products play the leading role.

The role of reductive activation in the antimicrobial action is well documented, thus it will be regarded as a fact and the presentation will deal more with its analysis than providing a formal proof for its existence. It should be stressed that direct quantitation of this process is not trivial, mainly because the nature of the active species is not known. Various investigators used different methods of quantitation, most of which are indirect. To mention a few: accumulation of the label of radiolabeled drugs in the target cells [16], measurement of the intracellular level of nitro-free radicals [13], estimation of reducing equivalents utilized in the reduction by monitoring metabolic fluxes [17], determination of the formation of end products of reduction [18], etc. Each of these provided important information but the interpretation of the results was never without some ambiguity.

In anaerobic protozoa and most susceptible anaerobic bacteria the main metabolic source of electrons donated to metronidazole is the oxidative decarboxylation of glycolytically formed pyruvate, a process catalysed by pyruvate:ferredoxin oxidoreductase [19, 20]. Reduction of the drug by intact cells can be inhibited by inhibitors of glycolysis [21]. In trichomonad flagellates but not in all anaerobic protozoa this enzyme is localized in hydrogenosomes, characteristic organelles of these organisms [22]. Subcellular fractions enriched in hydrogenosomes are able to reduce metronidazole if compounds needed for pyruvate:ferredoxin oxidoreductase activity are present [20]. It is of great significance that trichomonad flagellates and anaerobic bacteria lose their ability to activate metronidazole and become resistant to this drug when induced to lose their pyruvate:ferredoxin oxidoreductase activity [23–25].

In the absence of nitrocompounds the enzymatic systems responsible for nitroimidazole reduction transfer the electrons to other acceptors. Under anaerobic conditions in trichomonads and anaerobic bacteria the major acceptor is the proton, reduced to molecular hydrogen by the action of hydrogenase:

$$2e^- + 2H^+ \rightarrow H_2.$$

5-Nitroimidazoles effectively compete for the electrons and indeed the first clue to the mode of action of metronidazole was the observation that hydrogen production by trichomonad flagellates is inhibited by this compound [3, 4], an observation soon confirmed in bacteria [17]. Hydrogen production is, at least partly, restored after the drug added to the incubation mixture is reduced [17, 26]. This finding suggests that the antimicrobial action is not related to an irreversible inhibition of the hydrogenase. These observations show that a process normally leading to hydrogen production is intercepted by metronidazole that acts as an alternative electron sink. The interception by itself seems not to be deleterious for the organisms, only its consequence is, i.e. the reduction of the nitro-group.

Other electron acceptors can compete with metronidazole. 2,4-Dinitrophenol for instance inhibits the intracellular accumulation of the label of labeled metronidazole and the cytotoxic effect of the drug [27]. It acts as alternative electron sink, interrupts hydrogen production and is reduced to 2-amino-4-nitrophenol in a six-electron process. The most important alternative electron acceptor, however, is oxygen, the presence of which is expected in most therapeutic situations where metronidazole is being used.

Studies on the reduction of metronidazole with nonenzymatic electron donors (dithionite, reduced methyl viologen, or ferredoxin) and with isolated enzymes or subcellular organelles demonstrated that in all these systems, as expected from the redox properties of the components involved, oxygen is the preferred electron acceptor and in fact no reduction of metronidazole can be observed in its presence. It should be immediately added that again there is no good kinetic information on how the electron flow from the primary donor to oxygen is proportioned between direct oxidation of the donor and transfer via the nitro-free radical futile cycle. The fact that no steady state concentration of the nitro-free radical can be detected with EPR techniques [20, 14] by itself does not exclude the participation of the futile cycle. High concentrations of metronidazole stimulate H_2O_2 production by hydrogenosomes and ferredoxin-linked enzyme systems showing that under certain conditions the presence of oxygen does not inhibit completely the transfer of electrons to metronidazole but leads to electron flow through the futile cycle [20].

Metronidazole exerts activity on trichomonads and other anaerobic microorganisms under anaerobic and aerobic conditions. Its minimum lethal concentration (MLC) as determined in *in vitro* studies in *T. vaginalis* is higher under aerobic conditions than under anaerobic ones [7] (M. Müller *et al.*, submitted), but in *T. foetus* shows little difference [28]. Intracellular accumulation of the label of labeled metronidazole by trichomonads and *Entamoeba invadens* is also decreased under aerobic conditions [16, 29]. Differences between aerobic and anaerobic MLC values depend markedly on the assay conditions, however. Again no kinetic data on the electron flow are available. The fact that cytotoxicity is only decreased but is not suppressed entirely by aerobiosis indicates that metronidazole is being constantly reduced. EPR studies show that the nitro-free radical concentration in intact *T. vaginalis* cells

becomes undetectable at relatively low pO_2 values [14]. This suggests a high removal rate of the radicals by the futile cycle.

It is likely that the intracellular redox environment markedly influences the reductive activation of metronidazole. The localization of the major electron donating system in trichomonad flagellates in discrete organelles makes this proposition rather attractive. The intracellular redox state and its change in the presence of oxygen in trichomonads, or in other anaerobic microorganisms, is not known. One point is well established, however. As mentioned above, the metabolic source of electrons for metronidazole reduction is the oxidative decarboxylation of pyruvate by the hydrogenosomal pyruvate:ferredoxin oxidoreductase. The final product of this process is acetate, thus the rate of acetate production is a good indicator of the electron flow to ferredoxin. This process is not depressed by aerobiosis in *T. vaginalis* [29], indicating that hydrogenosomal electron generation is not really dependent on anaerobiosis even if oxygen can markedly alter the subsequent fate of these electrons. These considerations can at least partly explain the aerobic efficacy of metronidazole in trichomonads and the differences in the aerobic and anaerobic MLC values. No similar information is available for other anaerobic protozoa and bacteria in which pyruvate: ferredoxin oxidoreductase is not localized in organelles.

Trichomonad hydrogenosomes are located in a cytoplasm containing NADH and NADPH oxidases. These enzymes reduce oxygen with glycolytically generated reduced pyridine nucleotides as electron donors, thus can be regarded as oxygen scavenging enzymes. It has been suggested repeatedly that one of the results of their function could be a more reduced cytoplasm [30] (D. Linstead and S. Bradley, 1985, Abstr. Internat. Symp. Trichomonads and Trichomoniasis, Prague). Through these processes the hydrogenosomal environment could be rendered relatively oxygen-free, a condition favorable for the activation of metronidazole. It is likely that such mechanisms play an important role. That they are not exclusive is suggested by the observations that the level of NADH oxidase which can vary 40 fold from isolate to isolate is not correlated with the aerobic metronidazole susceptibility of *T. vaginalis* and that the level of NADPH oxidase shows only very small variations [29].

FERREDOXIN AND METRONIDAZOLE REDUCTION

It was recognized early that metronidazole interacts selectively with ferredoxin mediated metabolic processes, e.g. "phosphoroclastic" oxidation of pyruvate, catalysed by pyruvate:ferredoxin oxidoreductase, hydrogen production and photosynthesis. The interpretation given rested on the low midpoint potential of metronidazole ($E_7^1 = -486$ mV), stating that low redox potential electrons needed for reduction are only generated by ferredoxin-linked processes [3]. Pyruvate/acetate + CO_2 (−700 mV), $H_2/2H^+$ (−420 mV), and ferredoxin$^-$/ferredoxin (−420 mV for *Clostridium pasteurianum*) redox couples are more negative or similar to that of metronidazole$^-$/metronidazole couple, whereas the ubiquitous NADH/NAD^+ couple is more positive by more than 100 mV. These thermodynamic considerations are clearly part of the explanation.

Probing the reduction process further gave, however, somewhat unexpected results. All data supported the idea that the main source of electrons is indeed pyruvate oxidation. It was observed, however, that cell free extracts depleted of ferredoxin by DEAE–cellulose treatment largely lose their ability to reduce metronidazole even when their pyruvate:ferredoxin oxidoreductase activity is unchanged [17, 31, 32]. Thus an enzyme activity generating electrons of low redox potential proved to be a necessary but not sufficient condition for efficient reduction. Addition of purified ferredoxin (of homologous or heterologous origin) restored the reduction capacity of the system. The enhancement shows linear dependency on the concentration of added ferredoxin and is not saturable within the concentration limits feasibile. Similar results were obtained when H_2 was used as an electron donor inducing a reverse electron flow in hydrogenosomal preparations [32]. Nonenzymatic reduction of metronidazole by chemically reduced ferredoxin was also reported [33]. These observations demonstrate that for efficient activation of metronidazole, in addition to enzymatic processes generating electrons of low redox potential, ferredoxin is necessary as an electron carrier between the enzyme and metronidazole.

REDUCTION OF DIFFERENT NITROIMIDAZOLES

The success of metronidazole as an antimicrobial agent and its promise as a radiosensitizer in cancer chemotherapy led to the synthesis and testing of a large number of other nitroimidazole derivates and also of other nitroheterocyclic compounds. These compounds differ considerably in their antimicrobial spectrum and degree of cytotoxicity. In view of the critical role of reductive activation, a process likely to be dependent on the reducibility of the compounds [34], several authors explored the correlation of reducibility (as expressed by the one electron midpoint potential) with various biochemical and biological activities of the compounds. Extensive comparative material shows that all organisms tested can be divided into two major groups based on their response to nitroderivatives of different midpoint potentials. In facultative anaerobic and aerobic bacteria and aerobic eukaryotic cells cytoxicity and mutagenic action increases with increasing reducibility of the compounds [35–40]. No such correlation was observed, however, in anaerobic protozoa [38] and obligate anaerobic bacteria [41]. The conclusions seem to be valid for compounds with midpoint potentials more positive than −500 mV since more negative compounds show little biological activity in either anaerobic or aerobic organisms. The selectivity against anaerobic microorganisms of any given compound will depend to a large extent on its midpoint potential with highest selectivity expected among compounds with values somewhat more positive than −500 mV. This is the area where

metronidazole and other clinically utilized 5-nitroimidazoles lie. The biochemical basis of these relationships was sought, it seems correctly, in certain important metabolic differences between aerobic and facultative anaerobic organisms on one hand and anaerobic organisms on the other.

The correlation of the rate of *in vitro* reduction of nitroimidazoles and other nitroderivatives by various biological electron donors has been explored extensively. A positive correlation of the rate with midpoint potential can be demonstrated for reduction by reduced flavins [42], the flavoprotein oxidoreductase, xanthine oxidase [42, 43] and even ferredoxin depleted extracts of trichomonad hydrogenosomes containing pyruvate:ferrodoxin oxidoreductase [44].

Reduction by living facultative anaerobic bacteria [36] and cultured mammalian cells [36] also shows similar relationships. Reduction of nitroimidazoles by trichomonad ferredoxin, however, does not show any dependence on the midpoint potential of the compounds, as demonstrated by determining the differences in the reduction rates of 16 nitroimidazole derivatives by trichomonad pyruvate:ferredoxin oxidoreductase in the presence and absence of added ferredoxin [44]. This absence of dependence on midpoint potential separates ferredoxins from other biological electron donors involved in nitroreduction and renders them highly effective reductants for nitroimidazoles with relatively low midpoint potentials which are reduced by other donors only at very low rates. No other biological constituents are known today displaying a similar property. The physicochemical basis of this intriguing finding remains to be elucidated. In the present context, however, its significance is in contributing to our understanding of the marked differences in the response of different organisms to various nitroimidazole derivatives.

As mentioned above, cytotoxicity and mutagenicity in aerobic and facultative anaerobic organisms and cells shows a positive correlation with the midpoint potential of the compounds. In these organisms ferredoxin mediated processes play a minor role or none, thus reductive activation of the nitroimidazoles is probably due to direct or flavin mediated electron transfer from various oxidoreductases, processes which depend strongly on the midpoint potential. In anaerobic bacteria and protozoa where cytoxicity is not related to midpoint potential, ferredoxin-linked reactions play a major metabolic role thus reduced ferredoxin is available as an electron donor. Since reduction of nitroimidazoles by ferredoxin is midpoint potential independent, all compounds will be reduced by the ferredoxin-linked enzyme systems with similar rates. This does not exclude, however, that even in anaerobic organisms other enzymes and electron transfer components can donate electrons to nitroimidazoles.

These considerations show that the ratio of reduction in the absence and presence of ferredoxin will be a major determinant of the spectrum of action of any given nitroimidazole derivative. For more positive compounds the ratio will be high and these will have a broad spectrum of action. For more negative compounds the ratio will be low and these will show selective toxicity against anaerobic organisms with minimal cytotoxicity and mutagenicity in other cell types.

NITROIMIDAZOLE RESISTANCE

Clinical data on human vaginal trichomoniasis have long suggested the possibility of metronidazole resistance in *T. vaginalis*. Laboratory findings did not confirm, however, the increased drug tolerance in isolates from patients in whom treatment failed, thus the existence of resistance was dismissed until recently. Meingassner and Thurner reported in 1979 a case in which the isolate obtained showed marked tolerance to metronidazole and two other nitroimidazoles in an *in vivo* mouse assay [45]. These authors demonstrated that *in vitro* assays can detect resistance only if performed under aerobic conditions but not under anaerobic ones. Since their report several investigators obtained *T. vaginalis* isolates with lowered nitroimidazole susceptibility from clinical cases. The degree of resistance of these isolates shows a considerable variation and susceptibility values represent a continuum without sharp boundary between isolates from successfully treated patients and those from treatment failures (Lossick *et al.*, submitted). The latter, however, have on the average somewhat higher anaerobic and markedly higher aerobic MLC values resulting in high aerobic/anaerobic MLC ratios. These findings suggest that the observed lower clinical susceptibility of these isolates is due to an increased inhibitory effect of aerobiosis on metronidazole activation and not on a basic deficiency in the primary mechanism of activation. This conclusion is supported by the lack of any difference in the main processes of energy metabolism and in the activity of the pyruvate:ferredoxin oxidoreductase between susceptible and resistant organisms [29]. The increased effect of oxygen on the activation was demonstrated by determining the intracellular accumulation of label from radioactive metronidazole by the two groups of organisms [29] and also by observing an increased quenching by low concentrations of O_2 of metronidazole free-radical signal detected by EPR spectroscopy [14]. The molecular mechanisms underlying these effects remain obscure though recent results indicate that the more resistant organisms have a decreased tolerance to aerobiosis (J. Tachezy *et al.*, 1985. Abstr. Internat. Symp. Trichomonads and Trichomoniaisis, Prague), again pointing to an impaired ability of the organisms to handle oxygen. The clinical implications of these findings are beyond the scope of this presentation, suffice to state that clinically the resistance is relative and most patients harboring resistant organisms can be cured with increased dosage of metronidazole (J. Lossick *et al.*, submitted).

As mentioned above, in addition to this "aerobic" resistance, anaerobic 5-nitroimidazole resistance can also be induced in the laboratory in various microorganisms by prolonged metronidazole pressure or mutagenesis and selection in metronidazole containing media. Such strains were obtained in *T. foetus* [46], *T. vaginalis* (P. Demeš *et al.* and J. Kulda *et al.*, 1985. Abstr. Internat. Symp. Trichomonads and Trichomoniasis, Prague), *Bacteroides fragilis* [24]

and *Clostridium perfringens* [25]. All these organisms lack pyruvate:ferredoxin oxidoreductase activity thus resistance is due to the absence of the enzyme which generates electrons for nitroimidazole reduction. In fact these findings represent an elegant proof for the role of reductive activation in the action of metronidazole on anaerobic microorganisms.

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