Squalene epoxidase as a target for the allylamines

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Introduction

Squalene epoxidase (EC 1.14.99.7, squalene monoxygenase) occupies a key position in the biosynthetic pathway leading to ergosterol. The 2,3-oxidation of the 30-carbon hydrocarbon chain of squalene is essential for subsequent cyclization to the characteristic rigid 4-ring sterol skeleton which is required for membrane function in virtually all fungi. Squalene is the first lipophilic intermediate in the sterol pathway, and the epoxidase is the first enzyme in the pathway requiring molecular oxygen. Squalene epoxidase has been investigated in several fungal and mammalian squalene epoxidase systems, together with a discussion of the mechanisms by which enzyme inhibition is translated into antifungal action.

Inhibition of fungal squalene epoxidase

Squalene epoxidase has been investigated in several yeast-like fungi and has a number of features in common with the enzyme from rat liver [1], which is the only enzyme of this type reported to have been purified [11]. The membrane-bound (microsomal) enzyme requires molecular oxygen, NAD(P)H and FAD for activity. In contrast to the rat liver enzyme, no soluble cytoplasmic factors are required by the fungal epoxidase. Squalene epoxidase is clearly not of the cytochrome P-450 superfamily as it does not contain haem and is unaffected by carbon monoxide and other known cytochrome P-450 inhibitors.

The inhibitory effects of the allylamines have primarily been investigated using the microsomal squalene epoxidase system which was characterized in Candida albicans [5, 12]. Both naftifine and terbinafine cause a concentration-dependent inhibition of the C. albicans epoxidase, complete suppression of activity being attainable at relatively low inhibitor concentrations. Kinetic analysis of inhibition at various squalene concentrations provided evidence for a non-competitive type interaction. The K_i values for naftifine and terbinafine in this system are 1.1 mM and 0.03 mM respectively. It should of course be noted that classical kinetics do not strictly apply to a heterogeneous enzyme system with an insoluble substrate such as squalene. A non-competitive inhi-
bition is however, compatible with the observation that high levels of squalene accumulate in allylamine-treated fungi without release of the cells from the drug-induced growth inhibition. The co-factors NADH, NADPH, and FAD, as well as the soluble cytoplasmic fraction, are also completely non-competitive with respect to inhibition by naftifine and terbinafine. The inhibition is not time-dependent and is fully reversible. Qualitatively similar results have also been obtained using a microsomal squalene epoxidase system from C. parapsilosis. The enzyme from C. albicans can be solubilized by detergent action and remains equally sensitive to inhibition by terbinafine [13], indicating that the action of the drugs is not dependent on intact membrane structure.

A number of structurally related compounds have been found to inhibit the C. albicans squalene epoxidase in a manner similar to that of naftifine and terbinafine. Considerable structural variation is permitted while retaining potent epoxidase inhibition. Compounds with activity comparable with that of terbinafine include the chlorobenzthiophene SDZ 87-469 and the benzylamine derivative butenafine (structures shown in Fig. 1). Although the allylamine group was originally the characteristic chemical feature of these compounds, more recently it has become apparent that neither the nitrogen nor the double bond are actually required for epoxidase inhibition. Carbon analogues (lacking the allylamine nitrogen) can be effective epoxidase inhibitors but have little or no antifungal activity, apparently owing to failure to penetrate the fungal cell envelope [10].

The discovery of the mechanism of action of the allylamines stimulated a search for further inhibitors of squalene epoxidase. At high concentrations (0.1-1.0 mM) a number of lipophilic compounds and electron transport inhibitors cause a partial and probably non-specific inhibition of the epoxidase [1, 12]. In addition, the thiocarbamate antifungals, tolnaftate and tolciclate, which bear a superficial structural resemblance to naftifine, were found to be specific inhibitors of the fungal enzyme [14]. Some squalene analogues also inhibit the epoxidase in the micromolar range, as might be expected [1].

**Effects on mammalian squalene epoxidase**

The rat liver microsomal squalene epoxidase has been extensively investigated [1] and the components of the system have been purified [11]. The $K_m$ of the purified enzyme for squalene was 13 $\mu$M [11], which is similar to that of 11 $\mu$M measured in the microsomal system [5]. The rat liver microsomal epoxidase differs from the equivalent Candida system in that it has an almost complete requirement for the soluble cytoplasmic fraction, which can be satisfied by a combination of an anionic phospholipid and a protein termed Supernatant Protein Factor.

The effects of allylamines on mammalian squalene epoxidase have been examined in detail [5, 13, 15], especially in the case of terbinafine which is an orally active drug. Terbinafine proved to be a very weak reversible inhibitor of the rat liver microsomal enzyme with a $K_i$ of 77 $\mu$M, while the effect of naftifine is so weak as to be regarded as non-specific. Table 1 summarizes the activities of three allylamines against fungal and mammalian squalene epoxidases. This very high degree of selectivity is in agreement with findings in vivo that orally administered terbinafine has no significant effect on cholesterol biosynthesis in experimental animals or in humans. Inhibition of the rat liver epoxidase by terbinafine shows competitive kinetics with respect to the substrate squalene and also with respect to the soluble cytoplasmic fraction. Comparison of several mammalian liver squalene epoxidases showed the guinea pig enzyme to be particularly sensitive to allylamines (50% inhibition by about 5 $\mu$M terbinafine) [13]. There is, therefore, a signifi-
ties and high selectivity, it is unlikely that the allyl-inhibition. In view of their structure, kinetic proper-
types of inhibition are ruled out by the reversible
reductase supplying reducing equivalents from
to be at the level of the oxidase. Mechanism-based
allylamines, inhibition has been clearly established
as squalene analogues, but
side-chain and a strongly modified ring structure, is
also (in the case of the liver epoxidases) soluble
and also (in the case of the liver epoxidases) soluble
cytoplasmic fractions, demonstrated that differences
in sensitivity to terbinafine are not caused by
protection of the enzyme by the cytoplasmic frac-
tion [13]. The precise mechanism of selectivity is
thus not yet clear but appears to reside in intrinsic
differences in the respective epoxidase enzymes, as
discussed in the following section.

Very recently, the structure of the allylamine
derivative NB-598 has been published [16]. This
interesting compound (Fig. 1) with terbinafine-type
side-chain and a strongly modified ring structure, is
a potent inhibitor of mammalian squalene epoxi-
dase but has no antifungal activity [16]. This
reversed selectivity also appears to operate at the
enzymatic level.

**Mechanism of epoxidase inhibition**

Squalene epoxidation presents a complex target for
inhibition, consisting of the terminal oxidase, a
reductase supplying reducing equivalents from
NAD(P)H, the lipid environment of the complex,
and also (in the case of the liver epoxidases) soluble
non-catalytic protein factors. In view of this com-
plexity it is perhaps surprising that so few inhibitors of
the system have been identified. In the case of the
allylamines, inhibition has been clearly established
to be at the level of the oxidase. Mechanism-based
types of inhibition are ruled out by the reversible
non-competitive non-time-dependent kinetics, and
the allylamine nitrogen is clearly not required for
inhibition. In view of their structure, kinetic proper-
ties and high selectivity, it is unlikely that the allyl-
amines function simply as squalene analogues, but
an interaction with the substrate-binding site cannot
be ruled out. A recently suggested model for inhibition [17] postulates that the high affinity of the allyl-
amines for the fungal epoxidase may result from
entropic binding of the molecule to two separate
sites on the enzyme, namely the squalene-binding
site (naphthalene ring) and an adjacent lipophilic
pocket (side-chain). This model is compatible with
the available evidence, and also with the more
recent findings that naphthalene and 1-methyl-
naphthalene are weak inhibitors of the epoxidase
(50% inhibition at about 0.4 mM), while the terbina-
fine side-chain has no inhibitory effect at concentra-
tions up to 1 mM. There is good evidence for a
specific lipid-binding domain on squalene epoxi-
dase, and the fungal and rat liver enzymes display
different interactions with respect to deter-
genents, phospholipids and fatty acids [1]. Slight
differences in alignment of the two binding sites could
also provide the molecular basis for the selectivity
of the allylamines, and the reversed selectivity of
compounds such as NB-598.

**Mechanism of the antifungal action of
allylamines**

The potent inhibition of squalene epoxidase by
allylamines described in the previous sections is
reflected in a quantitatively similar inhibition of
fungal ergosterol biosynthesis in both cell-free
extracts and whole fungal cells. Epoxidase inhibi-
tion has been found to be an essential pre-requisite
for antifungal activity within this class of com-
ounds, although as mentioned previously, not all
epoxidase inhibitors have antifungal activity. Extensive
studies have shown that the squalene epoxidase
is the only enzyme of the ergosterol pathway which
is significantly inhibited by the allylamines, and that
growth inhibition of various pathogenic fungi corre-
lates with the sensitivity of cellular ergosterol bio-
synthesis [4, 6, 15]. However, physiological factors
which vary between different fungi also play an
important role in determining the extent to which
epoxidase inhibition is translated into actual
growth-inhibitory action in a specific fungus. Fil-
amentous growth is in general more susceptible to
inhibition by this mechanism, and the evidence
suggests that only partial inhibition of ergosterol
biosynthesis is required for full suppression of
growth in these fungi [6]. In C. albicans, which is
dimorphic, the filamentous form is about ten-fold
more susceptible than the yeast form to growth
inhibition by allylamines while there is little differ-
ence between ergosterol biosynthesis inhibition in
the two forms.
Fungal Sterol Biosynthesis and Inhibition

Inhibition of fungal squalene epoxidation leads to deficiency of ergosterol and accumulation of squalene in the cells, and both these events appear to play a role in blocking fungal growth. Since ergosterol is an essential component of the cell membrane, blocking its production would be expected to arrest growth. This results in a fungistatic action, as observed in *C. albicans* and some other yeasts, and is comparable with the action of other ergosterol biosynthesis inhibitors such as the azoles. The accumulation of squalene is thought to be responsible for the fungicidal action against many fungi which is characteristic of the allylamines. As observed in a large number of studies in several pathogenic fungi, the onset of cell death always coincides with the accumulation of high levels of squalene, but not necessarily with ergosterol deficiency (Fig. 2). The exact mechanism of induction of cell death is not clear but may involve disruption of membrane function and interference with cell wall biosynthesis.

The possibility of highly selective inhibition of fungal squalene epoxidase, the resultant primary fungicidal action, and the fact that the enzyme is not cytochrome P-450-dependent, combine to make squalene epoxidase an attractive target for development of antifungal agents.


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