Kinetically Controlled Drug Resistance

HOW PENCILLIUM BREVICOMPACTUM SURVIVES MYCOPHENOLIC ACID

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**Background:** Mycophenolic acid sensitivity varies by 10^3-fold among IMP dehydrogenases (IMPDHs) from different sources even though the drug-binding site is completely conserved.

**Results:** Resistant IMPDHs have a different kinetic mechanism compared with sensitive enzymes.

**Conclusion:** Resistance results from a failure to accumulate the drug-sensitive intermediate.

**Significance:** This is a novel mechanism of drug resistance.

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The filamentous fungus *Penicillium brevicompactum* produces the immunosuppressive drug mycophenolic acid (MPA), which is a potent inhibitor of eukaryotic IMP dehydrogenases (IMPDHs). IMPDH catalyzes the conversion of IMP to XMP via a covalent enzyme intermediate, E-IMP*-XMP*. MPA inhibits by trapping E-IMP*-XMP*. *P. brevicompactum* (Pb) contains two MPA-resistant IMPDHs, PbIMPDH-A and PbIMPDH-B, which are 17- and 10^3-fold more resistant to MPA than typically observed. Surprisingly, the active sites of these resistant enzymes are essentially identical to those of MPA-sensitive enzymes, so the mechanistic basis of resistance is not apparent. Here, we show that, unlike MPA-sensitive IMPDHs, formation of E-IMP*-XMP* is rate-limiting for both PbIMPDH-A and PbIMPDH-B. Therefore, MPA resistance derives from the failure to accumulate the drug-sensitive intermediate.

*Penicillium brevicompactum* produces a toxic small molecule called mycophenolic acid (MPA), which is used as an immunosuppressive drug (see Fig. 1A) (1). MPA is a potent inhibitor of eukaryotic IMP dehydrogenases (IMPDHs) and thus has antifungal activity (2). *P. brevicompactum* contains two genes for IMPDH, *PbIMPDH-A* and *PbIMPDH-B* (3). The *PbIMPDH-B* gene is located within the cluster that encodes the MPA biosynthetic proteins. Both *PbIMPDH-A* and *PbIMPDH-B* are more resistant to MPA compared with IMPDHs from fungi that do not produce the drug (e.g. *Aspergillus nidulans* IMPDH, *AnImdA*) (4, 5). However, *PbIMPDH-B* is remarkably resistant to MPA, with an ~1000-fold greater IC_{50} than that of typical eukaryotic IMPDHs. Curiously, the active site of *PbIMPDH-B* is essentially identical to MPA-sensitive IMPDHs (see Fig. 1B), so the mechanistic basis of drug resistance is not understood.

IMPDH catalyzes the conversion of IMP to XMP via two distinct chemical reactions (6). The first step is a hydride transfer reaction that produces NADH and the covalent intermediate E-IMP*-XMP* (Fig. 1B). NADH dissociates, and the enzyme switches from the open conformation (E-IMP*-XMP*_{open}) to the closed conformation (E-IMP*-XMP*_{closed}), where a mobile flap binds in the vacant cofactor site. This conformational change brings the catalytic Arg-429 into the active site to activate water for the hydrolysis of E-IMP*-XMP* (Chinese hamster IMPDH2 numbering will be used throughout unless noted otherwise.) Hydrolysis is at least partially rate-limiting in all IMPDHs studied to date (6), so E-IMP*-XMP* is the predominant enzyme form.

MPA is an uncompetitive inhibitor of IMPDH. MPA binds to E-IMP*-XMP*_{open} in the cofactor-binding site and directly competes with flap closure (7–9). MPA thereby traps IMPDH in a non-productive E-IMP*-XMP*-MPA complex that is unable to complete the hydrolysis reaction. MPA will also bind to E-IMP, but the affinity for this complex is >300-fold lower than that for E-IMP*-XMP* (10). Thus, the accumulation of E-IMP*-XMP*_{open} will be an important determinant of MPA sensitivity. Here, we show that the MPA resistance of *PbIMPDH-B* is elegantly conferred by a change in kinetic mechanism that reduces the accumulation of the drug-sensitive complex E-IMP*-XMP*_{open}.

**EXPERIMENTAL PROCEDURES**

**Materials**—IMP, MPA, 3-acetylpyridine adenine dinucleotide (APAD\(^3^-\)), ADP, Tris, and common chemicals were purchased from Sigma. NAD\(^+\) was purchased from Roche Applied Science. KCl and trichloroacetic acid were purchased from Fisher. D\(_2\)O and DCI were purchased from Cambridge Isotope Laboratories, Inc. (Andover, MA). [2-\(^3\)H]IMP was synthesized as described previously (11). [8-\(^14\)C]IMP was purchased from Moravek Biochemicals, Inc. Tiazofurin was obtained from NCI, National Institutes of Health.
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**Plasmids**—Constructs for the recombinant expression of N-terminally His-tagged AnImdA, PbIMPDH-A, and PbIMPDH-B in *Escherichia coli* were constructed as described previously (5). Site-directed mutagenesis of PbIMPDH-B R429A was performed using a method based on QuikChange mutagenesis (Stratagene, La Jolla, CA). (This is residue 440 in *Pb*IMPDH-B numbering.) Briefly, a T7 forward primer and reverse primer (GTC GTT CTC AGA GAA GTA GGC GGA AGC GCC AGC) were used to amplify by PCR a portion of the *Pb*IMPDH-B gene carrying the R429A mutation. This PCR product was used as the forward and reverse mutant primers to complete the remainder of the QuikChange procedure according to the manufacturer’s directions. Plasmids carrying the desired R429A mutation were verified by sequencing (GENEWIZ, South Plainfield, NJ).

**Expression and Purification of His-tagged Proteins**—Enzymes were expressed and purified as described previously (5). Briefly, plasmids were expressed in a ΔguaB derivative of *E. coli* BL21(DE3) (12); purified using a HisTrap affinity column (GE Healthcare); and dialyzed into buffer containing 50 mM Tris (pH 8.0), 100 mM KCl, 1 mM DTT, and 10% (v/v) glycerol. All enzymes were purified to >90% purity as determined by SDS-PAGE.

**Enzyme Concentration Determination**—Enzyme concentration was determined by the Bio-Rad assay according to the manufacturer’s instructions using IgG as a standard. The Bio-Rad assay overestimates IMPDH concentration by a factor of 2.6 compared with the concentration determined by active site titration using the irreversible inactivator 5-ethyl-1-β-D-ribofuranosylimidazole-4-carboximide 5'-monophosphate (13), so a correction factor of 2.6 was used.

**Enzyme Kinetics**—Standard IMPDH assay buffer consisted of 50 mM Tris (pH 8.0), 100 mM KCl, 1 mM DTT, and various concentrations of IMP and NAD⁺ or APAD⁺. Enzyme activity was measured by monitoring the absorbance increase at 340 nm corresponding to the production of NADH or at 363 nm corresponding to the production of reduced 3-acyctlypyridine adenine dinucleotide (APADH) on a Cary Bio-100 UV-visible spectrophotometer at 25 °C. Initial rates were calculated using ε₂₅₀ = 6.2 mM⁻¹ cm⁻¹ and ε₃₆₃ = 9.1 mM⁻¹ cm⁻¹. Initial velocity data were fit to either the Michaelis-Menten equation (Equation 1) or an uncompetitive substrate inhibition equation (Equation 2) using SigmaPlot (Systat Software).

\[
V = \frac{V_m[S]}{K_m + [S]} 
\]  
(Eq. 1)

\[
V = \frac{V_m}{1 + ([S]/K_m) + ([S]/K_i)} 
\]  
(Eq. 2)

MPA inhibition assays were performed at saturating IMP and half-saturating NAD⁺ concentrations to avoid complications arising from NAD⁺ substrate inhibition. The concentrations of IMP and NAD⁺ used for each enzyme are listed in the figure and table legends. MPA inhibition data were fit to either an uncompetitive inhibitor equation (Equation 3) or a noncompetitive/mixed inhibitor equation (Equation 4) using SigmaPlot.

\[
V = \frac{V_m[S]}{[S](1 + [I]/K_i) + K_m} 
\]  
(Eq. 3)

\[
V = \frac{V_m[S]}{[S](1 + [I]/K_i) + K_m(1 + [I]/K_i)} 
\]  
(Eq. 4)

**Multiple-inhibitor Experiment**—Experiments with tiazofurin and ADP were performed in standard IMPDH assay buffer with constant concentrations of IMP and NAD⁺ for each enzyme as follows: AnImdA, 500 μM IMP and 250 μM NAD⁺; PbIMPDH-A, 1.5 mM IMP and 500 μM NAD⁺; and PbIMPDH-B, 3 mM IMP and 1 mM NAD⁺. Data were fit to an equation for multiple inhibition (Equation 6) using SigmaPlot,
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RESULTS

Mechanism of Inhibition—MPA is a potent inhibitor of AnImdA (IC_{50} = 26 nM), a moderate inhibitor of PblIMPDH-A (IC_{50} = 430 nM), and a poor inhibitor of PblIMPDH-B (IC_{50} = 27,000 nM) (5). If MPA binds exclusively to E-XMP^*_{open}, then uncompetitive inhibition should be observed versus both substrates for all three enzymes. However, if MPA binds to additional enzyme forms, noncompetitive inhibition will be observed.

We examined the effects of varying substrate concentration on MPA action to determine the mechanism of inhibition for all three fungal enzymes. MPA concentrations approximated on MPA action to determine the mechanism of inhibition for all three fungal enzymes.

Primary Deuterium Isotope Effects—[2-^2H]IMP was used as the substrate in standard IMPDH assay buffer. Activity was measured by holding IMP at a constant saturating concentration and varying the concentration of NAD^+ or APAD^+.

Solvent Deuterium Isotope Effects—Assay buffer was prepared in either H_2O or D_2O. pH meter readings were corrected to pH by adding 0.4 units. Activity was assessed by holding IMP at a constant saturating concentration and varying the concentration of NAD^+ or APAD^+.

Measurement of E-XMP^*—Reaction mixtures contained 2 or 3 μM enzyme, 0.25 or 1 mM [8-^14C]IMP (for AnImdA and PblIMPDH-A/B, respectively), and 5 mM NAD^+ in standard IMPDH assay buffer at 25°C. Enzyme, [8-^14C]IMP, and NAD^+ were mixed to initiate the reaction, and the reaction was quenched during steady state by precipitation with TCA to a final concentration of 10% (w/v). Enzyme samples were collected using HA-nitrocellulose filters (Bio-Rad) and washed with 10% (w/v) TCA. Radioactivity was measured using a scintillation counter. Control reactions in which enzyme was omitted were included for each experiment.

FIGURE 2. Mechanism of MPA inhibition of fungal IMPDHs. A and B, AnImdA, [MPA] = 0 (○), 10 (□), 25 (■), and 50 (△) mM; E = 39 mM. A, versus NAD^+, [IMP] = 500 μM, and K_m = 10 μM. Data were fit to Equation 5. B, AnImdA versus IMP, [NAD^+] = 500 μM, and K_m = 170 μM. Data were fit to Equation 5. C and D, PblIMPDH-A, [MPA] = 0 (○), 200 (□), and 400 (△) mM; E = 76 mM. C, versus NAD^+, [IMP] = 2 mM, and K_m = 130 μM. Data were fit to Equation 3. D, versus IMP, [NAD^+] = 1 mM, and K_m = 340 μM. Data were fit to Equation 4. E and F, PblIMPDH-B, [MPA] = 0 (○), 10 (□), and 25 (△) mM; E = 71 mM. E, versus NAD^+, [IMP] = 5 mM, and K_m = 1.4 mM. Data were fit to Equation 3. F, versus IMP, [NAD^+] = 5 mM, and K_m = 0.79 mM. Data were fit to Equation 5. All fits were performed to nonlinear equations using SigmaPlot. Lineweaver-Burk plots are shown for inspection only.

\[ v = \frac{v_0}{1 + [I]/K_i + [J]/K_j + [I][J]/\alpha K_K} \]  

(Eq. 6)

where I and J are the inhibitors; v_0 is the uninhibited initial velocity; K_i and K_j are the inhibition constants for I and J, respectively; and α is the interaction constant between the two inhibitors.
TABLE 1
Inhibition of fungal IMPDHs

| Inhibitor | AnImdA | PbIMPDH-A | PbIMPDH-B | PbIMPDH-B R429A |
|-----------|--------|-----------|-----------|-----------------|
| MPA IC₅₀ (μM) | 26 ± 2 | 430 ± 30 | (2.7 ± 0.9) × 10⁻⁴ | (3.5 ± 0.4) × 10⁻¹ |
| MPA vs. IMP (μM) (mechanism) | Kᵢ = 25 ± 3 (UC) | Kᵢ = 450 ± 150 | Kᵢ = (2 ± 1) × 10⁻³ | ND² |
| MPA vs. NAD⁺ (μM) (mechanism) | Kᵢ = 22 ± 2 (UC) | Kᵢ = 500 ± 60 (UC) | Kᵢ = (1.4 ± 0.1) × 10⁻⁴ (UC) | ND |
| Tiazofurin IC₅₀ (μM) | 0.24 ± 0.4 | 4 ± 0.4 | 3.5 ± 0.4 | ND |
| ADP IC₅₀ (μM) | 6 ± 2 | 10 ± 1 | 3.3 ± 0.3 | ND |

a This is residue 449 in PbIMPDH-B numbering. The concentration of enzyme used for IC₅₀ determination was 500 nM. The concentrations of substrates were 1 mM IMP and 3 mM NAD⁺.

b Values are from Ref. 5.

¹ UC, uncompetitive; NC, noncompetitive.

² ND, no data.

³ IC₅₀ values for tiazofurin and ADP are the apparent Kᵢ values for tiazofurin and ADP obtained from the multiple-inhibitor experiment as described under "Experimental Procedures." The concentrations of substrates were as follows: AnImdA, 250 μM IMP and 0.5 mM NAD⁺; PbIMPDH-A, 3 mM IMP and 500 μM NAD⁺; and PbIMPDH-B, 3 mM IMP and 1 mM NAD⁺.

α = interaction constant between tiazofurin and ADP as described under "Experimental Procedures."

TABLE 2
Isotope effects

| Enzyme | kₐcat | D V | D V/Kₐcat(NAD⁺) | D²V | D²V/Kₐcat(NAD⁺) | Rate-limiting step(s) |
|--------|-------|-----|-----------------|-----|-----------------|----------------------|
| Human IMPDH2 | 0.31 ± 0.01⁴ | 1.0 ± 0.1² | 2.5 ± 0.2³ | 1.8 ± 0.1⁴ | ND⁵ | Hydrolysis |
| AnImdA | 0.74 ± 0.06¹ | 1.0 ± 0.3 | 2.5 ± 0.6 | 1.6 ± 0.1 | 1.1 ± 0.1 | Hydrolysis |
| PbIMPDH-A | 0.70 ± 0.08³ | 1.6 ± 0.2 | 1.6 ± 0.3 | 1.5 ± 0.1 | 1.2 ± 0.1 | Hydrolysis/transfer |
| PbIMPDH-B | 0.41 ± 0.02¹ | 2.5 ± 0.3 | 3.4 ± 0.2 | 1.1 ± 0.1 | 0.9 ± 0.2 | Hydrolysis |
| PbIMPDH-B R429A | (4.0 ± 0.3) × 10⁻⁴ | ND | ND | 4.4 ± 0.4 | ND | Hydrolysis |

a Values are from Ref. 21.

b ND, no data.

c Values are from Ref. 5.

d This is residue 449 in PbIMPDH-B numbering.

E-XMP⁺_closed and therefore provides an upper limit for the drug-sensitive E-XMP⁺_open species. Whereas most IMPDHs contain ~50% E-XMP⁺_open (6), 20 ± 1% of the active sites contain E-XMP⁺_open in PbIMPDH-A and only 7 ± 3% in PbIMPDH-B. These results suggest that MPA resistance may result from the failure to accumulate E-XMP⁺_open.

More E-XMP⁺_closed Cannot Account for Resistance—The above observations indicate that there is comparatively little E-XMP⁺_total and, therefore, very little E-XMP⁺_open during the reactions of PbIMPDH-A and PbIMPDH-B. The amount of E-XMP⁺_open would be further reduced if the conformational equilibrium favored E-XMP⁺_closed as has been observed in other MPA-resistant IMPDHs (15, 16). We performed a multiple-inhibitor experiment to probe the open/closed equilibrium (6). Tiazofurin binds to the nicotinamide subsite of the NAD⁺-binding pocket, and ADP binds to the adenosine subsite. If the closed conformation is favored, one inhibitor will pull the equilibrium toward the open conformation, resulting in enhanced binding of the second inhibitor. This will be reflected in the interaction constant α, which will be <1 if such synergism is observed. In contrast, if E-XMP⁺_open predominates, the two inhibitors will be independent, and α will be ~1.

The value of α is ~1 for both AnImdA and PbIMPDH-B (Table 1 and supplemental Fig. S1), indicating that the equilibrium favors the open form. In contrast, a synergistic interaction is observed for PbIMPDH-A (α = 0.4), which indicates that E-XMP⁺_closed predominates. The value of α approximates the fraction of E-XMP⁺_open. Because E-XMP⁺_total = 20% (see above), then E-XMP⁺_open can account for no more than 8% of enzyme in PbIMPDH-A.

Hydride Transfer Is Rate-limiting in Reactions of PbIMPDH-A and PbIMPDH-B—To further assess the accumulation of E-XMP⁺, we used isotope effects to identify the rate-limiting steps for each enzyme reaction. If hydrolysis is the rate-limiting step as typically seen, a solvent deuterium isotope effect will be observed. However, if hydride transfer is the slow step, there will be a primary deuterium isotope effect when [2-²H]IMP is used as the substrate.

No primary deuterium isotope effect is observed in the reaction of AnImdA, indicating that hydride transfer is fast, as observed for other IMPDHs (Table 2) (6). A small solvent deuterium isotope effect is observed on kₐcat indicating that hydrolysis is partially rate-limiting step, also as expected. Therefore, E-XMP⁺_open is the predominant enzyme form during the reaction of AnImdA, accounting for MPA sensitivity.

In contrast, PbIMPDH-A shows a primary deuterium isotope effect on kₐcat in addition to a solvent deuterium isotope effect, indicating that both steps are partially rate-limiting. This observation explains the relatively low amount of E-XMP⁺_total that accumulates during the reaction of PbIMPDH-A.

PbIMPDH-B also shows a primary deuterium isotope effect on kₐcat but no solvent deuterium isotope effect, indicating that hydride transfer is completely rate-limiting in this reaction. E-IMP-NAD⁺ is therefore the predominant enzyme form for PbIMPDH-B. This kinetic mechanism minimizes the accumulation of E-XMP⁺ in the steady state, rendering the enzyme resistant to MPA.

Increasing E-XMP⁺ Makes PbIMPDH-A and PbIMPDH-B More MPA-sensitive—If the MPA resistance of PbIMPDH-A and PbIMPDH-B results from the low accumulation of
**TABLE 3**

Effect of increasing E-XMP* on MPA inhibition

Conditions were as follows: AnlImdA, 500 μM IMP and 500 μM NAD*; and PbIMPDH-A and PbIMPDH-B, 3 mM IMP and 1 mM NAD.

| Enzyme         | Total E-XMP* | IC50(D2O)* | D2O/IC50 | IC50(APAD+)* | D2O/IC50 | IC50(D2O + APAD+)* | D2O/IC50 |
|----------------|--------------|------------|----------|--------------|----------|------------------|----------|
| AnlImdA        | ND           | 21 ± 3     | 1.2 ± 0.2 | 13 ± 1       |          | 2.0 ± 0.2        | 15 ± 2   |
| PbIMPDH-A      | 20 ± 1       | 75 ± 10    | 5.7 ± 0.9 | 97 ± 5       |          | 4.4 ± 0.4        | 45 ± 3   |
| PbIMPDH-B      | 7 ± 3        | (1.2 ± 0.1) × 10^4 | 2.2 ± 0.8 | (1.0 ± 0.1) × 10^4 | 2.7 ± 0.9 | 3400 ± 200       | 8.0 ± 2.6 |

* Parentheses indicate the substitutions of D2O for H2O and/or APAD+ for NAD*.

The amount of E-XMP* can also be increased with the NAD* analog APAD+. This dinucleotide has a greater reduction potential than NAD* (E0’ = −0.258 for APAD+ −0.320 for NAD*) (17) and a lower affinity for IMPDH (6, 18). These properties will shift the reaction toward E-XMP*open. Importantly, because the reduced cofactor must dissociate before MPA can bind, the structure of E-XMP* formed in the reaction with APAD+ will be identical to the E-XMP* formed in the reaction with NAD+. As predicted, all of the enzymes became more MPA-sensitive by factors of 2–4 (Table 3). When APAD+ and D2O were combined, the effects were approximately additive for all three enzymes (Table 3).

**DISCUSSION**

The MPA resistance properties of PbIMPDH-A and PbIMPDH-B derive from changes in the kinetic mechanism. Unlike all other IMPDHs characterized to date (6, 21), hydride transfer is at least partially rate-limiting for both enzymes. This simple kinetic switch minimizes the accumulation of the drug-sensitive species E-XMP*open, allowing these enzymes to evade MPA inhibition.

The resistance of PbIMPDH-A can be almost completely reversed by increasing the accumulation of E-XMP*. The remaining 3-fold resistance can be attributed to the equilibrium between the open and closed conformations of E-XMP*, which favors the closed conformation in PbIMPDH-A. This shift in equilibrium may be due to the presence of Ser-249. MPA-sensitive IMPDHs contain an Ala at this position (Fig. 1B). Ser at this position accounts for the MPA resistance of IMD2 in Saccharomyces cerevisiae (20). The substitution of Thr causes a 3-fold increase in MPA resistance in Candida albicans (15), shifting the equilibrium toward the closed conformation.

The resistance of PbIMPDH-B is only partially reversed by increasing E-XMP*. Like MPA-sensitive IMPDHs, PbIMPDH-B contains Ala-249. We have recently found that changes in residues 498–527 account for ∼7-fold of the 60-fold difference in MPA resistance between PbIMPDH-A and PbIMPDH-B; these residues form part of an allosteric site that binds K+ (8). However, many of the structural determinants of the MPA resistance of PbIMPDH-B remain a mystery.

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