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D-Penicillamine Causes Free Radical-Dependent Inactivation of Activator Protein-1 DNA Binding

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SUMMARY

D-Penicillamine (β,β-dimethyl cysteine) is an antirheumatic thiol drug with a poorly understood mechanism of action. On the basis that gold(I) thiolates and D-penicillamine are both capable of forming stable bonds with endogenous thiols, we sought a common target of action. Cysteine residues in the basic DNA binding domains of Jun and Fos, members of the activator protein-1 (AP-1) transcription factor family, have been identified as likely targets for the therapeutic action of antirheumatic gold(I) thiolates. The current study demonstrates that AP-1 DNA binding is inhibited by D-penicillamine in the presence of Fenton reagents (Fe^{2+}/EDTA and H_2O_2) but not with either agent alone. The effect is biphasic, with maximum inhibition in the concentration range of ~100-250 μM. Cysteine has qualitatively similar properties, although the effect is less pronounced. In contrast, glutathione and thiomalate do not inhibit AP-1 DNA binding, even in the presence of Fenton reagents. Mutant proteins were used to identify the cysteine residues within the DNA binding domains of Jun and Fos that are essential for the inhibitory action of D-penicillamine. The results suggest that D-penicillamine is distinguished from other thiols by its formation of sulfur-containing radicals capable of inhibiting AP-1 DNA binding by a mechanism involving the cysteine residues of Jun and Fos.

D-Penicillamine is a thiol drug that was first used in the treatment of rheumatoid arthritis for its ability to dissociate the disulfide bonds of IgM rheumatoid factor *in vitro* (1). A corresponding fall in rheumatoid factor titers was found to occur in patients administered D-penicillamine (2), and its clinical efficacy was demonstrated in 1973 by the British Multicentre Trial Group (3, 4). However, it became evident that symptom improvement was not related to its effects on rheumatoid factor, and the mechanism of action remains unknown.

A number of other thiol drugs have since been tested in rheumatoid arthritis, and some have either proven or probable efficacy. These include 5′-thiopyridoxine (5), captopril (6), tiopronin (7), and bucillamine (8). Not all thiol drugs have antirheumatic properties; e.g., sodium thiomalate, in the absence of gold(I), has no clinical efficacy (9). The possibility that antirheumatic thiol drugs may have a common mode of action has been suggested (10), and this hypothesis can be extended to include gold salts and antimalarials by considering that the common mode of action may involve reactivity with endogenous thiols rather than merely the possession of a thiol group. We recently identified thiol groups of the cysteine residues in the DNA binding domains of Jun and Fos as likely targets for the action of gold(I) thiomalate in the inhibition of AP-1 DNA binding and AP-1-mediated transcription (11). It is therefore hypothesized that D-penicillamine may also inhibit AP-1 DNA binding by acting at these same sites.

Jun and Fos proteins are proto-oncogene products that form Jun/Fos heterodimers and Jun/Jun homodimers (12, 13). These dimers, known collectively as AP-1, are phorbol ester-inducible transcription factors that bind a specific DNA sequence known alternatively as the AP-1 response element or the 12-*O*-tetradecanoyl-13-phorbol-acetate-response element. Several genes contain the AP-1 response element in their promoter regions, and their expression is activated when AP-1 is bound. Well-characterized examples include the human genes metallothionein II_A, collagenase, stromelysin, and interleukin-2 and viral genes simian virus 40 and polyoma (14–19). Inhibition of AP-1-dependent transcription has the potential to ameliorate the inflammatory response (11), consistent with the antirheumatic properties of gold and D-penicillamine.

DNA binding by Jun/Jun and Jun/Fos dimers to the AP-1 site is regulated *in vitro* by reduction-oxidation (redox) of a single conserved cysteine residue in the DNA-binding domains of the two proteins (20). Mutant Jun and Fos proteins, in which the conserved cysteine residue is substituted for

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ABBREVIATIONS: AP-1, activator protein-1; DTT, dithiothreitol; EMSA, electrophoretic mobility shift assay; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

serine, exhibit good DNA binding that is resistant to redox regulation (20). These results indicate that the conserved cysteine residue in the DNA binding domains of members of the Jun and Fos families must be in the reduced state for binding and, presumably, transcription to take place.

Thiol agents are capable of acting either as oxidizing or reducing agents, depending on their concentration, the presence of a free radical catalyst, and the nature of the thiol agent (21, 22). The enhancement of AP-1 DNA binding by DTT is attributed to the reducing nature of this thiol (20). In contrast a thiol agent, acting as an oxidant, may form a mixed disulfide with the cysteine residues in the DNA binding domains of Jun and Fos and therefore inhibit AP-1 DNA binding. D-Penicillamine is a candidate for such an oxidizing action because it would provide a common mode of action with gold salts and because its actions in several *in vitro* experiments imply oxidation reactions with free radical intermediates (22–24). The current study tests some endogenous and xenobiotic thiol agents for inhibition of AP-1 DNA binding *in vitro* in the presence or absence of free radicals provided by the Fenton reaction. Mutations of the conserved cysteine residues of Jun and Fos were used to determine the site of action of D-penicillamine in this system.

Materials and Methods

Cell culture. T-47D human breast cancer cells were supplied by the E. G. and G. Mason Research Institute (Worcester, MA). This cell line was used because it has been used in similar studies addressing the effects of metal ions on the binding of AP-1 (11) and steroid hormone receptors (25) to their DNA response elements. Stock cultures were maintained by weekly passage in RPMI 1640 medium supplemented with 6 mM glutamine, 10 μg/ml human insulin, and 10% fetal calf serum and buffered with 14 mM sodium bicarbonate and 20 mM HEPES. Stock cultures were free of antibiotics and *Mycoplasma* contamination. For most experiments, 20 μg/ml gentamicin was included.

Nuclear extract. Subconfluent T-47D cells were harvested with trypsin/EDTA, washed once in culture medium; washed once in homogenization buffer [15 mM Tris·HCl, pH 7.5, 1 mM EDTA, 2 mM DTT, 10% glycerol) and a mixture of protease inhibitors, including 1 μg/ml pepstatin A, 47 μg/ml leupeptin, 100 μg/ml bacitracin, and 77 μg/ml aprotinin (Sigma Chemical, St. Louis, MO)]; and kept on ice. Cell pellets were suspended in ~50 μl of homogenization buffer/10^7 cells and homogenized in a Teflon/glass Potter-Elvehjem homogenizer (Wheaton, Melville, NJ). Homogenates were centrifuged at 800 × *g* for 20 min; supernatants were discarded; and the nuclear pellet was washed twice in homogenization buffer by resuspension and centrifugation at 800 × *g*. Nuclear pellets were suspended in an equal volume of extraction buffer (20 mM HEPES, pH 7.9, 500 mM NaCl, 1.5 mM $MgCl_2$, 2 mM DTT, 20% glycerol), extracted on ice for 1 hr with repeated resuspension, and centrifuged at 100,000 × *g* at 4° for 60 min. Supernatants were frozen at −70° until required.

EMSA. Nuclear protein (5 μg) and 1 μg of poly(dI/dC)-poly(dI/dC) were preincubated for 20 min at 20° in binding buffer (50 mM sodium phosphate, pH 7.3, 5% glycerol, 5 mM $MgCl_2$, 0.1 μg/μl bovine serum albumin) and test compounds in a volume of 18 μl. Test thiol compounds, D-penicillamine, sodium thiomalate, cysteine, or glutathione (Sigma Chemical), were added immediately before Fenton reagents (10 μM $FeCl_2$, 10 μM EDTA, and 0.5 mM H_2O_2, unless otherwise stated). In some experiments, purified, truncated Jun and Fos proteins (~1 ng of each) were included. DTT (1 mM) was added to the DNA binding reactions of the specified lanes in the experiment shown in Fig. 1. In all other experiments, the only DTT present in the DNA binding reaction was that carried over in the nuclear extract, calculated to be 0.1 mM. A ^{32}P-labeled probe (0.5 ng) corresponding to the metallothionein II_A AP-1 site (20) was added, and the final volume of 20 μl was analyzed by nondenaturing 5% (1:29 cross-link) polyacrylamide gel electrophoresis in Tris-glycine buffer. Sequence-specific binding of retarded complexes was demonstrated by the addition of excess unlabeled oligonucleotide competitor. The presence of Jun and Fos in the sequence-specific AP-1 complex was determined by the addition of 1 μl of polyclonal rabbit antibody (1 μg/μl) raised against peptides corresponding to sequences within c-*jun* and c-*fos*, respectively (Caltag Laboratories, Burlingame, CA). An antiprogesterone antibody was used as a control (26). Retarded complexes were excised from the dried gel and quantified by Cerenkov counting. The purified, truncated Jun and Fos proteins and their mutants (20) were the kind gift of S. Xanthoudakis and T. Curran (Roche Institute of Molecular Biology, Nutley, NJ).

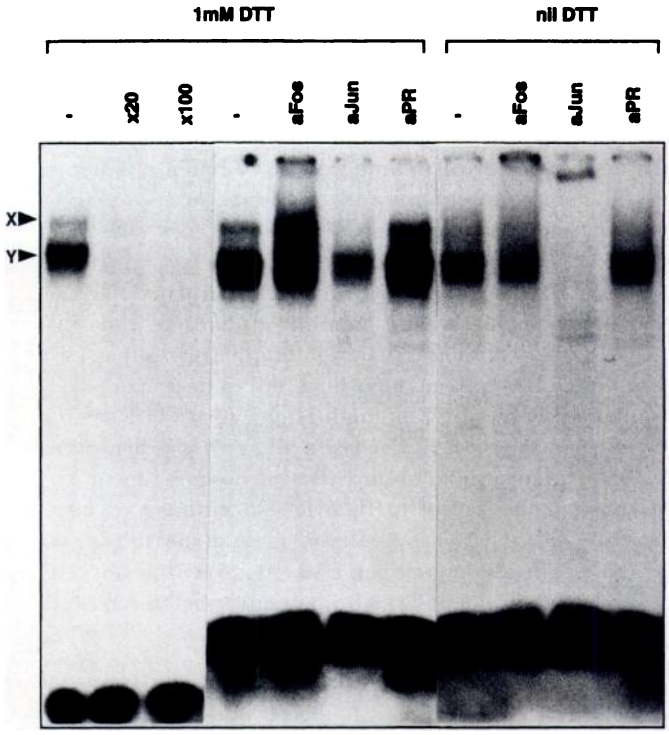

Fig. 1. Characterization of EMSA with T-47D nuclear extract and AP-1 probe. Nuclear protein (5 μg) and 1 μg of poly(dI/dC)-poly(dI/dC) were incubated with ^{32}P-labeled double-stranded oligonucleotide corresponding to the AP-1 consensus sequence and analyzed by nondenaturing polyacrylamide gel electrophoresis. *First seven lanes*, 1 mM DTT was added to the DNA binding reaction. *Remaining lanes*, no DTT was added to the DNA binding reaction, although a final concentration of 0.1 mM DTT was expected from the contribution of the nuclear extract. Sequence-specific binding of complexes X and Y was demonstrated by competition with 20- and 100-fold excess of unlabeled probe. Where indicated, nuclear extracts were preincubated with antibodies against Jun, Fos, and a control [progesterone receptor (*PR*)].

Results

Characterization of AP-1 DNA-binding activity. AP-1 DNA binding was measured by EMSA. Experiments performed with nuclear extract of T-47D cells revealed two sequence specific complexes, designated X and Y, which were efficiently competed with unlabeled AP-1 oligonucleotide (Fig. 1). The resolution and intensity of the specific complexes were enhanced in the presence of DTT in the DNA binding buffer. Anti-Jun antibodies directed against the DNA binding domain of Jun inhibited both of the specific

complexes. Anti-Fos antibodies caused trailing and diminution of the faster migrating complex (Y) without affecting the slower complex. An unrelated monoclonal antibody (antiprogesterone receptor) had no effect. It is concluded that the slower complex (X) represents the Jun homodimer and the faster complex (Y) represents a heterodimer of Jun and other proteins, including Fos. The incomplete effect of the anti-Fos antibody, even in the absence of DTT, may be due to the presence of Fos-related proteins: FosB, Fra-1, or Fra-2. Increasing the amount of anti-Fos did not significantly alter the effect, suggesting that sufficient antibody was present for saturation of its epitope.

Inhibition of AP-1 DNA binding by D-penicillamine requires Fenton reagents. The Fenton reaction was used as a source of free radicals to initiate the oxidation of thiols. In this system, Fe^{2+}/EDTA and H_2O_2 generate $^{\cdot}OH$ (27), which can oxidize thiols to form reactive thiyl radicals and, subsequently, disulfides. Ascorbate, which is sometimes used in this system (22), had no effect in these experiments with D-penicillamine (data not shown).

Nuclear extracts of T-47D cells were incubated with D-penicillamine and Fenton reagents (10 μM $FeCl_2$, 10 μM EDTA, 0.5 mM H_2O_2) and subjected to EMSA in the presence of radiolabeled AP-1 oligonucleotide (Fig. 2). Sequence-specific AP-1 DNA binding was inhibited by D-penicillamine only in the combined presence of Fe^{2+}/EDTA and H_2O_2. This effect occurred at 1 or 0.5 mM but not at 0.1 mM H_2O_2 (data not shown). In the absence of other agents, 0.5 mM H_2O_2 had only a slight inhibitory action that was not enhanced by D-penicillamine alone or by Fe^{2+}/EDTA alone. Furthermore, D-penicillamine and Fe^{2+}/EDTA, either individually or in combination, had no effect in the absence of H_2O_2. The necessity for all three components, D-penicillamine, Fe^{2+}/EDTA, and H_2O_2, strongly suggests that the thiyl radical of D-penicillamine is critical to the inhibitory effect on AP-1 DNA binding.

Comparison of D-penicillamine, cysteine, glutathione, and thiomalate. Other thiol agents were investigated for properties similar to those found for D-penicillamine. Sodium thiomalate (not shown) and glutathione (Fig. 3) did not inhibit AP-1 DNA binding but rather caused a slight increase in binding at concentrations of >100 μM, even in the presence of Fenton reagents. To confirm the opposite effects of different thiols, D-penicillamine and glutathione were compared in the same experiment under identical conditions in the presence of Fe^{2+}/EDTA and H_2O_2 (Fig. 3). Glutathione slightly enhanced AP-1 DNA binding in a concentration-dependent manner. D-Penicillamine markedly inhibited AP-1 DNA binding, with maximal effect in the concentration range of ~100-250 μM. This is a demonstration of the opposite effects that thiols are capable of exerting, depending on which thiol is used and whether free radicals are present. Cysteine had a biphasic inhibitory effect on AP-1 DNA binding in the presence of Fenton reagents, although the magnitude of inhibition was less pronounced than that occurring with D-penicillamine.

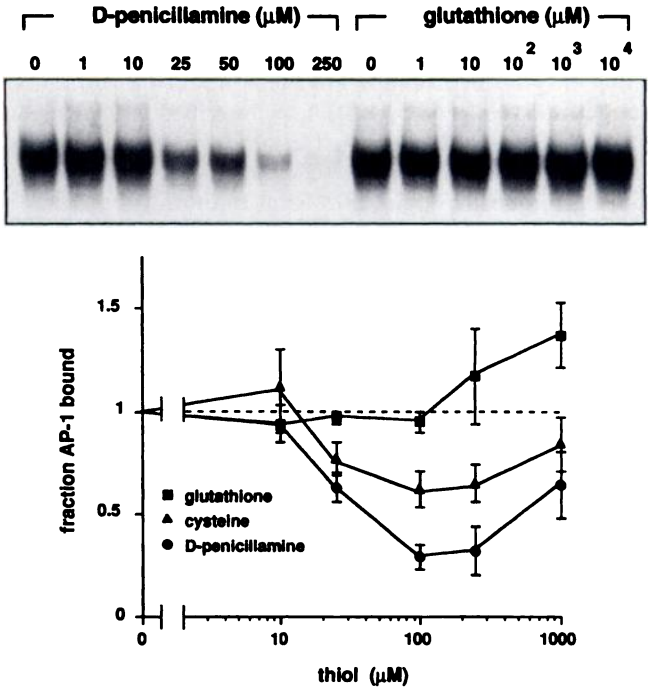

Fig. 3. Effect of different thiols on AP-1 DNA binding in the presence of Fenton reagents. *Top*, nuclear extracts were incubated with the indicated concentrations of D-penicillamine or glutathione in the presence of 10 μM $FeCl_2$/EDTA and 0.5 mM H_2O_2 and analyzed by EMSA with an AP-1 probe. *Bottom*, quantification of the concentration-dependent effects of glutathione, cysteine, and D-penicillamine on AP-1 DNA binding were determined by EMSA experiments. These results are the mean ± standard error of three independent experiments for each thiol agent.

Glutathione antagonized the effect of D-penicillamine in the presence of Fe^{2+}/EDTA and H_2O_2 (Fig. 4). The significant inhibition of AP-1 DNA binding by 100 μM D-penicillamine was antagonized in a concentration-dependent manner by coincubation with glutathione in the range of 10 μM to 1 mM.

Cysteine residues in the DNA binding domains of Jun and Fos are necessary for inhibition of AP-1 DNA binding by D-penicillamine. Each of the basic DNA binding domains of Jun and Fos possesses a cysteine residue.

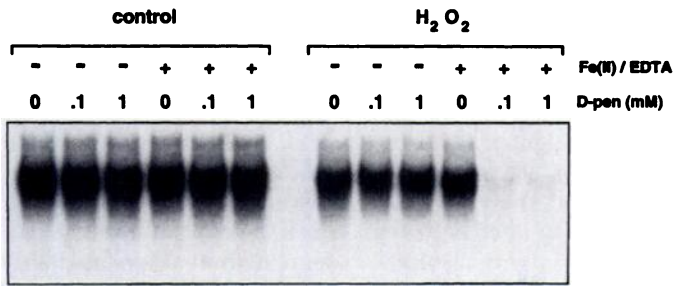

Fig. 2. D-Penicillamine (*D-pen*) inhibits AP-1 DNA binding only in the presence of Fenton reagents. Nuclear extracts were incubated with the indicated combinations of D-penicillamine (0.1 or 1 mM), 10 μM $FeCl_2$/EDTA, and 0.5 mM H_2O_2 and analyzed by EMSA with an AP-1 probe. Only the retarded complexes are shown.

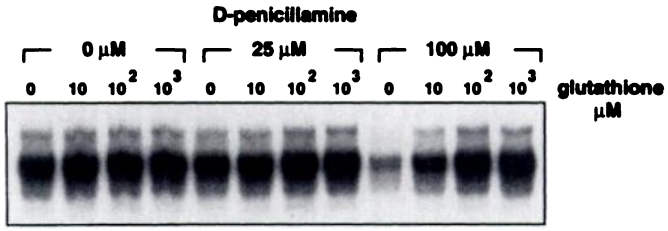

Fig. 4. Antagonism of D-penicillamine by glutathione. Nuclear extracts were incubated with the indicated combinations of D-penicillamine and glutathione in the presence of 10 μM $FeCl_2$/EDTA and 0.5 mM H_2O_2 and analyzed by EMSA with an AP-1 probe.

Truncated Jun and Fos proteins without mutations are designated J and F, and those with cysteine-to-serine mutations at these sites are designated JC272S and FC154S, respectively (20). Truncated Jun and Fos were mixed with nuclear extract and analyzed by EMSA as described previously (11). In these experiments, the faster-migrating AP-1 DNA binding complex contains truncated dimers, and the slower-migrating complex contains full-length Jun and Fos from the nuclear extract. As expected, D-penicillamine in the presence of Fenton reagents, Fe^{2+}/EDTA and H_2O_2, had a similar inhibitory effect on DNA binding by the full-length Jun/Fos complex and the truncated J-F complex (Fig. 5, *top*). In contrast, the complex containing the cysteine-to-serine mutant dimer JC272S/FC154S was resistant to the effects of D-penicillamine in the presence of Fenton reagents (Fig. 5, *bottom*). Therefore, cysteine residues in the DNA binding domains of Jun and Fos are the targets of D-penicillamine action in this system.

Discussion

D-Penicillamine is an end product of the metabolism of penicillin, and its *in vivo* reactions are predominantly a series of thiol/disulfide exchanges (28–30) that are analogous to the ligand exchanges of gold(I). Gold(I) and D-penicillamine can therefore be regarded as nondegradable agents that bind strongly, but reversibly, to endogenous thiols. D-Penicillamine and gold(I) thiomalate also have many similar clinical properties with regard to efficacy, adverse effects, and slow kinetics of response in the treatment of rheumatoid arthritis (31). The results of the current study with D-penicillamine extend our previous work with gold(I) thiolates (11) and provide a potential similarity in the molecular mode of action of these two drugs.

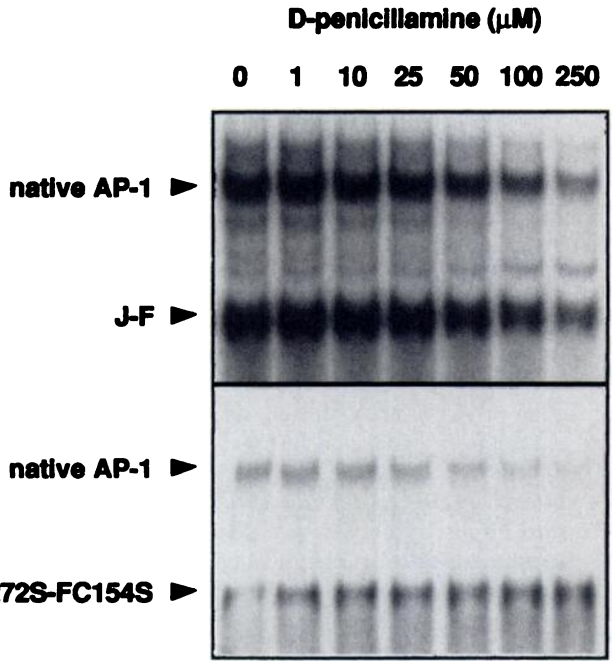

Fig. 5. Effect of D-penicillamine on truncated Jun and Fos mutants. Truncated proteins with the native sequence are designated J and F, and those with cysteine to serine mutations at Cys272 of Jun and Cys154 of Fos are designated JC272S and FC154S, respectively. The truncated proteins were mixed with T-47D nuclear extract in EMSA experiments. The slower-migrating complexes contain endogenous AP-1 DNA binding proteins, Jun and Fos, and the faster-migrating complexes contain truncated proteins (11). *Top*, truncated dimer J-F. *Bottom*, mutant truncated dimer JC272S-FC154S. Fenton reagents, 10 μM $FeCl_2$/EDTA and 0.5 mM H_2O_2, were present in all incubations. D-Penicillamine was present in increasing concentrations as indicated. Values are representative of four experiments with similar results.

The ability of thiols to act both as pro-oxidants and antioxidants has been well documented (21). At low concentrations of some thiols, the pro-oxidant effect may predominate, whereas at high concentrations, even though the rate of production of oxidizing thiyl free radicals may increase, sufficient reduced thiol is available for a net reducing effect (27). Some authors have sought to explain the mode of action of thiol drugs, including D-penicillamine, on the basis of reducing properties of thiols (32), whereas the experiments reported in the current study indicate that D-penicillamine can inhibit AP-1 DNA binding *in vitro* by an oxidative process. Much of the therapeutic activity could be explained if D-penicillamine is capable of inhibiting AP-1 DNA binding *in vivo* in the oxidative environment of inflammation. In patients receiving chronic D-penicillamine therapy of 750 mg/d, the serum concentration of D-penicillamine reaches 100 μM (33). Significant inhibition of AP-1 DNA binding *in vitro* occurs at this concentration of D-penicillamine (Fig. 3, *bottom*).

D-Penicillamine was distinguished from other thiols that have reducing or biphasic effects on AP-1 DNA binding. The potential for D-penicillamine to have a greater oxidizing effect than other thiols was reported in experiments testing the ability of drugs to protect α_1-antiproteinase from hydroxyl radical inactivation (22). Oxysulfur radicals of D-penicillamine were thought to be likely mediators of this effect.

Mutant Jun and Fos proteins have previously been used to demonstrate that binding of Jun/Jun and Jun/Fos dimers to the AP-1 site is inhibited *in vitro* by oxidation of the conserved cysteine residues in the DNA binding domains of Jun and Fos (20). We used these mutant proteins to verify that the targets for D-penicillamine action are the same cysteine residues in the Jun/Fos heterodimer. The same result is expected for the Jun/Jun homodimer. A common property of DNA binding proteins is a concentration of positively charged residues within their DNA binding domains. In the case of Jun and Fos, the cysteine residues in the DNA binding domains are each flanked by the positive residues lysine (K) and arginine (R), forming a KCR sequence. The pK_a and reactivity of a cysteine residue are influenced by electrostatic forces from neighboring charged amino acids. By application of the Debye-Huckel relationship, the effective charge at a cysteine residue in a random coil protein is calculated to approximate the sum of the charges of the cysteine residue and its two immediate neighbors (34). At an ionic strength of 20 mM, rate constants for disulfide formation with a cysteine residue having two positive neighbors, one positive and one neutral neighbor, or two neutral neighbors are 132,000, 3,350, and 367 sec^{-1} M^{-1}, respectively. In practice, this means that the cysteine residues in the DNA binding domains of Jun and Fos are expected to have greater sensitivity to oxidation and disulfide formation than other available cysteine residues, thus providing a potential physicochemical basis for the specificity of action of D-penicillamine. Other transcription factors with cysteine residues within their basic DNA binding domains, including nuclear factor-κB (35), are also candidates for such an action.

A ubiquitous heat labile nuclear redox factor that stimulates AP-1 DNA binding (36, 37) through the conserved cysteine residues has been identified and named Ref-1 (38). This reducing enzyme copurifies with Jun and Fos, indicating active maintenance of the reduced state and suggesting the notion that redox regulation of AP-1 DNA binding may be a physiological phenomenon. Pharmacological control of the redox state of Jun and Fos opens the possibility of regulating the expression of AP-1-dependent genes, particularly in situations in which endogenous thiols and other antioxidant defenses are depleted. Other transcription factors, particularly nuclear factor-κB, are regulated by redox state and may also be influenced by D-penicillamine. We have previously shown that gold(I) thiomalate inhibits the expression of a transfected AP-1-dependent reporter gene (11). Furthermore, AP-1 DNA binding *in vitro* is inhibited by gold(I) thiomalate and gold(I) thioglucose but not by thiomalate alone. These effects were mediated via the conserved cysteine residues and, unlike the action of D-penicillamine, were unaffected by the absence or presence of Fenton reagents. The results of the current study extend these findings with gold(I) by demonstrating that D-penicillamine has similar *in vitro* properties that may contribute to its mechanism of action *in vivo*.

Acknowledgments

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References

1. Hess, E. V., and M. Ziff. Effect of penicillamine on the titre of rheumatoid factor: a historical perspective. *Ann. Rheum. Dis.* **51**:422-423 (1992).
2. Jaffe, I. A. Comparison of the effect of plasmapheresis and penicillamine on the level of circulating rheumatoid factor. *Ann. Rheum. Dis.* **22**:71-76 (1963).
3. Multicentre Trial Group. Controlled trial of D(-)penicillamine in severe rheumatoid arthritis. *Lancet* **1**:275-280 (1973).
4. Golding, J. R., F. M. Andrews, V. Camp, A. T. Day, A. M. Freeman, D. N. Golding, A. G. Hill, W. H. Lyle, and A. G. Mowat. Controlled trial of penicillamine in severe rheumatoid arthritis. *Ann. Rheum. Dis.* **32**:385-386 (1973).
5. Huskisson, E. C., I. A. Jaffe, J. Scott, and P. A. Dieppe. 5-Thiopyridoxine in rheumatoid arthritis: clinical and experimental studies. *Arthritis Rheum.* **23**:106-110 (1980).
6. Martin, M. F., K. E. Surrall, F. McKenna, J. S. Dixon, H. A. Bird, and V. Wright. Captopril: a new treatment for rheumatoid arthritis? *Lancet* **1**:1325-1328 (1984).
7. Pasero, G., P. Pellegrini, U. Ambanelli, M. L. Ciompi, V. Colamussi, G. Ferraccioli, P. Barbieri, M. R. Mazzoni, G. Menegale, and D. Trippi. Controlled multicenter trial of tiopronin and d-penicillamine for rheumatoid arthritis. *Arthritis Rheum.* **25**:923-929 (1982).
8. Ishikawa, K., and M. Sakaguchi. SA96 (N-(2-mercapto-2-methylpropanoyl)-1-cysteine) in rheumatoid arthritis. *Scand. J. Rheumatol.* **15**: 85-90 (1986).
9. Rudge, S. R., D. Perrett, and M. Kelly. Failure of oral thiomalate to act as an alternative to intramuscular gold in rheumatoid arthritis. *Ann. Rheum. Dis.* **47**:224-227 (1988).
10. Bacon, P. A., and M. Salmon. Modes of action of second-line agents. *Scand. J. Rheumatol. Suppl.* **64**:17-24 (1987).
11. Handel, M. L., C. K. W. Watts, A. deFazio, R. O. Day, and R. L. Sutherland. Inhibition of AP-1 binding and transcription by gold and selenium involving conserved cysteine residues in Jun and Fos. *Proc. Natl. Acad. Sci. USA* **92**:4497-4501 (1995).
12. Rauscher, F. J., III, D. R. Cohen, T. Curran, T. J. Bos, P. K. Vogt, D. Bohmann, R. Tjian, and B. R. Franza Jr. Fos-associated protein p39 is the product of the jun proto-oncogene. *Science (Washington D. C.)* **240**:1010-1016 (1988).
13. Chiu, R., W. J. Boyle, J. Meek, T. Smeal, T. Hunter, and M. Karin. The c-Fos protein interacts with c-Jun/AP-1 to stimulate transcription of AP-1 responsive genes. *Cell* **54**:541-552 (1988).
14. Lee, W., A. Haslinger, M. Karin, and R. Tjian. Activation of transcription by two factors that bind promoter and enhancer sequences of the human metallothionein gene and SV40. *Nature (Lond.)* **325**:368-372 (1987).
15. Haslinger, A., and M. Karin. Upstream promoter element of the human metallothionein-IIA gene can act like an enhancer element. *Proc. Natl. Acad. Sci. USA* **82**:8572-8576 (1985).
16. Karin, M., A. Haslinger, A. Heguy, T. Dietlin, and R. Imbra. Transcriptional control mechanisms which regulate the expression of human metallothionein genes. *Exper. Suppl. (Basel)* **52**:401-405 (1987).
17. Scholer, H., A. Haslinger, A. Heguy, H. Holtgreve, and M. Karin. *In vivo* competition between a metallothionein regulatory element and the SV40 enhancer. *Science (Washington D. C.)* **232**:76-80 (1986).
18. Angel, P., M. Imagawa, R. Chiu, B. Stein, R. J. Imbra, H. J. Rahmsdorf, C. Jonat, P. Herrlich, and M. Karin. Phorbol ester-inducible genes contain a common cis element recognized by a TPA-modulated trans-acting factor. *Cell* **49**:729-739 (1987).
19. Angel, P., I. Baumann, B. Stein, H. Delius, H. J. Rahmsdorf, and P. Herrlich. 12-O-Tetradecanoyl-phorbol-13-acetate induction of the human collagenase gene is mediated by an inducible enhancer element located in the 5′-flanking region. *Mol. Cell. Biol.* **7**:2256-2266 (1987).
20. Abate, C., L. Patel, F. J. Rauscher III, and T. Curran. Redox regulation of fos and jun DNA-binding activity *in vitro*. *Science (Washington D. C.)* **249**:1157-1161 (1990).
21. Takagi, Y., M. Shikita, T. Terasima, and S. Akaboshi. Specificity of radioprotective and cytotoxic effects of cysteamine in HeLa S_3 cells: generation of peroxide as the mechanism of paradoxical toxicity. *Radiat. Res.* **60**:292-301 (1974).
22. Aruoma, O. I., B. Halliwell, J. Butler, and B. M. Hoey. Apparent inactivation of α_1-antiproteinase by sulphur-containing radicals derived from penicillamine. *Biochem. Pharmacol.* **38**:4353-4357 (1989).
23. Lipsky, P. E. Immunosuppression by D-penicillamine *in vitro*: inhibition of human T lymphocyte proliferation by copper- or ceruloplasmin-dependent generation of hydrogen peroxide and protection by monocytes. *J. Clin. Invest.* **73**:53-65 (1984).
24. Matsubara, T., and K. Hirohata. Suppression of human fibroblast proliferation by D-penicillamine and copper sulfate *in vitro*. *Arthritis Rheum.* **31**:964-972 (1988).
25. Handel, M. L., A. deFazio, C. K. W. Watts, R. O. Day, and R. L. Sutherland. Inhibition of DNA binding and transcriptional activity of a nuclear receptor transcription factor by aurothiomalate and other metal ions. *Mol. Pharmacol.* **40**:613-618 (1991).
26. Clarke, C. L., R. J. Zaino, P. D. Feil, J. V. Miller, M. E. Steck, B. M. Ohlsson-Wilhelm, and P. G. Satyaswaroop. Monoclonal antibodies to human progesterone receptor: characterization by biochemical and immunohistochemical techniques. *Endocrinology* **121**:1123-1132 (1987).
27. Munday, R. Toxicity of thiols and disulphides: involvement of free-radical species. *Free Radical Biol. Med.* **7**:659-673 (1989).
28. Joyce, D. A., D. N. Wade, and B. R. Swanson. The pharmacokinetics of albumin conjugates of D-penicillamine in rats. *Drug Metab. Dispos.* **17**: 208-211 (1989).
29. Joyce, D. A., and R. O. Day. D-Penicillamine and D-penicillamine-protein disulphide in plasma and synovial fluid of patients with rheumatoid arthritis. *Br. J. Clin. Pharmacol.* **30**:511-517 (1990).
30. Joyce, D. A. *In vitro* mechanism of oxidation of D-penicillamine in plasma. *J. Pharm. Sci.* **80**:289–292 (1991).
31. Thomas, M. H., N. O. Rothermich, V. K. Philips, W. Bergen, and S. W. Hedrick. Gold vs D-penicillamine double blind study and follow up. *J. Rheumatol.* **11**:764-767 (1984).
32. Huck, F., M. R. de, A. Lussier, G. Dupuis, and P. Federlin. Reducing property of some slow acting antirheumatic drugs. *J. Rheumatol.* **11**:605-609 (1984).
33. Muijsers, A. O., R. J. van de Stadt, A. M. A. Henrichs, H. J. W. Ament, and J. K. van der Korst. D-Penicillamine in patients with rheumatoid arthritis. *Arthritis Rheum.* **27**:1362-1369 (1984).
34. Snyder, G. H., M. J. Cennerazzo, A. J. Karalis, and D. Field. Electrostatic influence of local cysteine environments on disulfide exchange kinetics. *Biochemistry* **20**:6509-6519 (1981).
35. Toledano, M. B., and W. J. Leonard. Modulation of transcription factor NF-*kappa* B binding activity by oxidation-reduction *in vitro*. *Proc. Natl. Acad. Sci. USA* **88**:4328-4332 (1991).
36. Abate, C., D. Luk, and T. Curran. A ubiquitous nuclear protein stimulates the DNA-binding activity of fos and jun indirectly. *Cell Growth Differ.* **1**:455-462 (1990).
37. Frame, J. C., N. M. Wilkie, A. J. Darling, A. Chudleigh, A. Pintzas, J. C. Lang, and D. A. Gillespie. Regulation of AP-1/DNA complex formation *in vitro*. *Oncogene* **6**:205-209 (1991).
38. Xanthoudakis, S., and T. Curran. Identification and characterization of Ref-1, a nuclear protein that facilitates AP-1 DNA-binding activity. *EMBO J.* **11**:653-665 (1992).

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