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Studies on the Inhibition of Bovine Liver Dihydrofolate Reductase by Pyrimidine Compounds

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ABSTRACT

A series of 6-alky1-2,4-diaminopyrimidines (compounds 1-5) and 6-methylamino-2,4-diaminopyimidines (compounds 6-12), prepared as nonclassical folate antimetabolites, were subjected to inhibition study on bovine liver dihydrofolate reductase. The 2,4-diaminopyrimidine compounds showed IC_{50}'s at 10^{-7}-10^{-9}M, with compound 5 as the most acitve showing comparable activity to that of methotrexate. The triaminopyrimidine analogues 6-12 were much less active, with IC_{50}'s ranged between 10^{-4}-10^{-6}M.

Key words : 6-Alky1-2,4-diaminopyrimidines, 6-Methylamino-2,4-diaminopyrimidines, Inhibition of dihydrofolate reductase, Folate antimetabolites.

INTRODUCTION

Methotrexate (MTX), a classical dihydrofolate reductase (DHFR) inhibitor as an antitumor agent, has been used clinically for a long time.[1-3] However, its clinical efficacy is diminishing with development of drug resistance. The resistance occurrs in part, because of the reluctance of membrane transport protein in carrying MTX through the membrane.[4] To overcome the pharmacological deficiency and to treat cancer more effectively, the structure of MTX was modified and a series of 6-alky1-2,4-diamino-

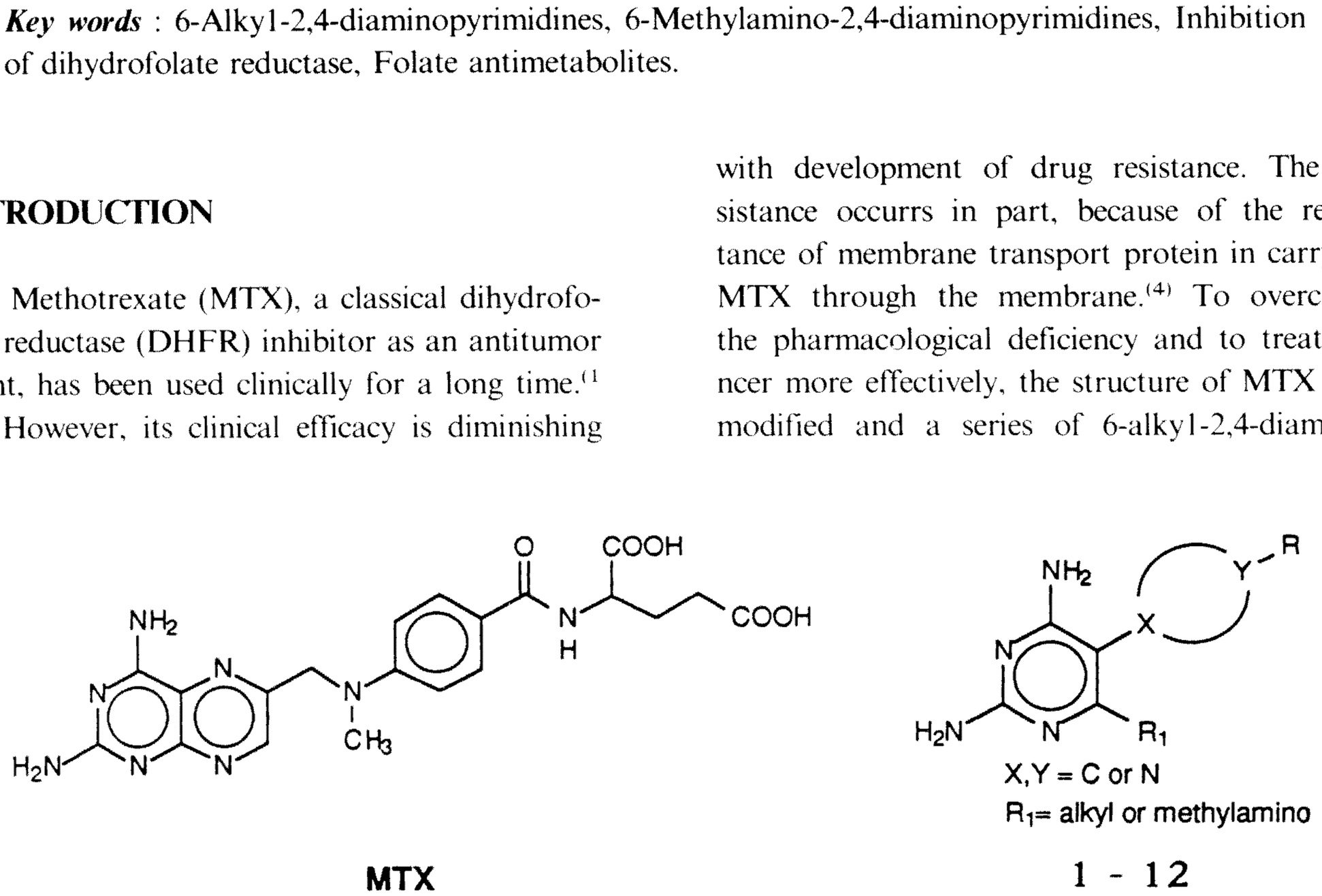

Figure 1. Structures of methotrexate and its open-ring analogues 1-12.

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pyrimidines (compounds 1-5)[5] and 6-methylamino-2,4-diaminopyrimidines (compounds 6-12) [6] (Figure 1) were prepared. These compounds were considered as open-ring analogues of MTX. The introduction of pyrimidinyl moiety into the molecule in replacing the pteridine ring of MTX was thought to eliminate conformational rigidity and, in consequence, to enhance the accessibility of the molecules into the active site of the target enzyme. The higher lipophilicity of these compounds compared to that of pteridine analogues is beneficial in terms of overcoming the drug resistance problem associated with MTX transport through the cell membrane. This report describes the inhibition study of compounds 1-12 on the target enzyme, using bovine liver dihydrofolate reductase as study model.

MATERIALS AND METHODS

I. *Materials*

Bovine liver dihydrofolate reductae, dihydrofolic acid and NADPH were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). One unit of enzyme is defined as the quantity of protein which converts 1.0 μmole of 7,8-dihydrofolic acid and NADPH to tetrahydrofolic acid and $NADP^+$ per minute at 20°C. The test compounds 1-12 were prepared previously.[5,6] Methotrexate was a gift from the Pharmacy Departement, National Taiwan University Hospital. Other chemicals, reagents and solvents were from E. Merck, Aldrich or Wako Companies and were of analytical grade.

II. *Preparation of Stock Solutions*

The following stock solutions were prepared prior to the assay: Stock solution A consisted of 0.01 M 2-mercaptoethanol in a 0.10 M pH 7.0 phosphate buffer. Stock solution B consisted of 0.01 M 2-mercaptoethanol and 5% (v/v) DMSO in a 0.10M pH 7.0 phosphate buffer. Stock solution C, designated as the substrate/cofactor solution, consisted of 6.75×10^{-4} M dihydrofolic acid and 1.10×10^{-3} M NADPH in stock solution A. Enzyme stock solution D was prepared by diluting 1 unit enzyme suspension in stock solution A which corresponded to 1-2 $\times 10^{-3}$ enzyme unit per assay solution. The inhibitor solutions were prepared by dissolving test compounds 1-12 and methotrexate, respectively, in a minimum amount of DMSO and then serially diluted with stock solution A and stock solution B so that the concentration of DMSO in the inhibitor solution was 4% (v/v).

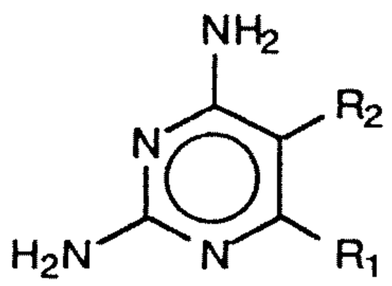
III. *Dihydrofolate Reductase Inhibition Assay*[7]

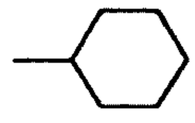
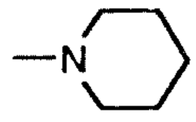
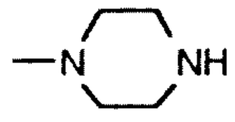
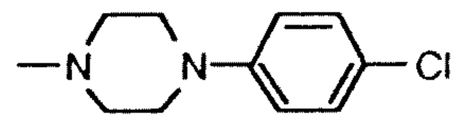
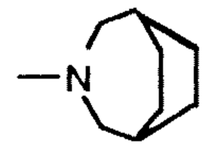
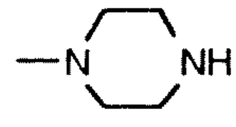
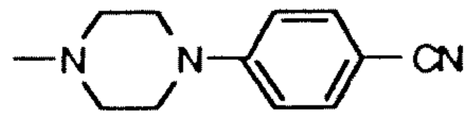
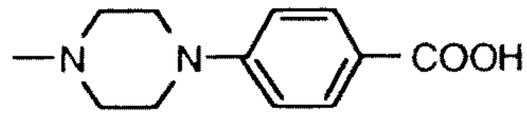
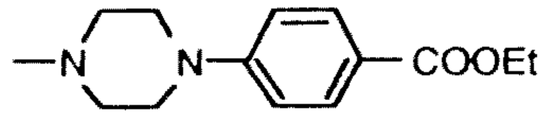
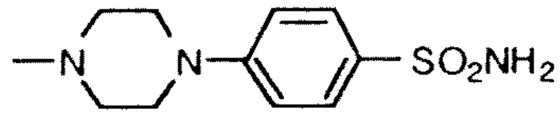
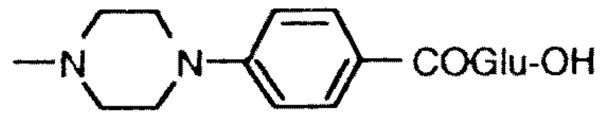
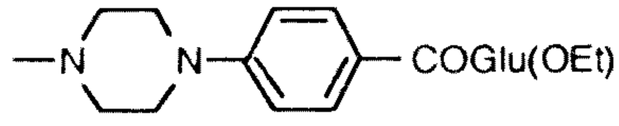
The assay system was a slight modification of that described by Burchall.[7] The absorption of the reaction mixture was performed in a Gilford Model 240S recording spectrophotomer at room temperature. A solution containing 0.1 mL of enzyme stock solution, x mL of inhibitor solution and (0.8-x) mL (x ranged from 0.1 to 0.8) of the stock solution A in a quartz cuvette was preincubated for five minutes. The substrate/cofactor stock solution, 0.1 mL was then added to initiate the reaction, and the absorbance at 340 nm was recorded for ten minutes. Therefore, the final assay solution contained, in a total volume of 1.0 mL, 0.10 M of phosphate buffer, 6.75×10^{-5} M dihydrofolic acid, 1.10×10^{-4} M of NADPH, 0.01 M of mercaptoethanol, 4% (v/v) DMSO, the enzyme and the test compounds. $\triangle A_{340}$ was defined as the absorbance change from zero to ten minutes of incubation. $\triangle A^{\circ}_{340}$ was the absorbance change of control assays, which contained all the components except the inhibitor, performed before and after the inhibition assay. The inhibition assays were repeated three to six times at at least six different concentrations evenly spaced over the concentration range that would cause 20%-80% of relative absorbance change (i.e. $\triangle A_{340}/\triangle A^{\circ}_{340}=0.2$-0.8).

IV. *Data Analysis*

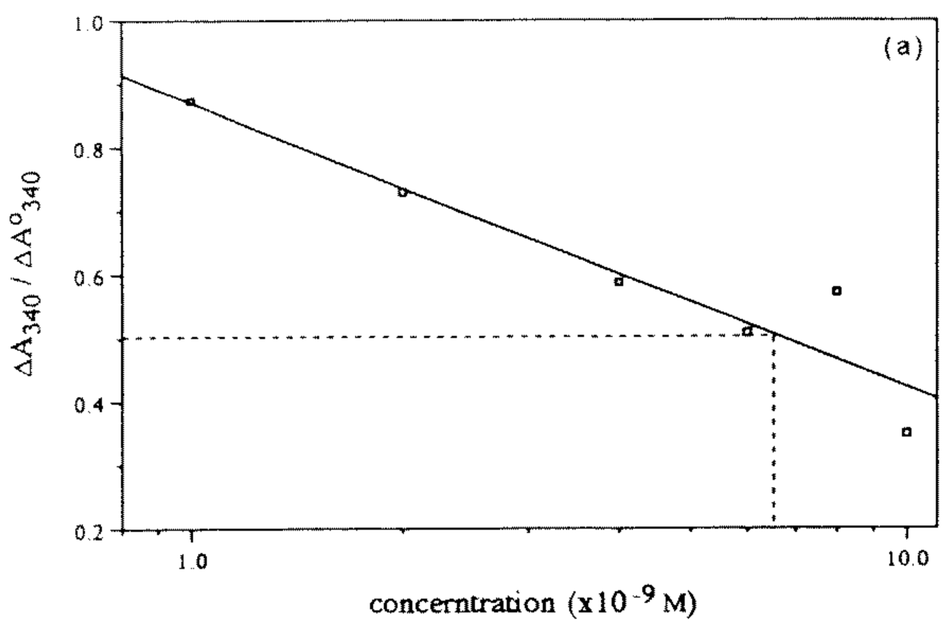
Relative absorbance change (i.e. $\triangle A_{340}/\triangle A$

Table 1. Summary of the inhibitory activity of compounds 1-12 and methotrexate on bovine liver dihydrofolate reductase.



compounds	R_1	R_2	IC_{50} (M)
1	CH_3		$5.17X10^{-7}$
2	CH_3		$6.13X10^{-8}$
3	CH_3		$6.23X10^{-8}$
4	C_2H_5		$3.76X10^{-8}$
5	C_2H_5		$6.73X10^{-9}$
6	$NHCH_3$		$3.23X10^{-5}$
7	$NHCH_3$		$1.10X10^{-4}$
8	$NHCH_3$		$5.90X10^{-6}$
9	$NHCH_3$		$4.03X10^{-6}$
10	$NHCH_3$		$4.34X10^{-4}$
11	$NHCH_3$		$4.49X10^{-6}$
12	$NHCH_3$		$8.22X10^{-6}$
MTX	—	—	$7.42X10^{-9}$

*Glu denotes glutamic acid residue.



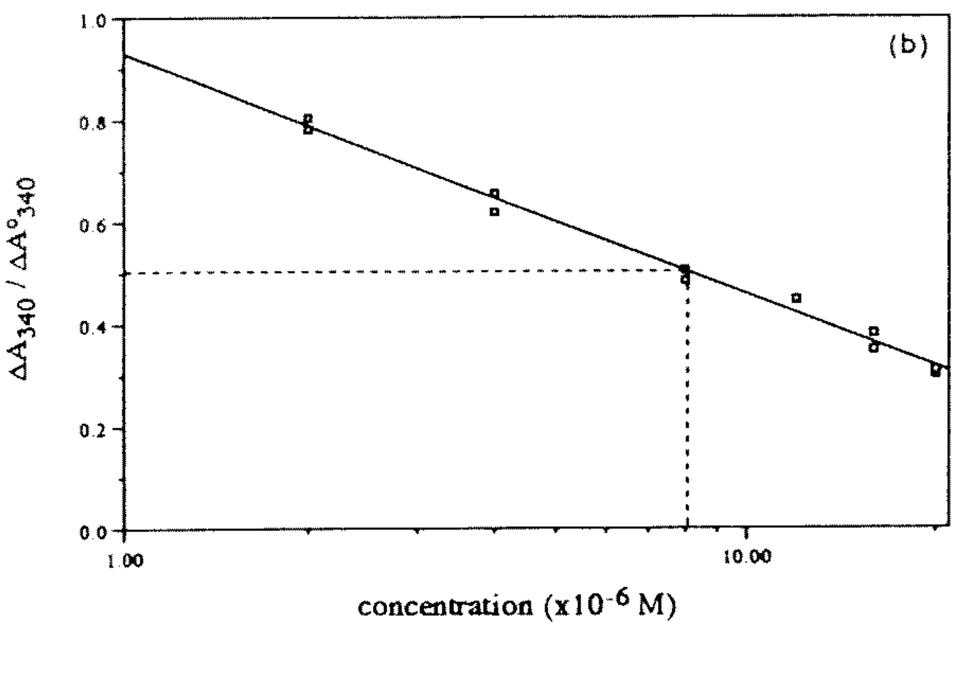


Figure 2. The inhibition profiles of (a) compound 5 and (b) compound 12 on DHFR.

$^{\circ}_{340}$) was plotted against the logarithm of the inhibitor concentration for each test compound. IC_{50} values were obtained from equation $\triangle A_{340}/\triangle A^{\circ}_{340} = a \log C + b$ derived from linear regression of the plot.

RESULTS AND DISCUSSION

Dihydrofolate reductase catalyzes the reversible reduction of 7,8-dihydrofolic acid to 5,6,7,8-tetrahydrofolic acid by NADPH, as shown below :

$$NADPH + H + 7,8\text{-dihydrofolic acid} \rightleftharpoons NADP^{+} + 5,6,7,8\text{-tetrahydrofolic acid}$$

The enzyme activity was determined by virtue of measuring the decrease in absorbance of NADPH at 340 nm (i.e. $\triangle A_{340}$). The correlation coefficient (R^2) from linear regression of the plot of $\triangle A_{340}/\triangle A^{\circ}_{340}$ versus the logarithm of inhibitor concentration ranged from 0.86 to 0.99 for the twelve compounds and MTX tested. Figure. 2a and 2b for compounds 1-12 obtained from equation $\triangle A_{340}/\triangle A^{\circ}_{340} = a \log C + b$ were listed in Table 1. As shown in Table 1, the 6-alkyl-2,4-diaminopyrimidine series of compounds 1-5 were more active in inhibiting the enzyme than the 6-methylamino-2,4-diaminolpyrimidine analogues 6-12. Among the series tested, the azabicyclo analogue 5 was the most active with activity comparable to that of methotrexate.

The inhibition of folate antimetabolites on dihydrofolate reductase has been studied extensively. The interaction between MTX and DHFR at molecular basis has been disclosed by the crystallographic structures of the MTX-DHFR (E. coli) binary complex[8] and the MTX-NADPH-DHFR (L. casei) ternary complex.[9] It has been demonstrated that the diaminopyrimidine portion of MTX is important for binding to the Asp-27 residue within the active site of the enzyme and the two carboxyl groups on the glutamate moiety were involved in a charge-charge interaction respectively with the His-28 and the Arg-57 residues of the enzyme. It is worth noting that even in the absence of glutamic acid side chain, our 6-alkyl-2,4-diaminopyrimidine series of compounds 1-5 showed high inhibitory activities. This result was very similar to the report on the interaction between DHFR and trimethoprim, a lipophilic diaminopyrimidine antifolate.

Based on the knowledge that the C-2 amino and the N-3 region of the pteridine ring on MTX was important for enzyme binding, the 2,4,6-triaminopyrimidine analogues 6-12 was prepared, with the thought that methylamino substitution at the C-6 position would increase the basicity of the pyrimidine ring and consequently would enhance the binding. However, the inhibitory acitvity of the series of compounds was much less than that of the analogues where an alkyl substituent was on the C-6 position. As IC_{50}'s were compared, the inhibitory activity of compound 6 was 10^3 fold lower than that of its methyl analogue, compound 3. These results

prompt an interesting question for further investigation on the interaction between the inhibitors and the target enzyme at the molecular level.

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嘧啶類化合物對二氫葉酸還原酶之抑制活性研究

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摘　要

本研究以一系列6-烷基-2,4-二胺基嘧啶化合物1-5以及6-甲胺基-2,4-二胺基嘧啶類化合物6-12作牛肝二氫葉酸還原酶之抑制試驗。結果顯示6-烷基-2,4-二胺基嘧啶化合物具有良好的抑制活性，其IC_{50}在10^{-7}-10^{-9}之間。其中以化合物5之活性最高，其活性與methotrexate相當。6-甲胺基-2,4-二胺基嘧啶類衍生物對該還原酶之抑制效果則很低，其IC_{50}在10^{-4}-10^{-6} M之間。