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Specific inhibition of Type II inosine monophosphate dehydrogenase activity of $Tmolt_4$ T cell human leukaemia cells by 3-methoxy and di-benzohydroxamic acids, maleic hydrazide and malonic acids

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Abstract

Small-molecular-weight benzohydroxamic and malonic acids and maleic hydrazide proved to be potent inhibitors of the activity of human $Tmolt_4$ leukaemia Type II IMP (inosine monophosphate) dehydrogenase (IMPDH) activity. They were competitive inhibitors with respect to IMPDH demonstrating K_i values in the range 2.57–41.3 μM, less than half the values of the IC50 (μM) for the inhibition of Type II IMPDH. The IC50 μM values positively correlated with the ability of each compound to inhibit crude IMPDH activity, de-novo purine and DNA syntheses and growth of the T leukaemia cell line. Compounds were not inhibitors of Type I IMPDH. Type I IMPDH predominates in normal resting cells compared with Type II which is found in rapidly proliferating cells. Discovery of agents which would selectivity target IMPDH found in proliferating cells should eliminate any antineoplastic therapeutic toxic effects in normal cells of the body.

Introduction

Benzohydroxamic acids and aliphatic dicarboxylic acids (i.e., malonic acids), are effective cytotoxic agents in murine and human tumours (Hall et al 1998, 1999). The agents inhibited in-vivo Ehrlich ascites carcinoma growth in CF_1 male mice at 8 mg kg^{-1} daily intraperitoneally, L1210 DNA and RNA syntheses, purine synthesis and IMP dehydrogenase (IMPDH) activity. Neither type of derivative was specific for these metabolic pathways at the exclusion of the other effects on nucleic acid metabolism. Nevertheless, IMPDH appears to be one of the targets where inhibition of activity by the malonic acids and the benzohydroxamic acids was $\sim 70\%$ and ~ 50–80%, respectively. The human IMPDH enzyme exists in two isoforms (Senda & Natsumeda 1994). Type I IMPDH predominates in resting normal cells while Type II IMPDH is induced in proliferating cells (Nagai et al 1991, 1992). These isoforms of IMPDH are indistinguishable in their catalytic activities, substrate affinities and K_i of known inhibitors (Wang et al 1996). Known IMPDH inhibitors, mycophenolic acid and ribavirin, did not differentiate between the two isoforms of IMPDH in $Tmolt_4$ cells (Dayton et al 1994; Hager et al 1995); however, diazabicyclohexanediones (Barnes et al 2000) are able to differentiate between the two isoforms of IMPDH, inhibiting only Type II. Thus, the different isoforms appear to be a good target for proposed new antineoplastic drugs (Natsumeda et al 1993). Since cloned human $Tmolt_4$ T cell leukaemia Type I and II IMPDH enzyme are available, the effects of these compounds on the enzymes were examined.

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Materials and Methods

Materials

Compounds **1**–**4** were purchased commercially. Compounds **5** and **6** were synthesized and reported previously (Hall et al 1998). All radioisotopes were purchased from New England Nuclear (Boston, MA) unless otherwise indicated. Substrates and cofactors were obtained from Sigma Chemical Co. (St Louis, MO). The pET (I) and pET(II) plasmids containing the previously cloned human Type I and II IMPDH from $Tmolt_4$ leukaemia cells were provided by Dr Beverly Mitchell (Departments of Pharmacology and Internal Medicine, UNC, Chapel Hill, NC).

Cytotoxicity

Compounds **1**–**6** (Figure 1) were prepared for cytotoxic activity by homogenizing a 50 μM solution in 0.05% Tween 80–H_2O (Table 1). $Tmolt_4$ acute lymphoblastic T cell leukaemia cells were maintained by literature techniques and the growth media and conditions were according to American Type Culture Collection protocols (Geran et al 1972). Cell numbers were determined by the trypan blue exclusion technique after three days incubation. The protocol of Geran et al (1972) was used to assess the suspended cell cytotoxicity of the compounds and standards. Values for cytotoxicity were expressed as ED50 (μM), the concentration of the compound inhibiting 50% of growth. A value $\leqslant 4\ \mu g\ mL^{-1}$ was required for significant inhibition of growth (Geran et al 1972).

Incorporation studies

The effects of agents **1**–**6** at 10–60 μM on the incorporation of ^{3}H-thymidine (24.1 Ci mmol^{-1}) into DNA were determined from 60-min incubations in 10^6 human $Tmolt_4$ cells (Hall et al 2000b). After inactivation with 10% perchloric acid, the acid-insoluble radiolabelled DNA was collected on Whatman filters GF/A which were counted in a Packard β counter. Radioactivity was determined in Fisher Scintiverse scintillation fluid with correction for quenching. The incorporation of ^{14}C-glycine (20 Ci mmol^{-1}) into purines was obtained via the method of Cadman et al (1981). The final purines synthesized were separated by TLC from starting components using the appropriate standard nucleoside bases and counted.

Inosine-monophosphate dehyrogenase crude enzyme assay

The effects of compounds **1**–**6** on IMPDH activity were determined (10–60 μM) after 60-min incubations of 10^6 human $Tmolt_4$ cells homogenized 10 × with a Dounce

Table 1 Effects of compounds **1**–**6** on human $Tmolt_4$ T cell leukaemia growth, DNA and purine syntheses and IMP dehydrogenase (IMPDH) activity (n = 6).

Compound (%)	ED50	DNA synthesis IC50	Purine synthesis IC50	IMPDH crude IC50	Type II IMPDH		Increase in Type I IMPDH activity
					IC50	K_i	
1	4.36	51.7	43.7	47.8	63.6	41.3	216
2	3.81	41.9	36.9	35.4	48.5	18.0	201
3	3.27	20.8	16.7	17.1	28.3	2.57	216
4	5.01	42.4	53.2	49.2	44.4	13.1	204
5	5.27	38.9	56.7	52.4	33.3	5.67	187
6	3.54	20.9	14.3	23.3	12.6	3.33	216
Ribavirin	12.1			81	79	37	–
6-Aza UMP	4.78				38	15	–
Mycophenolic acid	8.93				0.082	37	–
6MP	17.5						165

The ED50, IC50 and K_i values are estimates from graphs with ~ 3.5% error. Allopurinol was used as a negative standard in the enzyme assays. ED50, IC50 and K_i values are reported as μM. ED50 values were obtained at 3 days. IC50 values were determined out to 60 min. ED50 values (μM) for standards: cytosine arabinoside, 9.7; 5-fluorouracil, 21.1; and etoposide, 3.26.
Type I IMPDH data is reported for 100 μM of inhibitor. Percentage increase relative to control = 100%. 6 Aza-uridine is a competitive inhibitor of Type I with an IC50 of 41 μM and K_i 16 μM which is consistent with Hager et al (1995).

homogenizer. IMPDH activity was analysed by separating the starting material, 8-^{14}C-IMP (54 mCi mmol^{-1}; Amersham, Arlington Heights, IL), from the produced ^{14}C-xanthosine-5′-monophosphate (^{14}C-XMP) by TLC on PEI plates (Becker & Lohr 1979). The isolated ^{14}C-XMP was then counted in a Packard β counter as described above. Protein content was determined for the enzymatic assays (Lowry et al 1951). IC50 values were then estimated from a semilog plot of inhibitor concentration vs percent inhibition of enzyme activity for Type II IMPDH.

IMPDH enzyme assays

Recombinant human Type I and II IMPDH were prepared as described previously (Barnes et al 2000; Hall et al 2000b) and had specific activities of 0.88 and 0.70 μmol min^{-1} (mg protein)$^{-1}$, respectively, (> 99% purity) in the standard assay buffer at 37°C. The standard assay buffer consisted of 50 mM Tris-HCl (pH 8.0), 100 mM KCl, 3 mM EDTA, 1 mM DTT, 10–200 μM IMP, 30–500 μM NAD and enzyme. IMPDH activity was determined spectrophotometrically by monitoring the formation of NADH at 340 nm ($\epsilon_{340} = 6200$ M^{-1}cm^{-1}) with a Bausch & Lomb Spectronic 1001. Reactions were performed in 1-cm cuvettes and were initiated by the addition of purified enzyme.

Type-I and -II IMPDH enzyme inhibition

IC50 values for compounds were determined using purified recombinant Type I and II IMPDH. Inhibitors were tested against Type II activity over a concentration range of 5–50 μM prepared in 0.05% Tween 80–water by Dounce homogenization. Type I activity was tested for inhibition by the compounds at 100 μM. Analysis of inhibitor activity was performed with the standard assay buffer as described above consisting of 50 μM IMP, 170 μM NAD and Type II (20 μg), or 14.2 μM IMP, 42 μM NAD and Type I (6 μg) IMPDH enzyme, and inhibitors. Initial velocities were determined from continuous time courses by extrapolation of the linear portions of the curves to zero time so that the rates reflect conversion of less than 10% of the limiting substrate. Steady-state apparent kinetic parameters were evaluated by the direct fit of the Michealis-Menten equation to the initial velocity data vs substrate concentration using Prism 3 graphics (GraphPad, San Francisco, CA).

Kinetic analysis for Type-II IMPDH inhibition

Kinetic studies were conducted at 37°C in the standard assay buffer but with variable concentrations of inhibitor and IMP, while the NAD concentration was either at or below saturation. In addition, studies were conducted with IMP as the non-variable substrate. The type of inhibition (e.g., competitive, uncompetitive or noncompetitive; Figure 2) and K_m values were determined from Lineweaver-Burke plots at saturating and subsaturating concentrations of the non-variable substrate and increasing concentrations of inhibitor. K_i determinations (Figure 3) for inhibitor binding were obtained from linear plots of apparent K_m vs inhibitor concentration.

Results and Discussion

All of the compounds demonstrated potent inhibition of the growth of human Tmolt$_4$ leukaemia cells over 3 days and were generally better than known standards (Table 1). The IC50 values for the inhibition of Tmolt$_4$ DNA synthesis, purine synthesis and crude IMPDH activities for 60 min were generally of similar magnitudes. The IC50 values for the inhibition of isolated Type II IMPDH activity were similar to those values for inhibiting crude IMPDH activity. All six agents inhibited Type II IMPDH activity in a competitive manner with K_i values less than half of the IC50 value for each compound. These results were lower than that obtained for ribavirin (79 μM) and equal to that for 6-azaUMP (38 μM) but higher than that for mycophenolic acid (82 nM). As expected, ribavarin and 6-aza-UMP are competitive inhibitors of Type I IMPDH activity (IC50 = 121 μM and 41 μM, respectively). Surprisingly, the compounds (100 μM) did not inhibit, but significantly increased, Type I IMPDH activity, as did 6MP.

The correlation between the EC50 values for inhibition of Tmolt$_4$ leukaemia cell growth and the IC50 values for the inhibition of DNA synthesis was positive ($r^2 = 0.90$). In addition, the cell growth inhibition correlated positively with inhibition of purine synthesis ($r^2 = 0.89$). The correlation between the IC50 values for the inhibition of DNA and purine synthesis was also positive with $r^2 = 0.94$. The correlation between the IC50 values for the inhibition of purine synthesis and crude IMPDH activity was positive ($r^2 = 0.73$). These correlation results indicate that the modes of action of these compounds on the three metabolic events are linked and that a major target is the IMPDH enzyme, which suggests that they are functioning as antimetabolites of

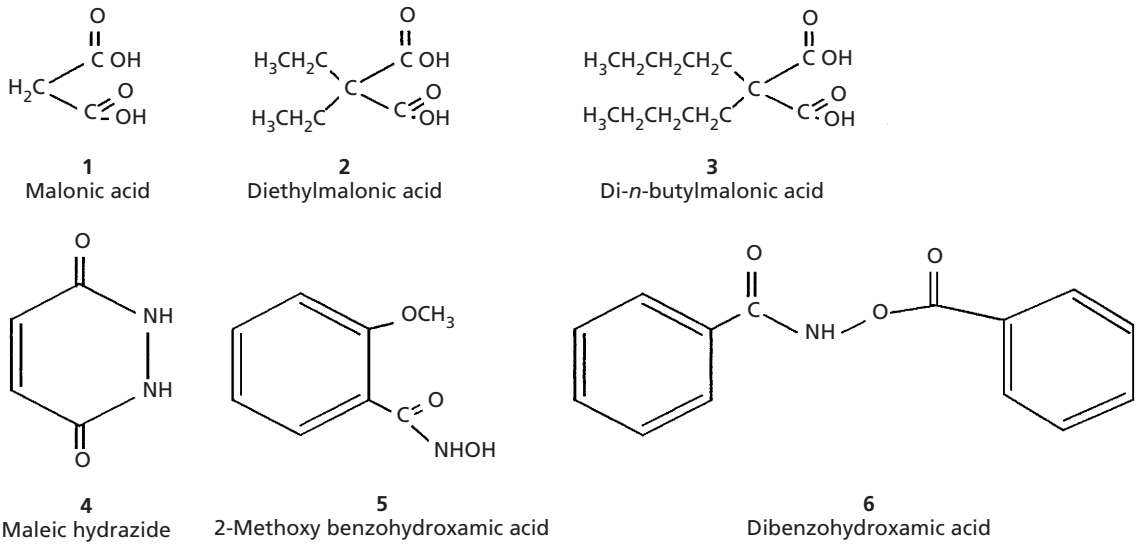

Figure 1 Structures of compounds **1**–**6**.

the de-novo purine pathway. Comparison of Type II IMPDH to crude IMPDH inhibition was also positive ($r^2 = 0.85$). Correlating K_i values for the competitive inhibitors with the IC50 values for Type II IMPDH inhibition resulted in an r^2 value of 0.79.

IMPDH inhibitors can be designed which are specific for the Type II isoform. Furthermore, agents can be designed which do not bind at the NAD site or are nucleotides that are more specific in their feedback regulation of the IMPDH enzyme. Mycophenolic acid, an uncompetitive inhibitor, was effective against both isoforms I and II at 82 and 100 nM, respectively, with a K_i of 37 μM for Type II. Recently in $Tmolt_4$ cells, 1,5-diazabicyclo(3.1.0)hexane-2,4-diones (Barnes et al 2000) were shown to be specific for Type II IMPDH with IC50 values of 22–63 μM and K_i values of 5–44 μM. The cyclic imides, phthalimide, saccharin, succinimide and indandiones, were shown to be inhibitors of Type II IMPDH with IC50 values of 9–125 μM and K_i values of 2.15–62.7 μM (Hall et al 2000a). Also, dihydrophthalazine-1,4-diones, indazolones, 3-imino-1-oxoisodolines, homo-phthalimides, napthalidimides, diphenamides, and dihydro-dibenz(c,e)azepines produced IC50 and K_i values from 28.5–112 μM and 1.96–48.9 μM, respectively (Hall et al 2000c). Triazolidinediones, azetidinediones, isoxazolidine diones and pyrazolidinediones gave values for IC50 of 4.2–38.1 μM and K_i of 0.74–38.5 μM (Hall et al 2000b). All of these derivatives actually elevated Type I activity, as did 6MP. The observed increase in Type I IMPDH activity was unexpected, although an increase has been previously demonstrated by the 3,3-disubstituted-1,5-diazabicyclo-(3.1.0)hexane-2,4-diones (Barnes et al 2000) by 10–15 % and 3,3-disubstituted-6,6-pentamethylene-1,5-diazabicyclo(3.1.0)hexane-2,4-diones. Results from molecular modelling studies analysing binding of the 1,5-diazabicyclo(3.1.0)hexane-2,4-diones to the Chinese hamster IMPDH crystal structure reveal that selectivity of these agents to the Type II IMPDH isoform, in particular, may be due to binding in a region extending outside the IMP phosphate binding site (Barnes et al 2000). Analysis of the amino-acid sequences of human Type I and Type II IMPDH indicates that a number of substitutions exist in the secondary layer surrounding the IMP active site of the enzyme, but not within the actual binding site for IMP. It has been hypothesized that binding in this region may lead to alterations in protein folding, thus globally changing the geometry of the active sites of the two enzymes, allowing ligands to bind in different manners (Barnes et al 2000) resulting in both agonist and antagonist actions. Computer modelling studies are underway with these new agents to investigate the possibility of differences in binding to the two isoforms of IMPDH. Like the benzohydroxamic acids, maleic hydrazide and malonic acids, certain IMPDH inhibitors appear to target the Type II isoform found in tumour cells without inhibiting Type I IMPDH activity. This may be of advantage in clinical cancer therapy in that normal cells, bone marrow and gastrointestinal mucosa would not be affected by the agents, whereas the agents would block exclusively tumour cell proliferation.

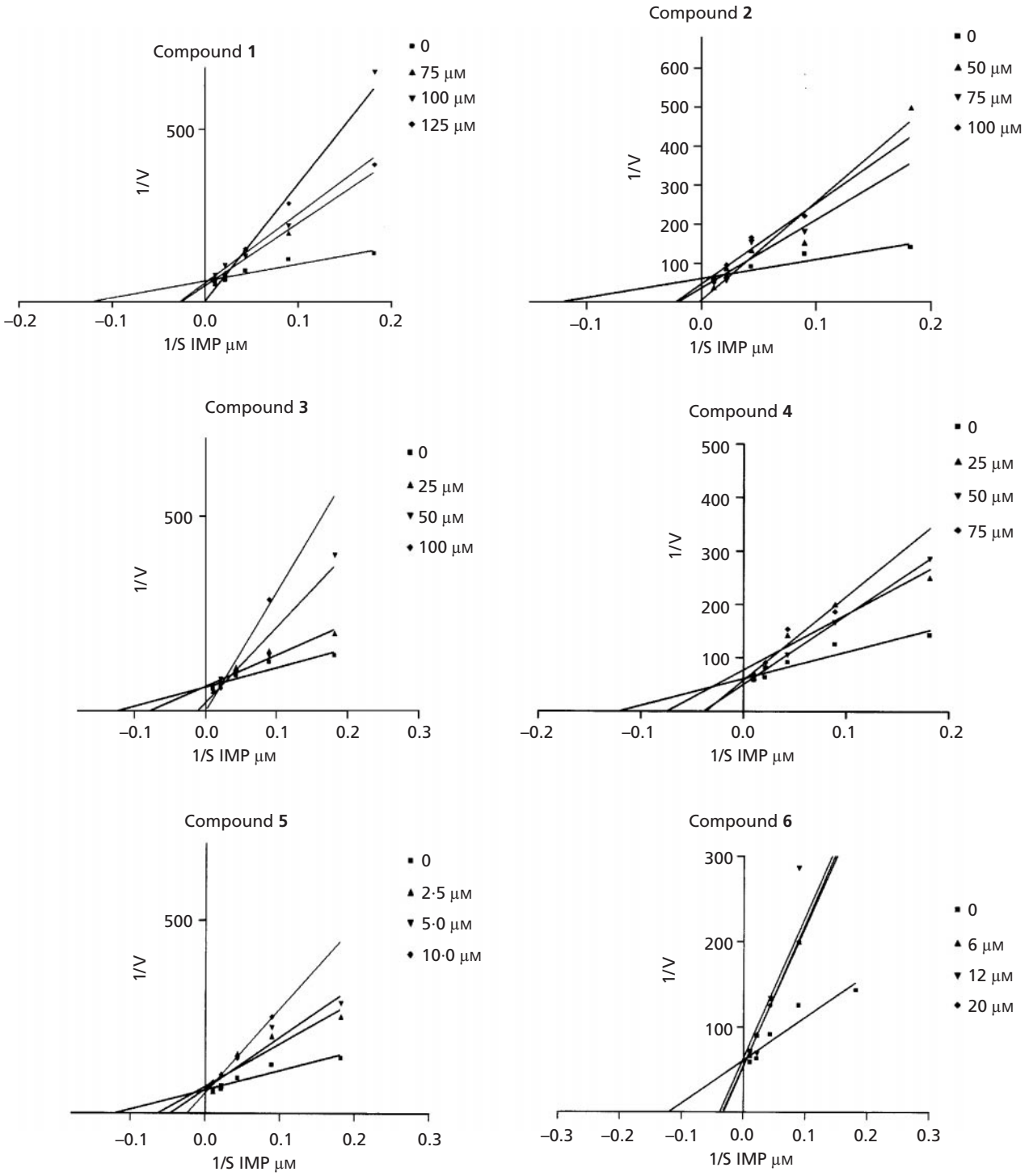

Figure 2 Lineweaver-Burke plots for the inhibition of Type II IMPDH activity by 3-methoxy and di-benzohydroxamic acids, maleic hydrazide and malonic acids. 1/v (O. D. density units (μg protein)$^{-1}$ min^{-1}) vs 1/S (μM substrate IMP).

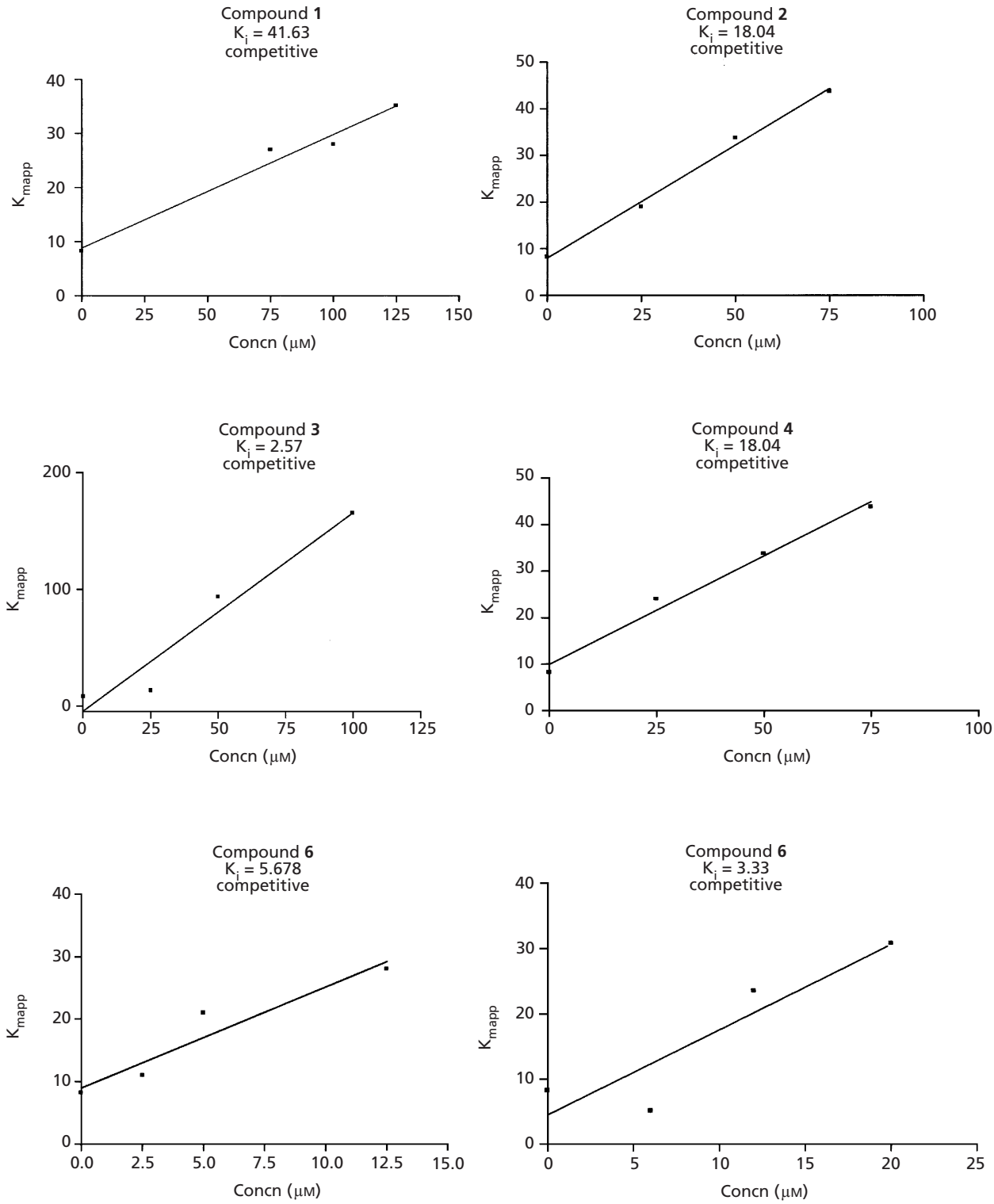

Figure 3 K_i (μM) plots for 3-methoxy and di-benzohydroxamic acids, maleic hydrazide and malonic acids. K_m apparent (μM) vs inhibitor concentration (μM).

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