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DISTRIBUTION AND METABOLISM OF THE PERIODATE OXIDATION PRODUCT OF 9-β-D-RIBOSYL-6-METHYLTHIOPURINE*

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Abstract—The periodate oxidation product of 9-β-D-ribosyl-6-methylthiopurine (MMPR-OP) was labeled with ^{35}S and compared with MMPR in distribution studies in mice. MMPR-OP was excreted faster than MMPR and was not converted to its nucleotide, but was bound to tissues and cleaved to methylthiopurine. The cleavage of MMPR-OP was largely due to chemical interactions with amino acids and cell constituents, as demonstrated in L1210 ascites tumor cells, immunologically-stimulated spleen cells and normal spleen cells. MMPR-OP inhibited the incorporation of glycine-2-^{14}C into the nucleic acids and proteins of spleen cell suspensions, although Schiff base formation with glycine-2-^{14}C complicated the results as shown by a comparison of two extraction procedures.

9-β-D-RIBOSYL-6-METHYLTHIOPURINE (MMPR§), a nucleoside analog with both carcinostatic[1] and immunosuppressive activity,[2] was oxidized with periodic acid to produce MMPR-OP, a compound that was active against a 6MP-resistant L1210 ascites tumor and that exhibited more selective immunosuppressive activity than did MMPR itself.[3] An L1210 line resistant to both 6MP and MMPR, however, was cross-resistant to MMPR-OP.[4]

Among the metabolic blockades produced by MMPR-OP,[3] a specific, sequential blockade in the incorporation of thymidine into the DNA of an Ehrlich ascites tumor was demonstrated.[5]

In connection with more detailed studies on the effects of MMPR-OP and other analogs on the humoral antibody response[6] and transplantation immunity,[7] the distribution and metabolism of ^{35}S-labeled MMPR-OP were studied in normal, neoplastic and antibody-synthesizing mouse tissues. This investigation compared the chemical and biological properties of MMPR-OP with those of MMPR.

MATERIALS AND METHODS

The L1210/MP-MMPR ascites tumor line was kindly supplied by W. R. Laster,

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§Abbreviations used: MMPR, 9-β-D-ribosyl-6-methylthiopurine; MMPR-OP, the periodate oxidation product of 9-β-D-ribosyl-6-methylthiopurine; MMP, 6-methylthiopurine; TCA, trichloroacetic acid; PCA, perchloric acid; 6MP, 6-mercaptopurine; Ara-6-MP, 9-β-D-arabinofuranosyl-6-mercaptopurine; SRBC, sheep red blood cells.

Jr., Head, Cancer Screening Division, Southern Research Institute, Birmingham, Ala. This line and the L1210/MP line were maintained by weekly transplantation in female AKD_2F_1 mice. These mice, as well as female AKR mice, were obtained from Jackson Laboratories, Bar Harbor, Me. Female Swiss mice were obtained from Simonsen Laboratories, Gilroy, Calif. All injections were given intraperitoneally. Data presented in the tables are the averages ± the deviations.

Chromatography on Whatman 3MM paper by the descending technique was performed with the following solvents: Solvent A: 5% Na_2HPO_4; Solvent B:5% KH_2PO_4; Solvent C: 95% ethanol–saturated NaB_4O_7–5 M NH_4OAc, pH9·5–0·25 M EDTA (660:240:60:3); Solvent D: 44% propionic acid–93·8% *n*-butanol mixed 1:1 before use.

Radioactivity on chromatograms was scanned by counting successive 1-cm strips in a toluene scintillator. A Nuclear-Chicago liquid scintillation counter was used.

Synthesis and properties of ^{35}S-labeled MMPR-OP. The thione group was exchanged with rhombic ^{35}S by the following method:[8] A suspension of 6MPR (240 mg, 0·845 μmole) and rhombic ^{35}S (24 mg, 0·685 milliatom; sp. act. 19 μc/μatom) was heated to reflux in 11 ml of dry pyridine for 3 hr, cooled and evaporated to dryness. The residue was taken up in 10 ml of hot water, the ^{35}S was filtered off and the filtrate was evaporated to dryness; this process was repeated two more times to ensure removal of all free ^{35}S. Crystallization from the final filtrate gave 205 mg of colorless ^{35}S-labeled 6MPR. This material was methylated according to the procedure of Fox *et al.*[9] to yield 196 mg of ^{35}S-labeled MMPR (4×10^6 cpm/μmole). After oxidation as previously described,[3] 155 mg of the lyophilized, amorphous ^{35}S-labeled MMPR-OP was isolated. Analyses were carried out on an unlabeled sample. The u.v. spectrum at pH 7 revealed maxima at 292 mμ (E = 18,800) and 223 mμ (E = 11,400). The u.v. and i.r. spectra of MMPR-OP were essentially the same as those of MMPR. The lack of carbonyl absorption in the i.r. spectrum of MMPR-OP in a nujol mull indicated a masking of the aldehyde groups, possibly by the mole of water present.

Anal. Calcd. for $C_{11}H_{12}N_4O_4S \cdot H_2O$(314·3); C, 42·04; H, 4·49; N, 17·83; S, 10·18. Found: C, 42·42; H, 4·16; N, 17·81; S, 9·96.

Chromatography of MMPR-OP-^{35}S in Solvent C showed that there was no MMPR-^{35}S present. However, chromatography in Solvents A and B revealed 0·6 and 0·2%, respectively, of MMP-^{35}S, apparently the result of cleavage during chromatography since this contaminant was not found in the MMPR-^{35}S.

Distribution studies. The ^{35}S-labeled drugs were given to the BDF_1 mice as single doses. Blood was removed from the heart and the various organs were rapidly chilled, weighed and extracted with 7 vol. of cold 5% TCA. An aliquot of each extract was neutralized for counting in Bray's scintillator.[10] Each value is the average of determinations on 2 mice and has been corrected for quenching. Urine from each of the mice was collected on filter paper; the radioactivity was eluted with water and an aliquot was counted. Another aliquot was chromatographed in Solvent A to assay for cleavage to MMP.

For determination of the amounts of TCA insoluble radioactivity bound to the tissues of AKR mice, each tissue was washed twice with 7 vol. of cold TCA and once with 7 vol. of ethanol; while still damp, each tissue residue was dissolved in hyamine hydroxide for counting in a toluene scintillator.[11] Each value is the average of determinations on 2 mice and has been corrected for quenching.

L1210 ascites tumor cells were washed four times with 10 vol. of Krebs–Ringer phosphate buffer (pH 7·4) at 0°. Then a 7-vol. ethanol extract and a 7-vol. 5% TCA extract were successively made and an aliquot of each taken for counting. The cell residues, extracted again with TCA and then ethanol, were dried, weighed and counted. The results were expressed as cpm/g of cell residue. Chromatographic analyses of the TCA and ethanol extracts from the L1210 cells were carried out in Solvents A, C, and D to assay for metabolites from MMPR-^{35}S and MMPR-OP-^{35}S. The R_f values were compared with those for MMPR-phosphate isolated from Ehrlich TGR II cells.[12]

Binding in vitro *to mouse tail skin.* Strips of tail skin (1·5 × 5 mm) from female AKD_2F_1 mice were incubated with various ^{35}S-labeled nucleoside analogs in Krebs–Ringer phosphate buffer (pH 7·4) for 0·5 hr at 37° in a Dubnoff shaking incubator. The skin slices were washed with cold Krebs–Ringe rbuffer until eluted radioactivity reached a minimum (five washes). The slices were blotted, weighed, homogenized in hyamine hydroxide and an aliquot counted in Bray's scintillator.

Studies in vitro *with spleen cells.* Preparation of spleen cell suspensions from Swiss mice, incubation with drugs, glycine-2-^{14}C ($5·5 \times 10^{-3}$M, 1 μc/μmole), glutamine (1×10^{-3}M) and glucose ($5·5 \times 10^{-3}$M) and the extraction procedure using 5% TCA at 100° have been described.[3] The extraction procedure using 10% NaCl at 100° is the same as that used by Gisler and Bell[7] except that the extraction was carried out at pH 7·4. Some mice were sensitized to tanned SRBC by the method of Nathan *et al.*[13] Cleavage of MMPR-OP-^{35}S to MMP-^{35}S in spleen cell suspensions from normal and SRBC-sensitized mice was studied in vessels containing $1·1 \times 10^{-2}$M glucose, 125 mg (wet weight) of cells, and 10^{-3}M MMPR-OP-^{35}S in 5 ml of Krebs–Ringer phosphate buffer, pH 7·4. After incubation with MMPR-OP-^{35}S for 1 hr at 37°, the cells were separated from the reaction solutions and washed once with cold Krebs–Ringer buffer. A 10-vol., 2% PCA extract was made at 0° and immediately neutralized to the brom cresol green end point (pH 5·4) with 2 M KOH. The precipitated $KClo_4$ was removed and the supernatant was evaporated *in vacuo* over H_2SO_4 and NaOH flakes. The residue was chromatographed for 5 hr in Solvent A along with MMP and MMPR-OP as carriers; either the fluorescent spots were cut out for counting or the whole chromatogram was cut into strips and counted. Percentage of cleavage was calculated as (cpm in MMP/cpm in MMPR-OP + metabolites) × 100 and is relative to that occurring during the extraction procedure.

In some experiments, part of the neutralized PCA extract was chromatographed in Solvent C along with carrier 6MP to check for the conversion of MMP to 6MP.

Cleavage studies in vivo. Four hr after a single injection of MMPR-OP^{35}S, the spleens of the treated mice were removed, weighed and then extracted as in the studies *in vitro*. L1210/MP-MMPR ascites cells (5-day growths) and ascitic fluid from MMPR-OP-treated AKD_2F_1 mice were extracted in the same way.

RESULTS AND DISCUSSION

Distribution studies. A comparison of the levels of radioactivity in the tissues and urine of BDF_1 mice for up to 3 hr after a single injection of MMPR-^{35}S or MMPR-OP-^{35}S is presented in Table 1. The following observations can be made:

(1) MMPR uptake is consistently higher than that of MMPR-OP in all tissues except the kidney, in which MMPR-OP is higher during the early part of the

TABLE 1. DISTRIBUTION OF MMPR-^{35}S AND MMPR-OP-^{35}S IN NORMAL MOUSE TISSUES AND URINE*

Time	Blood		Spleen		Liver		Kidney		Small intestine		Bone marrow		Urine	
	MMPR	MMPR = OP	MMPR	MMPR = OP	MMPR	MMPR = OP	MMPR	MMPR = OP	MMPR	MMPR = OP	MMPR	MMPR = OP	MMPR	MMPR = OP
3 min	0·9	2·1	14·5	4·8	9·5	3·9	4·4	6·4	8·0	4·8	0·4	0·4		
15 min	8·5	2·8	13·3	4·0	33·2	3·1	5·8	7·7	10·2	6·4	1·0	0·9	2·2	12·3
30 min	11·4	2·3	7·8	2·0	27·3	2·3	2·8	5·0	8·3	3·0	1·3	0·8	2·8	30·5
1 hr	13·8	1·6	9·8	1·2	32·3	1·2	2·8	2·1	9·2	1·1	2·2	0·5	2·8	45·5
3 hr	15·7	1·0	9·2	0·5	29·0	0·5	2·6	0·6	9·2	4·5	2·6	0·5	11·6	63·3

*BDF_1 mice (23 g) were given MMPR-^{35}S, 50 mg/kg, or MMPR-OP-^{35}S, 50 mg/kg, and sacrificed at the specified times. The figures for the tissues are given as per cent of dose per g wet weight. The figures for the urine are given as per cent of dose in the total urine collected.

TABLE 2. COMPARISON OF THE DISTRIBUTION AND METABOLISM OF MMPR-^{35}S AND MMPR-OP-^{35}S IN L1210/MP AND L1210/MP-MMPR ASCITES CELLS *IN VIVO**

Time (hr)	% of Dose in total ascitic fluid		% of Dose/g cell residue					
			EtOH extract		TCA extract		Cell residues	
	L1210/MP	L1210/MP = MMPR	L1210/MP	L1210/MP = MMPR	L1210/MP	L1210/MP = MMPR	L1210/MP	L1210/MP = MMPR
				95 mg/kg MMPR-^{35}S				
1	2	12	60	24	127	15	0·6	0·4
5	0·8	1	28	2	64	4	0·6	0·4
20	0·5	1	27	1	48	1	0	0
				100 mg/kg MMPR-OP-^{35}S				
1	20	24	48	70	13	14	52	66
5	9	14	24	20	12	5	53	63
20	2	5	7	3	6	2	27	15

*Tumor bearing mice were given either MMPR-^{35}S or MMPR-OP^{35}S in equimolar amounts and sacrificed for analysis at the specified times. Each value is the average of determinations on two mice. The tumors were used 5 days after transplantation, at which time approximately 0·3 ml of ascites cells were present in each mouse.

period. This correlates with the higher rate of urinary excretion of MMPR-OP as compared to MMPR. Chromatographic analysis of the urine from MMPR-OP-^{35}S-treated mice showed that 95 per cent of the radioactivity was due to MMPR-OP-^{35}S, and 5 per cent was due to MMP-^{35}S.

(2) The levels of MMPR were the highest in the liver, a tissue in which it is most efficiently phosphorylated.[14]

(3) Although MMPR levels vary from tissue to tissue due to different levels of phosphorylation,[14] MMPR-OP levels do not show much variation.

(4) Whereas MMPR levels in tissues are maintained over the period tested, MMPR-OP levels gradually fall.

Further correlations between phosphorylation and tissue distribution are brought out in Table 2, which present the results on 2 L1210 ascites tumors—one resistant only to 6MP (L1210/MP) and the other resistant to both 6MP and MMPR (L1210/MP-MMPR). The L1210/MP tumor is sensitive to MMPR-OP[3] while the L1210/MP-MMPR tumor is resistant to MMPR-OP.[4] Lower MMPR levels in the ascitic fluid of mice bearing MMPR-treated L1210/MP cells indicate greater drug uptake by these cells than by L1210/MP-MMPR cells. In contrast, the levels of radioactivity in the ascitic fluid of mice bearing either of the two MMPR-OP treated L1210 cell lines were nearly the same. MMPR levels were higher in ethanol extracts taken from MMPR-treated L1210/MP cells than in those from MMPR-treated L1210/MP-MMPR cells. The same was true in TCA extracts and chromatographic analysis revealed that the radioactivity from the L1210/MP cells was present as MMPR-phosphate; the total from both extracts was 1·5 μmoles/hr/ml of cells. The rate of phosphorylation was only 5 per cent of this level in L1210/MP-MMPR cells; this is similar to findings in other MMPR-resistant mammalian cells.[14] These differences were not found in the extracts of MMPR-OP-treated cells, in which levels of radioactivity were not consistently higher in L1210/MP cells than in L1210/MP-MMPR cells; paper chromatograms revealed only MMP and MMPR-OP; no ^{35}S-labeled materials was found in areas to which a phosphate derivative would be expected to move.

In addition, cell-free extracts of L1210/MP cells,[12] which produced 0·5 mμmoles of MMPR-phosphate/min/mg of protein, did not produce any metabolites from MMPR-OP-^{35}S. Thus, the data presented support the conclusion that MMPR-OP is not phosphorylated, but that some of it is cleaved to MMP. Conversion of MMP to MMPR-phosphate was not detected.

Binding of MMPR-OP-^{35}S to mouse tissues. A difference between MMPR and its oxidation product was found in the amounts remaining on L1210 cell residues after the TCA extractions (Table 2): there was a significant amount of radioactivity bound to the residues of MMPR-OP-^{35}S-treated L1210 cells, which may be due to the ability of aldehydes to form Schiff base derivatives with free amino groups on proteins and nucleic acids. This binding was not found in MMPR-^{35}S-treated cells. A low level of radioactivity, both acid-soluble and acid-insoluble, was still present in the tissues of AKR mice 4 days after the injection of MMPR-OP-^{35}S (Table 3). Of special relevance to skin graft studies in these mice[7] was the presence of radioactivity in their tail skin. Binding to tail skin slices could be demonstrated *in vitro*; the data in Table 4 show that the amount of MMPR-OP remaining after thorough washing with pH 7·4 buffer was three to four times that of either MMPR or Ara-6-MP. Low levels of

Ara-6-MP have been previously shown to be bound to mouse proteins *in vivo*,[15] and sheep erythrocyte membranes *in vitro*.[16]

Binding in vitro *of MMPR-OP-^{35}S to spleen cells and effects of glycine metabolism.* Since the binding of certain ketoaldehydes to cell constituents has been shown to be related to antiviral activity,[17] studies with mouse spleen cells *in vitro* were carried out in connection with studies on the immunosuppressive activity of MMPR-OP on

TABLE 3. LONG TERM DISTRIBUTION OF MMPR-OP-^{35}S IN AKR TISSUES*

	% of Dose/g of tissue (wet wt.) 4 days after injection	
	Acid = Soluble	Acid = Insoluble
Whole blood	0·64	0·07
Ascitic fluid	0·12	0·36
Thymus	0·11	0·32
Spleen	0·94	0·70
Kidney	0·34	0·61
Small intestine	0·12	0·60
Liver	0·11	0·18
Tail skin	0·31	0·76
Bone marrow	1·21	0·57

*AKR mice (24–31 g) were given MMPR-OP-^{35}S, 65 mg/kg. After 4 days the mice were sacrificed and the TCA extractions were carried out as in Table 1.

TABLE 4. COMPARISON OF THE BINDING OF NUCLEOSIDE ANALOGS TO MOUSE TAIL SKIN *IN VITRO*

Labeled nucleoside	Specific activity (cpm/μmoles)	mμmoles Bound/mg skin (wet wt.)
Ara-6-MP-^{35}S	$2{\cdot}39 \times 10^6$	$0{\cdot}572 \pm 0{\cdot}069$
MMPR-^{35}S	$4{\cdot}58 \times 10^6$	$0{\cdot}574 \pm 0{\cdot}130$
MMPR-OP^{35}S	$4{\cdot}82 \times 10^6$	$1{\cdot}97 \pm 0{\cdot}10$

*Each nucleoside was incubated at 2×10^{-3}M for $\frac{1}{2}$ hr. Each value is the average of analyses of three skin slices. The difference between MMPR and MMPR-OP binding is significant at the 0·1 per cent level.

these cells.[6] MMPR-OP at 1×10^{-3}M was previously reported to have inhibited the incorporation *in vitro* of glycine-2-^{14}C into spleen cell proteins yet apparently to have stimulated incorporation into nucleic acids isolated by extraction with 5 per cent TCA at 100°.[3] The data in Table 5 show this stimulatory effect to be an artifact of the procedure, possibly due to the binding of MMPR-OP-^{35}S to the various fractions isolated. When the nucleic acids were extracted with 10% sodium chloride at 100°, MMPR-OP was found to inhibit the incorporation of glycine-2-^{14}C into nucleic acids

TABLE 5. EFFECTS OF MMPR-OP ON THE INCORPORATION OF GLYCINE-2-^{14}C INTO THE NUCLEIC ACIDS AND PROTEINS OF SPLEEN CELLS; A COMPARISON OF EXTRACTION PROCEDURES AND THE DISTRIBUTION OF MMPR-OP-^{35}S IN THE FRACTIONS ISOLATED*

Isolation of nucleic acids with hot TCA			Isolation of nucleic acids with hot NaCl		
Fraction	Incubation with MMPR-OP-^{35}S and glycine (mμmoles ^{35}S/25 mg cells)	Incubation with MMPR-OP and glycine-2-^{14}C (% of control cpm)	Fraction	Incubation with MMPR-OP^{35}S and glycine (mμmoles ^{35}S/25 mg cells)	Incubation with MMPR-OP and glycine-2-^{14}C (% of control cpm)
First TCA wash	74		First TCA wash	71	
Second TCA wash	8		Second TCA wash	7	
			First ethanol wash	36	
			Second ethanol wash	6	
			100°C NaCl extract:		
			(a) Supernatant	25	
100° TCA extract (nucleic acids)	65	343	(b) EtOH precipitated sodium nucleates	1	41
TCA wash	6		100° TCA extract	27	
Ethanol wash	4		Ethanol wash	2	
Residual proteins	38	22	Residual proteins	18	37
Total	113		Total	115	

*Swiss mouse spleen cells (25 mg wet weight) were incubated 15 min at 37° with MMPR-OP-^{35}S (1×10^{-3}M)) before glycine was added. After another 45 min of incubation, 0·22 ml of 50% TCA was added to the 2-ml reaction mixture at 0° and the supernatant was discarded. The indicated extracts were then carried out at 0° unless otherwise noted. Incubations with glycine = 2-^{14}C and MMPR-OP (1×10^{-3}M) were carried out in triplicate and the results were averaged; control vessels not containing MMPR-OP were incubated 15 min before adding glycine-2-^{14}C.

and proteins to the same extent. No radioactivity from cell-bound MMPR-OP-^{35}S was present in the ethanol precipitated nucleic acids as opposed to the large amounts found in the 5% TCA used originally for nucleic acid extraction at 100°.

If spleen cell suspensions from Swiss mice, treated 3 days before with 65 mg per kg MMPR-OP, were incubated 1 hr with glycine-2-^{14}C, an apparent stimulation of the incorporation of glycine-2-^{14}C into the nucleic acids and proteins isolated by the hot TCA procedure was observed; if the hot sodium chloride procedure was used instead, glycine-2-^{14}C incorporation was the same as that in spleen cells from saline-treated mice. Thus, the radioactivity found in mouse spleens several days after injection with MMPR-OP-^{35}S (Table 3) was not effecting the nucleic acid or protein synthesis of the spleen cells *in vitro*.

When the spleen cell suspensions, which had been treated with MMPR-OP-^{35}S as in Table 5, were washed with Krebs–Ringer buffer until no more radioactivity was eluted (four washes) and then laked in distilled water according to the method of Kimball *et al.*,[16] there were 52 mμmoles of intracellular drug and 38 mμmoles of membrane-bound drug present. These results show that the radioactivity appearing in the extracts listed in Table 5 resides on the surface as well as inside spleen cells.

Interaction in vitro *of MMPR-OP with glycine.* Since it is possible that much of the MMPR-OP on the surfaces of spleen cells incubated with glycine-2-^{14}C could be bound through one aldehyde group, allowing the other aldehyde group to react with free glycine-2-^{14}C, one should find this glycine-2-^{14}C artifact in the hot TCA extract of Table 5. Evaporation of this extract and chromatography in Solvent A showed that free glycine-2-^{14}C was not present. Attempts to isolate the glycine-2-^{14}C by reaction with fluorodinitrobenzene according to the procedure of Perrone[18] failed, probably due to the presence of MMPR-OP and its degradation products.

Evidence for a reaction between glycine and MMPR-OP-^{35}S under physiological conditions was obtained by incubation of these two chemicals in Krebs–Ringer phosphate buffer (pH 7·4) at 37° (Table 6). MMPR-OP-^{35}S was stable alone, but varying amounts of glycine caused the appearance of a new component as well as smaller amounts of MMP-^{35}S. Since this component at R_f 0·68 contained glycine, as shown by parallel experiments with glycine-2-^{14}C and MMPR-OP, it is reasonable to assume that this product is the mono- or di-Schiff base derivative of MMPR-OP. Evidence that this is the intermediate that yields MMP and sugar aldehyde fragments comes from studies on the liability of the periodate oxidation products of nucleosides in glycine buffers;[19] the purine or pyrimidine base, as well as glyceraldehyde and glyoxal, were produced.[19]

Cleavage of MMPR-OP-^{35}S by cells in vitro *and* in vivo. Considerable cleavage of MMPR-OP to MMP was found to occur in both L1210 ascites cells and spleen cells. Solvent A or B was used to separate MMP from MMPR-OP and Solvent C or D was used to separate any 6MP from MMP. 6MP has not been found in MMPR-OP-treated cells either *in vitro* or *in vivo*.

A 100-mg/kg dose of MMPR-OP-^{35}S was given to AKD_2F_1 mice bearing L1210/MP-MMPR ascites cells and the radioactivity remaining in the L1210 cells 1 hr later was analyzed for MMP-^{35}S; 18 per cent of the radioactivity inside these cells was MMP-^{35}S while only 0·2 per cent of the radioactivity in the surrounding ascitic fluid was MMP-^{35}S. These percentages, as found in PCA extracts, have been corrected for 2·4 per cent cleavage of MMPR-OP-^{35}S to MMP-^{35}S in 2% PCA alone,

which is increased to 8·7 per cent cleavage when either L1210 ascites cells, the ascitic fluid surrounding these cells, or spleen cells are added.

It appears that the cleavage of MMPR-OP *in vivo* could also be due to nonspecific interactions with cell constituents, as supported by the cleavage studies *in vitro* with glycine and MMPR-OP at pH 7·4 (Table 6). It was thought, therefore, that cleavage would be higher in tissues more actively engaged in protein synthesis. However, in

TABLE 6. INTERACTION OF GLYCINE AND MMPR-OP-^{35}S AT pH 7·4*

Ratio of glycine to MMPR-OP-^{35}S	% Cleavage to MMP-^{35}S	% Formation of a ^{35}S-intermediate at R_f 0·68
1:20	0·15	0
1:2	0·65	1·50
1:1	1·18	3·52
5:1	3·82	13·1

*MMPR-OP^{35}S, 1×10^{-3}M, was incubated in Krebs–Ringer buffer 1 hr at 37° with the indicated relative amounts of glycine. Results are calculated as

$$\frac{\text{cpm in product}}{\text{cpm in MMPR-OP + products}} \times 100$$

and are relative to the amounts produced during paper chromatography in Solvent A. MMPR-OP-^{35}S appears at R_f 0·58 and MMP-^{35}S appears at R_f 0·32.

TABLE 7. CLEAVAGE OF MMPR-OP-^{35}S BY SPLEENS CELLS *IN VIVO* AND *IN VITRO**

Treatment	% Cleavage to MMP-^{35}S	
	Normal	SRBC-stimulated
65 mg/kg MMPR-OP-^{35}S *in vivo*, analyze 4 hr later	17·1 ± 5·9	8·6 ± 3·8
1×10^{-3}M *in vitro*, incubate 1 hr	14·0 ± 0·1	14·0 ± 0·2

*Each result *in vivo* is the average of determinations on seven Swiss mice. The difference between the two values *in vivo* is significant at the 5 per cent level. The amount of MMPR-OP per g of tissue (wet wt.) remaining in either normal or SRBC-stimulated spleens after 4 hr was 0·36% of the dose. The results *in vitro* are the average of analyses of 3 vessels. Swiss mice of matched weights were given either saline or 0·25 ml of a 30% suspension of tanned SRBC on day 0, and the cleavage studies were done on day 3.

comparative studies with normal and SRBC-stimulated spleen cells, this was not found to be the case. As shown in Table 7, an injection of MMPR-OP-^{35}S was cleaved *in vivo* more by normal spleens than by immunologically active spleens, yet the per cent of the dose remaining was the same. When normal and SRBC-stimulated spleen cell suspensions were treated with MMPR-OP *in vitro*, the amounts of cleavage were identical.

CONCLUSIONS

Oxidation of the ribosyl moiety of MMPR with periodic acid changes its mode of action as well as its stability. The distribution studies show that MMPR-OP is not selectively concentrated in tissues, but is rapidly excreted in the urine, most of it unchanged. However, a small portion is retained in a bound form in tissues for at least 4 days. The long-range effects of MMPR-OP after cessation of treatment[6, 7] must be due to its binding capacity as well as to that of its degradation products, since MMPR does not have these properties. The remnants of ^{35}S-labeled material still bound to the TCA insoluble fractions of tissues at 4 days (Table 3) would include unlabeled glyceraldehyde and glyoxal,[19] depending on the extent of cleavage to MMP. Glyoxal and higher homologs block DNA replication and protein synthesis and cross-link guanine–cytidine base pairs in DNA.[20] Of the degradation products of MMPR-OP, daily doses of glyoxal at 700 mg/kg[21] or MMP at 100 mg/kg[22] had little or no effect against L1210 ascites tumors. We found no demethylation of MMP to 6MP or conversion of MMP to MMPR-phosphate in the present study. The lack of conversion of MMP to MMPR-phosphate has been demonstrated in other mammalian tumors.[1, 23] Intact MMPR-OP must, therefore, be the active agent.

Both binding and cleavage of MMPR-OP are nonspecific and appear to occur in all tissues whether they are normal, neoplastic, or producing anti-SRBC antibodies. Not only has the selective activation of MMPR by phosphorylation been lost in MMPR-OP, but the increased chemical reactivity of MMPR-OP is also nonselective in nature. The activity of MMPR-OP against L1210/MP ascites cells[3] may be due in part to binding to ascites cell membranes after intraperitoneal injection. Rapid excretion of the unbound drug would spare the host.

The chemical reactivity of MMPR-OP, as demonstrated *in vitro* with spleen cells and glycine-2-^{14}C, can cause complications in studies on the effects of MMPR-OP on glycine metabolism (Table 5). When a procedure was used which gave a more purified nucleic acid fraction, it was found that MMPR-OP inhibited both nucleic acid and protein synthesis of spleen cells *in vitro*, similar to the effects of MMPR-OP on L1210 ascites cells *in vivo*.[3]

The immunosuppressive activity of MMPR-OP[3] may be related in part to nonspecific binding to cell membranes which could delay the lymphatic connection between graft and host as well as inhibit cytotoxic lymphocyte-target cell interactions during graft rejection. The studies in this paper show that there is no selective concentration or metabolism of MMPR-OP in mouse spleens at the peak of their response to antigen (Table 7). Further immunological studies also show that the metabolic effects of MMPR-OP are not sufficient to completely suppress immunocompetent cell activity.[6, 7]

The future usefulness of MMPR-OP as an anticancer or immunosuppressive drug will depend on exploitation of its binding capacity. Pretreatment of the donor tissue would be one way of avoiding toxicity in the recipient. Treatment with the drug bound to certain serum proteins might provide a more narrow range of action.

Note added in proof: Significant prolongation in the survival of mice bearing an L1210 ascites tumor was recently reported using glyceraldehyde, another possible degradation product of MMPR-OR, at daily doses of 600 mg/kg or more [M. A. APPLE and D. M. GREENBERG, *Cancer Chemother. Rep.* **52**, 687 (1969)].

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