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2,3-DIHYDRO-1*H*-IMIDAZO[1,2-*b*]PYRAZOLE
A NEW INHIBITOR OF DEOXYRIBONUCLEIC ACID SYNTHESIS

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Abstract—2,3-Dihydro-1*H*-imidazo[1,2-*b*]pyrazole has been shown to inhibit DNA synthesis in animal cells growing in culture, but has no effect on bacterial cells. The inhibition of DNA synthesis due to the drug is prevented and reversed by deoxyguanosine, but is potentiated by deoxycytidine. The mode of action of the compound is different from that observed using other known inhibitors of DNA synthesis and therefore the drug may be useful as an alternative method to stop DNA synthesis.

A LARGE number of antitumor agents and other compounds inhibit DNA synthesis in a variety of different organisms.[1–9] These drugs have been useful in studying DNA synthesis in intact cells and *in vitro* and in elucidating the interrelationships between DNA synthesis and the formation of other macromolecular components of the cell.[1,2,10]

The present investigation describes some studies on 2,3-dihydro-1*H*-imidazo [1,2-*b*]pyrazole (IMPY), a new inhibitor of DNA synthesis in animal cells. This compound has been shown to have sedative and antipyretic[11] as well as antitumor activity.‖ The structural formula is shown in Fig. 1.

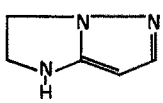

FIG. 1. Structural formula of 2,3-dihydro-1*H*-imidazo[1,2-*b*]pyrazole (IMPY).

MATERIALS AND METHODS

Cells. L cells growing in suspension culture in Eagle's MEM suspension medium (Microbiological Associates, Bethesda, Md.) with a doubling time of approximately 24 hr at 37° were used. All experiments *in vitro* were performed with cells at a concentration of 3–5 × 10^5 cells/ml. Similar results were obtained using HeLa cells growing as a monolayer. A series of experiments were also performed using S-180 ascites tumor cells growing in Swiss mice.

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‖ J. J. Wang and O. S. Selawry, Unpublished observations, using L1210 growing in DBA/2 mice and S-180 growing in Swiss mice.

Radioisotopes and biochemicals. Uridine-2-^{14}C (30 mc/m-mole), L-leucine-1-^{14}C (23 mc/m-mole), thymidine-2-^{14}C (50 mc/m-mole), thymidine-methyl-^{3}H (15 mc/m-mole) and orotate-6-^{14}C (6·1 mc/m-mole) were purchased from New England Corp., Boston, Mass. 2,3-Dihydro-1*H*-imidazo[1,2-*b*]pyrazole (Ciba 21,381-Ba) was a gift from Dr. Prosper Loustalot of Ciba Ltd., Basle, Switzerland. It was dissolved in water before use. All other chemicals were the best grade commercially available.

Measurement of incorporation of radioactivity into RNA, DNA and protein. In all experiments using L cells, aliquots of actively growing cells were washed once with fresh warm medium and inoculated in 10 ml of fresh medium at about 3–5 $\times$ 10^5 cells ml in 25- or 50-ml stoppered Erlenmeyer flasks. The flasks were gassed with an air–CO_2 mixture (95%:5%, v/v). The cells were shaken at 80 rev/min for 30–60 min at 37° in a New Brunswick gyratory shaker. The drug was then added and the incubation was continued for an additional 10 min. The indicated radioactive compound was added and the incubation was continued for 2 additional hr. Eight ml was taken, mixed with 5 ml of cold Earle's salt solution, centrifuged at 1000 *g* for 10 min, and the cells were collected.

For determination of incorporation of [^{14}C]leucine into protein, the precursor was present at a concentration of 52 μg/ml (0·2 μc/ml). The collected cells were suspended in 2 ml water and then 2 ml of 1 N NaOH was added. The dissolved cells were incubated at 37° for 30 min. Then 0·5 ml of 1 N HCl and 2 ml of 10% trichloroacetic acid were added to duplicate 1-ml aliquots. The resulting precipitates were filtered through Millipore filters (0·45 μ pore size) and were washed with 5 ml of 5% trichloroacetic acid.

For uridine (1·6 μg, 0·2 μc/ml) incorporation into RNA (and DNA), the cells were treated as above except that 0·1 ml of 1 N HCl was added to neutralize the cooled solution. One sample was immediately precipitated with cold trichloroacetic acid and filtered as above (Total). The other sample in 0·5 N NaOH was incubated for 2 hr at 37°, then neutralized and precipitated with trichloroacetic acid and filtered as above (DNA). The difference between the radioactivity incorporated into the Total (RNA + DNA) and that incorporated into the DNA gives the radioactivity present in RNA.[12]

Thymidine (0·8 μg, 0·1 μc/ml) incorporation into DNA was determined as for leucine incorporation into protein.

The dried filters were pasted to aluminium planchets and counted, using a Nuclear-Chicago thin-window low background counter. The tritium content was determined by dropping the dried filters into Omnifluor (New England Nuclear Corp.) and counting using a Packard scintillation spectrometer.

Determination of synthesis of macromolecules in S-180 tumor bearing mice. On the seventh day after intraperitoneal inoculation of Swiss mice with 0·1 ml containing 2–4 $\times$ 10^8 Sarcoma 180 ascites tumor cells, the animals were injected intraperitoneally with 600 mg/kg of IMPY (LD_{10}) or a comparable volume of saline. Two hr after drug administration, either leucine-1-^{14}C (0·01 μc/g), thymidine-methyl-^{3}H (1 μc/g) or orotate-6-^{14}C (0·1 μc/g) was injected intraperitoneally, and 1 hr later the ascites cells were drawn and divided into 0·1-ml aliquots for cell count and isolation of tumor cells. Ascites were first washed with 0·45% saline to remove contaminated red blood cells and immediately centrifuged for 10 min at 1000 *g* to collect the cells. Incorporation of the radioactive precursors by the cells into the respective macromolecules was determined according to the procedure outlined for use with L cells.

RESULTS

Effect of IMPY on DNA, RNA and protein synthesis. The effect of IMPY on the synthesis of macromolecules was studied. In these and subsequent experiments, L cells were used. However, similar results were also obtained using HeLa cells. As seen in Fig. 2, IMPY had no effect on RNA and protein synthesis, but markedly

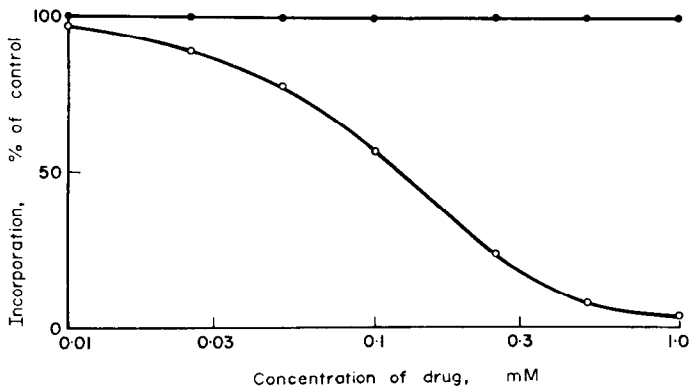

FIG. 2. Effect of IMPY on thymidine, uridine and leucine incorporation into L cells growing in suspension culture. The procedure is that outlined in Materials and Methods. Incorporation into the respective macromolecule was determined after 2 hr of incubation. The data are an average of 3 separate experiments. ○, Thymidine incorporation; ●, leucine or uridine incorporation. The control cultures incorporated the following amounts of radioactivity (counts/min/ml): thymidine, 7875; uridine 6340; leucine, 1030.

inhibited thymidine incorporation into DNA. Incorporation of uridine into DNA was similarly inhibited, showing that IMPY does not inhibit by interfering with thymidine uptake. The kinetics of this inhibition are shown in Fig. 3. The drug had no effect on RNA and protein synthesis for at least 9 hr after its addition to the cells, whereas DNA synthesis was inhibited during this period.

IMPY also inhibited DNA synthesis in S-180 ascites tumor cells growing in Swiss mice. As with L cells, RNA and protein synthesis were unaffected by concentrations of the drug which almost completely inhibited DNA synthesis (Table 1).

TABLE 1. EFFECT OF IMPY ON DNA, RNA AND PROTEIN SYNTHESIS BY S-180 ASCITES TUMOR CELLS GROWING IN SWISS MICE*

Radioactive compound used	Counts/min Incorporated/10^6 cells (Mean ± S.E.)		% of Control (uninhibited)
	Treated	Control	
Thymidine	3810 ± 360	57,320 ± 10,000	7
Orotic acid	8160 ± 1210	7070 ± 710	115
Leucine	53,540 ± 4670	54,310 ± 8420	99

* Incorporation of thymidine into DNA, orotic acid into RNA, and leucine into protein was determined as described in Materials and Methods. Six animals were used for each of the above values obtained. The P value for the inhibition of thymidine incorporation is $< 0{\cdot}001$. The differences obtained between treated and control for the orotic acid and leucine incorporation were not significant.

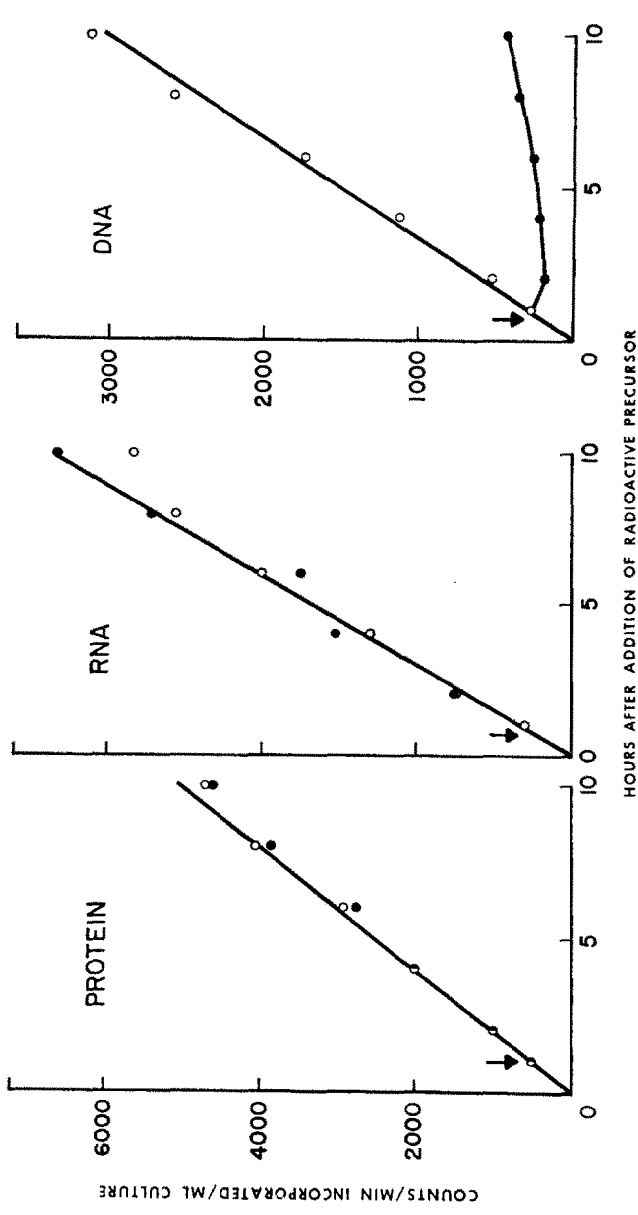

FIG. 3. Effect of IMPY on kinetics of incorporation of thymidine, uridine and leucine incorporation into L cells growing in suspension culture. The experimental procedure used is the same as that outlined in the legend to Fig. 2. IMPY (1 mM final concentration) was added (arrow) 1 hr after the radioactive precursor was added. This is one of two experiments done. ○, Control (no drug); ●, plus drug.

IMPY had little effect on DNA synthesis in the bacteria *Bacillus subtilis* and in *Escherichia coli*, either infected with bacteriophage T4 or uninfected. Figure 4 shows the data obtained using bacteriophage T4 infected *E. coli* cells. As can be seen, the inhibition of DNA synthesis in the presence of 1 mM IMPY is only 18 per cent. Data obtained with uninfected *E. coli* or *B. subtilis* cells are the same (data not shown).

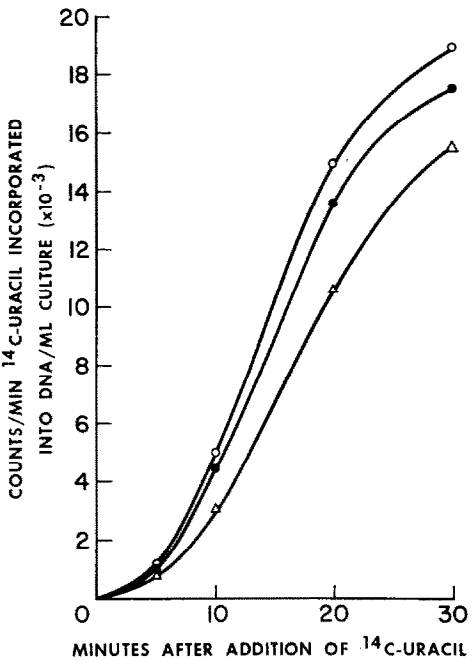

FIG. 4. Effect of IMPY on DNA synthesis in bacteriophage T4-infected *E. coli* cells. The cells were infected at a multiplicity of 6 as previously described.[13] The indicated concentrations of IMPY were added to separate 10-ml cultures 5 min after infection, and the incubation was continued for an additional 5 min. Then, [^{14}C]uracil (0·1 μc/ml; mg/ml final concentration) was added to each culture. The incorporation in DNA was determined as previously described.[12] ○, Control (no drug); ●, 0·1 mM IMPY; △, 1·0 mM IMPY.

Recovery of cells from treatment with IMPY. In this experiment, it was determined whether inhibition of DNA synthesis by IMPY was reversible by washing the inhibited cells free of drug. L cells were treated with 1 mM IMPY for 23 hr. During this time, growth of the cells was completely inhibited. Control untreated cells doubled during the same period. The treated and control cells were washed as indicated in the legend to Fig. 5, reinoculated at equal cell numbers into fresh media without drug, and incorporation of thymidine into DNA was determined. The incorporation into DNA was the same in control and treated cells, indicating complete reversibility by washing cells free of drug.

Prevention and reversal of IMPY inhibition by deoxynucleosides. The ability of deoxynucleosides to prevent inhibition by IMPY was determined. In these experiments, IMPY and the indicated deoxynucleoside were added to growing L cells at the same time. The incorporation of thymidine into DNA was determined in each culture at 2 hr and compared to that of the control untreated culture. (The control

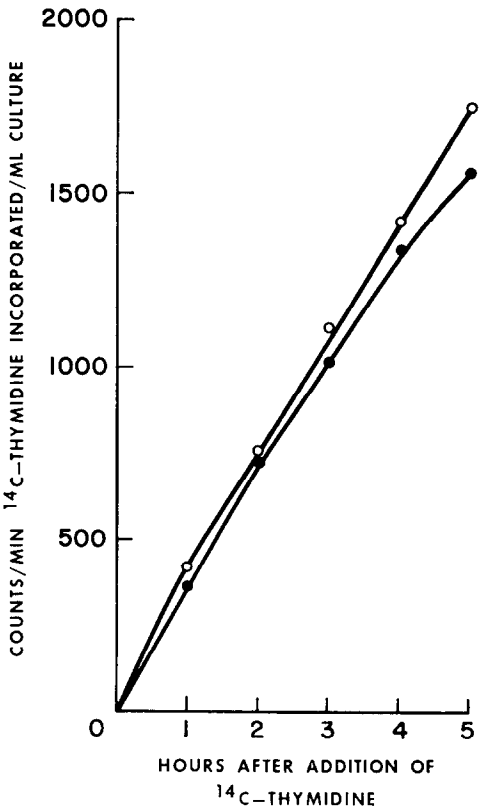

FIG. 5. Recovery of L cells treated with IMPY. Two 50-ml cultures of L cells growing in suspension were used. To one, IMPY (1 mM final concentration) was added, and to the other, nothing (control). Both cultures were started containing 1×10^5 cells/ml. After 23 hr of incubation at 37°, the cells were harvested by centrifugation and washed three times with fresh-warmed medium. The control culture had grown to 2×10^5 cells/ml, whereas the culture containing IMPY was still at 1×10^5 cells/ml. The washed cells from each culture were separately inoculated at 1×10^5 cells/ml into 40 ml of fresh medium without drug, and incubated with shaking at 37°. Thymidine incorporation was determined at the indicated intervals, as outlined in Materials and Methods. ○, Control (not pretreated with drugs); ●, pretreated with IMPY.

cultures contained the given concentration of deoxynucleoside, because these alone at high concentration gave significant inhibition of thymidine incorporation.) As can be seen in Table 2, deoxyguanosine in high concentration could prevent the inhibition of DNA synthesis by IMPY. On the other hand, very low concentrations of deoxycytidine could potentiate the inhibitory activity of IMPY. Thymidine may also reverse the inhibition due to IMPY, but higher concentrations of this deoxynucleoside could not be used because incorporation of the thymidine was severely depressed by the addition of a large excess of nonradioactive thymidine. In other experiments not reported here, we have also shown that deoxyguanosine can reverse the inhibition due to IMPY, even when added 2 hr after the drug was allowed to act. The four nucleosides (0·2 mM) had no effect on either reversal or potentiation of IMPY inhibition of DNA synthesis.

TABLE 2. PREVENTION BY DEOXYNUCLEOSIDES OF IMPY INHIBITION*

Deoxynucleoside added	Concn of deoxynucleoside (mM)	% of Control (uninhibited)
None (control, no IMPY present)		100
None (IMPY present, 0·1 mM)		53
Deoxyguanosine	0·1	66
	0·2	88
	0·3	84
Deoxycytidine	0·002	27
	0·05	24
	0·1	26
Deoxyadenosine	0·2	52
Thymidine	0·1	65
	0·2	71
Mixture of all above	0·1 each	48
Deoxyuridine	0·1	55

* Incorporation of thymidine into DNA was determined as outlined in Materials and Methods. IMPY and deoxynucleoside tested were added at the same time to the suspension of L cells. At high concentration, the deoxynucleosides themselves interfere with the incorporation of thymidine into DNA. Consequently, in each experiment the effect of the deoxynucleoside itself on thymidine incorporation was determined and taken into account when figuring out the effect of the deoxynucleoside on inhibition due to IMPY. The data are averages of up to nine separate experiments. In the control, 6310 counts/min of thymidine were incorporated per ml of culture.

TABLE 3. POTENTIATION OF IMPY ACTIVITY BY DEOXYCYTIDINE*

Concn of IMPY (mM)	% Control	
	No deoxycytidine added	Deoxycytidine added
0·0	100	100
0·01	94	84
0·025	86	73
0·05	71	55
0·10	52	27

* The procedure is the same as that described in the legend to Table 2. The concentration of deoxycytidine used was 0·02 mM. IMPY and the deoxycytidine were added at the same time to the L cell suspension. The data are an average of two separate experiments. In the control, 8250 counts/min of thymidine were incorporated per ml of culture.

Table 3 describes in detail the potentiation of IMPY activity by deoxycytidine (0·02 mM) at various concentrations of IMPY. The inhibition of DNA synthesis in the deoxycytidine and IMPY-treated cells was about twice that observed in cells incubated with only IMPY.

DISCUSSION

This study presents information concerning the mode of action of IMPY, a novel inhibitor of DNA synthesis. The inhibition of DNA synthesis due to IMPY can be prevented and reversed by deoxyguanosine, indicating that the drug may be interfering with dGTP synthesis, although we have no direct information concerning this point. Furthermore, the inhibitory action of IMPY can be potentiated by deoxycytidine. The mechanism of this synergism is not clear.

What we have shown in this study is that IMPY is a good, reversible inhibitor of DNA synthesis, in animal cells (but not bacterial cells), whose site of action is not yet known. The drug is another addition to the group of specific inhibitors of DNA synthesis which have been used successfully in the past to dissect the biochemical reactions involved in DNA synthesis and to study the interrelationships among the synthesis of macromolecules.[1,2,10] Since its action is probably different from those of other known inhibitors,[1–9] IMPY may be another alternative compound which can be used to stop DNA synthesis.

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REFERENCES

1. M. L. EIDINOFF and M. A. RICH, *Cancer Res.* **19,** 521 (1959).
2. R. R. RUECKERT and G. C. MUELLER, *Cancer Res.* **20,** 1584 (1960).
3. H. B. BREWER, J. P. COMSTOCK and L. ARONOW, *Biochem. Pharmac.* **8,** 281 (1961).
4. J. P. LAYDE and R. BASERGA, *Br. J. Cancer* **18,** 150 (1964).
5. C. W. YOUNG and S. HODAS, *Science, N.Y.* **146,** 1172 (1964).
6. Y. KURODA and J. FURUYAMA, *Cancer Res.* **23,** 682 (1963).
7. J. H. KIM, S. H. KIM and M. L. EIDINOFF, *Biochem. Pharmac.* **14,** 1821 (1965).
8. J. H. KIM and M. L. EIDINOFF, *Cancer Res.* **25,** 698 (1965).
9. M. KARON, P. HENRY, S. WEISSMAN and C. MEYER, *Cancer Res.* **26,** 166 (1966).
10. L. S. COHEN and G. P. STUDZINSKI, *J. Cell Biol.* **69,** 331 (1967).
11. J. DRUEY, P. SCHMIDT and K. EICHENBERGER, U.S. Patent 2,989,537 as cited in, *Chem. Abstr.* **55,** entry 25990 g (1961).
12. D. B. ROODYN and H. G. MANDEL, *Biochim. biophys. Acta* **41,** 80 (1960).
13. P. S. COHEN and H. L. ENNIS, *Virology* **27,** 282 (1965).