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VIRAZOLE (1-β-D-RIBOFURANOSYL-1,2,4-TRIAZOLE-3-CARBOXAMIDE; A CYTOSTATIC AGENT

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Abstract—Virazole (Ribavirin) strongly inhibits the cell proliferation of L5178y cells. These cells were not infected with DNA- or RNA viruses. Starting with 3×10^3 cells/ml and an incubation period of 72 hr, the drug reduces the cell proliferation to 50 per cent in a concentration of 4.7 μM. Virazole acts cytostatically up to 3 times the ED_{50} concentration. The cytostatic effect of Virazole can be abolished by guanosine, xanthosine and inosine but not by adenosine. Both the incorporation rates of precursors of DNA- and RNA synthesis, as well as of protein synthesis are in intact cells inhibited by Virazole. Already 2 hr after incubation with the drug the amount of polyribosomes decreases. In the presence of Virazole, the pool sizes of dGTP and GTP are drastically reduced.

Virazole 5'-triphosphate does not inhibit the following enzymes: Eukaryotic DNA polymerase α and β, eukaryotic RNA polymerase I and II and eukaryotic poly(A) polymerase.

The synthetic triazole nucleoside Virazole (1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide; Ribavirin; ICN 1229) has been synthesized by Witkowski *et al.* [1] and was found to be a broad spectrum antiviral agent [review: 2]. X-ray studies could establish that the geometry of Virazole is similar to that of guanosine [3].

For a future clinical application it seems to be necessary to clarify whether Virazole is a specific antiviral drug or whether it also affects non-infected eukaryotic cells. The present paper is an approach to solve this question. The studies were performed with mouse lymphoma cells in culture.

MATERIALS AND METHODS

Compounds. The following materials were obtained: Adenosine, guanosine, inosine and xanthosine from Serva, Heidelberg (Germany); Methyl[^{3}H] thymidine (sp. act., 19 Ci/m-mole), [^{3}H]uridine (generally labeled; sp. act., 6.3 Ci/m-mole), L[4.5-^{3}H] lysine (sp. act., 6.6 Ci/m-mole), [^{3}H]dCTP (sp. act., 9.5 Ci/m-mole), [^{3}H]CTP (sp. act., 20.3 Ci/m-mole), [^{3}H]dGTP sp. act., 6.2 Ci/m-mole), [^{3}H]GTP (sp. act., 10 Ci/m-mole) and [^{3}H]ATP (sp. act., 11 Ci/m-mole) from the Radiochemical Centre, Amersham (England); poly[d(G-C)], unlabeled ribo- and deoxyribonucleoside triphosphates, DNA-dependent RNA polymerase (*E. coli*; isolated according to Burgess *et al.*, [4]) and DNA-dependent DNA polymerase (*E. coli* pol I; isolated according to Jovin *et al.*, [5]) from Boehringer, Mannheim (Germany); poly[d(I-C)] and oligo $d(pA)_{10}$ from P-L Biochemicals, Milwaukee, WI (U.S.A.).

Herring sperm DNA, isolated according to Zahn *et al.* [6] was a gift of H. Mack, Illertissen (Germany). We thank Dr. R. W. Sidwell and Dr. J. P. Miller (ICN Nucleic Acid Research Institute, Irvine, CA USA) for the samples of virazole and virazole 5'-triphosphate.

Cell culture. L5178y cells were grown in Fisher's medium for leukemic cells, supplemented with 10% horse serum (Grand Island Biological Co., Grand Island, N.Y.) in suspension cultures [7].

Cell concentrations and volume distributions were determined with a Model B Coulter Counter with a size-distribution plotter (Coulter Electronics, Hialeah, Fla.). The "mean window", a characteristic of a cell vol. distribution curve, which represents the window into which all cells would fall if the total cell vol. and number of cells were unchanged, was computed according to the method of Brecher *et al.* [8]. The absolute cell vol. was determined as described (8).

For the dose–response experiments, the cultures (5 ml) were routinely initiated by inoculation of 3×10^3 cells/ml and incubated at 37° in roller tubes for 72 hr; the controls reached a cell concentration of about 3×10^5 cells/ml. Per inhibitor concentration 10 parallel assays were performed. The ED_{50} was estimated by Logit regression. The number of doublings was calculated by a formula, published earlier [9].

The viability of the cells after incubation with Virazole was tested as follows: After incubation the cells were washed twice with fresh medium to remove the drug, diluted to 3×10^3 cells/ml and incubated in the standard roller tube assay for 72 hr. The viability is expressed by the ratio of the plating efficiency of the treated cells to the plating efficiency of untreated cells.

Incorporation studies. Suspensions (vol. 5 ml) of exponentially growing cells at 2×10^5 cells/ml were supplemented with Virazole 2 hr prior to the addition of the labeled precursors 25 μCi [^{3}H]dThd, 25 μCi [^{3}H]Urd, or 25 μCi [^{3}H]Lys. The incubations were continued routinely for 30 min. Samples of 5 ml were

analyzed for acid-insoluble radioactivity in DNA, RNA or protein [10].

Isolation of polysomes. For the isolation of both membrane-bound and free polysomes [11] from L5178y cells on sucrose gradients (0.5 to 1.5 M) procedures previously described [12] were followed with small modifications [13]. One ml of the homogenate contained 0.02 mg DNA, 2.3 mg RNA and 7.6 mg protein.

dGTP and GTP pool sizes. The method applied based on published procedures [14, 15]. Cultures (100 ml) containing 2×10^7 cells were centrifuged (10 min; 2°; 10,000 *g*) and the resulting pellet extracted with 4 vol. of 2 M $HClO_4$ at 4°. After neutralization with 2 M KOH to pH 7.4 the $KClO_4$ salt formed was removed by centrifugation. The extract was freeze-dried and the residue dissolved in 100 μl H_2O. The dGTP and GTP concentrations were determined by the enzymatic assay described [14, 15]. A standard curve, made with known dGTP or GTP concentrations was linear in the concentration range between 5 mM and 25 mM.

Enzyme preparations. DNA-dependent DNA polymerase α was isolated from L5178y cells according to Rohde *et al.* [16]; the Sephadex G-200 fraction with a sp. act. of 65 nmoles of labeled substrate/hr/mg protein was used. DNA-dependent DNA polymerase β was isolated from L5178y cells as described by Chang and Bollum [17]: the phosphocellulose fraction with a sp. act. of 210 nmoles of nucleotide incorporated/hr/mg protein was taken.

DNA-dependent RNA polymerase I and II were isolated from mouse liver according to Müller *et al.* [18]. The fractions after elution from DEAE-Sephadex were used. The sp. act. were: RNA polymerase I 0.21 nmoles nucleotide incorporated/15 min/mg protein and enzyme II 0.36 nmoles/15 min/mg protein.

Poly(A) polymerase was extracted from quail oviduct according to Müller *et al.* [19]; Fraction I:3 was used. This preparation had a sp. act. of 83 nmoles AMP incorporated/30 min/mg protein.

Enzyme assays. For the determination of DNA-dependent DNA polymerase activities 10 μl enzyme was combined with 50 μl polymerase α or β mixture. The DNA polymerase α mixture consisted of varying amounts of [^{3}H]dGTP (80 cpm/pmole), 0.1 mM each of dCTP, dATP, dTTP, 20 mM K-phosphate buffer (pH 7.2), 1 mM 2-mercaptoethanol, 8 mM $MgCl_2$ and 0.5 A_{260} units of activated herring sperm DNA; the DNA polymerase β mixture was identical to the polymerase α reaction mixture, except that the buffer was 50 mM ammediol (pH 8.8) rather than K-phosphate. The reaction was carried out at 37° for 30 min; 50 μl were placed on GF/C discs and processed as described [20].

The standard reaction mixture for RNA polymerases contained the following components in 100 μl; Varying amounts of [^{3}H]GTP (50 cpm/pmole), 0.1 mM each of ATP, CTP, UTP, 50 mM Tris-HCl pH 7.8, 3 mM $MnCl_2$, 2 mM 2-mercaptoethanol, 2 mM creatine phosphate, 20 μg/ml creatine phosphokinase, 5 μg bovine serum albumin and 20 μg native or heat-denatured DNA. The concentrations of $(NH_4)_2SO_4$ in the incubation mixture for assaying RNA polymerase I were adjusted to 50 mM; for Form II 150 mM $(NH_4)_2SO_4$ were added. Usually the reaction mixture was incubated at 37° for 15 min after addition of 40 μl of enzyme preparation. After incubation, aliquots of 50 μl were tested on GF/C filters for acid-insoluble radioactivity [20].

The standard assay for poly(A) polymerase (vol. 100 μl) contained 100 mM Tris-HCl, pH 8.2; 5 mM $MgCl_2$; 0.2 mM dithiothreitol; varying amounts of [^{3}H]ATP (100 cpm/pmole), 5 μg oligo $(pA)_{10}$ and 20 μl enzyme. After incubation for 30 min at 37°, a 40 μl aliquot was taken to determine the acid-precipitable radioactivity on GF/C filters [20].

Miscellaneous methods. DNA was determined by the method of Kissane *et al.* [21], RNA by the orcinol reaction [22], and protein according to the method of Lowry *et al.* [23]. Herring sperm DNA was activated according to Aposhian *et al.* [24].

RESULTS

Influence of Virazole in intact cell systems

Influence on cell proliferation. Virazole strongly inhibits the proliferation of L5178 y cells. In the standard dose-response experiments, starting with 3×10^3 cells/ml and an incubation period of 72 hr, the drug reduces the cell proliferation to 50 per cent ($=ED_{50}$) at a concentration of 1.15 ± 0.08 μg/ml ($=4.7$ μM); (Fig. 1). With the dose-response experiments started with 30×10^3 or 100×10^3 logarithmically growing cells/ml, the corresponding ED_{50} concentrations have been determined as 2.93 ± 0.23 or 5.71 ± 0.41 μg/ml, respectively; the incubation period in these experiments was terminated after 24 hr.

Virazole acts cytostatically in a certain concentration range. The inhibition of the cell proliferation with 3 times the ED_{50} concentration for a period of 24 hr is perfectly reversible. At concentrations higher than 3 times the ED_{50} concentration the cells are affected cytotoxically to a considerable extent: at $4 \times ED_{50}$ or $5 \times ED_{50}$ the viability drops to 76 per cent or 43 per cent.

The average vol. of L5178y cells is not altered by Virazole. After incubation of the cells with $2 \times ED_{50}$ concentration for 24 hr, the cell vol. amounts to 1320 μm^3; the value of the controls is 1290 μm^3.

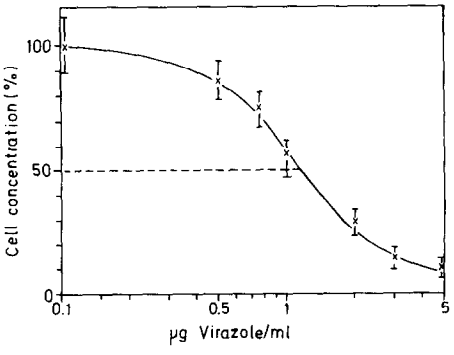

Fig. 1. Effect of Virazole on cell proliferation of L5178y cells. Each value (mean ± S.D.) comes from 10 parallel assays. The solid curve was obtained by semilogarithmic regression. – –, ED_{50} value. The assay conditions are as described in "Materials and Methods".

Table 1. Influence of natural nucleosides on cell proliferation inhibition by Virazole*

Virazole (μM)	Additional compound (μM)	Cell concentration after incubation (cells $\times 10^3$/ml)	Cell doublings	Alteration of the Virazole effect by additional compound (increase [+]/decrease [−]; in doubling steps)
—	—	320 ± 28	6.74	
1.15	—	165 ± 12	5.78	
3.30	—	70 ± 5	4.54	
1.15	adenosine: 2	159 ± 13	5.73	−0.05
3.30		73 ± 5	4.61	+0.07
1.15	guanosine: 40	264 ± 21	6.46	+0.68
3.30		178 ± 14	5.89	+1.35
1.15	inosine: 40	289 ± 21	6.58	+0.80
3.30		196 ± 14	6.01	+1.47
1.15	xanthosine: 40	259 ± 20	6.43	+0.65
3.30		183 ± 13	5.93	+1.39

Cultures were inoculated with 3×10^3 cells/ml in the presence of the compounds, indicated in the Table. The incubation of the cultures and the calculation of cell doublings was performed as described under Materials and Methods. The reduction of cell growth, caused by Virazole together with different compounds, was evaluated by subtracting the number of doublings with Virazole alone from the number of doublings with Virazole plus the nucleoside. Each value in the table represents the mean ± S.D. of 10 parallel assays.

Coincubation with natural nucleosides. Virazole was coincubated with natural nucleosides in the standard roller tube assay (Table 1). The natural nucleosides were added at concentrations that had no influence on cell proliferation. With the exception of adenosine, the concentrations of the natural compounds were 40 μM. Adenosine was used in a concentration of 2 μM because higher concentrations cause inhibition of cell proliferation. The inhibitory potency of Virazole can not be abolished by coincubation with adenosine while the addition of guanosine and xanthosine causes a reduction of the Virazole effect; the cell doublings increase in the presence of guanosine from 5.78 to 6.46 or from 4.54 to 5.89, depending on the Virazole concentration used. A similar influence is observed with inosine; this nucleoside also causes a reduction of the cytostatic activity of Virazole. Using a concentration of 2 μM of the natural nucleosides the same effect is observed, however to a lower extent. After incubation of the cells with 3.3 μM Virazole together with 2 μM guanine, the cell doublings increase from 4.54 to 5.01; in the case of inosine and xanthosine an increase to 5.32 and 4.95 is observed. From these data it seems to be reasonable to assume, that guanosine, inosine and xanthosine interfere directly with Virazole itself.

Influence on synthesis of macromolecules. The effect of Virazole on the incorporation rate of radio-labeled precursors into DNA, RNA and protein in exponentially growing L5178y cells is shown in Table 2. The incorporation rate, a first approach to determine the effect of this drug on macromolecular synthesis, of all three precursors used is strongly inhibited in the presence of Virazole. At the ED_{50} concentration (38 μM) the incorporation into DNA is reduced to 42 per cent, the one into RNA 57 per cent and that into protein 88 per cent. These studies were performed with cultures of a cell density of 2×10^5 cells/ml.

A sensitive method to estimate the influence of Virazole on mRNA synthesized is the determination of the amount of polysomes after incubation with the compound. Figure 2 demonstrates that the amount of extractable total polysomes decreases after incubation with Virazole. In the control experiments an amount of 0.39 A_{260} units (=100%) of polysomes can be isolated from 2×10^7 cells; after incubation with 38 or 76 μM Virazole the quantity of polysomes amounts to 0.19 A_{260} units (=49%) or 0.07 A_{260} units (=18%) respectively. From these data we have to conclude that the synthesis of mRNA is also strongly affected in the presence of Virazole.

Table 2. Influence of Virazole on the synthesis of DNA, RNA and protein in L5178y cells*

Virazole concentration (μM)	Influence on cell doublings		Incorporation into macromolecules/100,000 cells					
			[^{3}H]dThd		[^{3}H]Urd		[^{3}H]Lys	
	Doubling steps	per cent	cpm	per cent	cpm	per cent	cpm	per cent
0	0.15	100	17,400	100	3,680	100	2,230	100
38	0.07	46	7,300	42	2.110	57	1,970	88
76	0.03	18	4,800	28	1,650	45	1,840	83

* The incorporation studies were performed as described under Methods. Exposure time of the precursor was 30 min. Values represent means of 4 parallel experiments. The standard deviation does not exceed 10%. The influence of Virazole on cell proliferation is expressed in doublings.

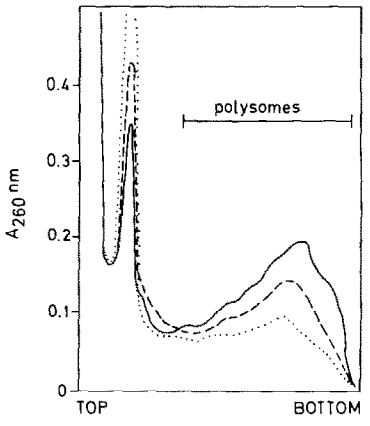

Fig. 2. Influence of Virazole on polysome formation in L5178y cells. 100 ml cultures containing 2×10^5 cells/ml were incubated for 2 hr with 0 (—), 38 (- -) and 76 μM Virazole (· · · · · ·); after incubation total polysomes were extracted and isolated in a sucrose gradient, as described under Methods. The total extract (1 ml) was applied onto the gradient.

dGTP and GTP pool sizes. The coincubation experiments showed that guanosine and xanthosine can abolish the potency of Virazole to inhibit cell proliferation, to some extent. Therefore it was very likely that Virazole inhibits the synthesis of dGTP and GTP in intact cell system. For that reason L5178y cells were incubated with Virazole and subsequently the pool size of dGTP and GTP in these drug-treated cells was determined. The results are summarized in Table 3. After incubation of exponentially growing cells for 3 hr with the ED_{50} concentration of Virazole, the dGTP pool size is reduced to 46 per cent and the GTP pool to 74 per cent. At higher Virazole concentrations the decrease in the pool sizes is even more pronounced.

Influence of Virazole 5'-triphosphate on polymerase systems

The effect of Virazole 5'-triphosphate was determined in isolated DNA- and RNA polymerizing enzyme systems. The enzyme assay systems for DNA-dependent DNA polymerase α and β from L5178y cells and the DNA-dependent RNA polymerase I and II from mouse liver consisted (among the other components mentioned under Methods) of 3 μM [^{3}H]dGTP or of 5 μM [^{3}H]GTP. As the labeled triphosphate precursor in the case of poly(A) polymerase 10 μM [^{3}H]ATP was used. The concentrations of labeled precursors in the assays are about half of the Michaelis constant of the respective enzymes. It was found, that the addition of 100 μM Virazole 5'-triphosphate has no influence on the incorporation rate of the different enzymes.

DISCUSSION

The data presented indicate that Virazole is a potent cytostatic agent as tested in mouse lymphoma cells L5178y. The ED_{50} concentration for this compound was found to be 4.7 μM. Under identical conditions, L5178y cell proliferation is inhibited by 50 per cent by 16 μM Formycin B [9], 2.9 μM 9-β-D-arabinofuranosyladenine [25], 13.1 μM Distamycin A [26] and 1 μM Bleomycin [13]. It remains to be studied whether Virazole affects normal cells with the same sensitivity as L5178y mouse lymphoma cells. Nevertheless the data presented in the present paper with L5178y cells show, that the synthetic triazole nucleoside acts not only as an antiviral drug, as published (review: 2] but also inhibitory on cell proliferation of uninfected cells. The presented observation, that the vol. of the drug treated cells is identical to the one of the controls is the first clue that Virazole does not selectively inhibit DNA synthesis; in other words Virazole does not cause "unbalanced growth" [27] of the cells. This assumption was supported by the presented *in vitro* studies determining the relative rate of synthesis of DNA, RNA and protein in intact cells, using the determination method of incorporation of precursors into these macromolecules. These experiments clearly demonstrated that in non-infected cells DNA- and RNA synthesis as well as protein synthesis are strongly affected by Virazole. The polysome formation, one parameter of mRNA synthesis, is also inhibited by Virazole. In a recent study [28], it has also been documented, that Virazole blocks cellular DNA synthesis. In addition the report of De Clercq *et al.* [29] indicates that the antiviral effect of Virazole might be a result of an inhibition of nucleic acid synthesis in infected cells. Taking these data together, we must conclude that Virazole is a chemotherapeutic agent with both a cytostatic and an antiviral activity.

The molecular biological mechanism of action of Virazole is not yet fully understood. An important finding came from Streeter *et al.* [30] who could show that Virazole is phosphorylated intracellularly to Virazole 5'-phosphate. This nucleotide is a potent inhibitor of IMP dehydrogenase from Ehrlich ascites tumor cells with a high relative affinity ($K_i : K_m$) of 0.014 [30]. This *in vitro* result suggested that the cytostatic effect is due to an inhibition of the synthesis of the guano-

Table 3. Alteration of dGTP and GTP pool sizes in dependence on Virazole*

Virazole concentration (μM)	Influence on cell doublings		Pool size of nucleotide (pmoles/10^6 cells)	
	Doubling steps	per cent	dGTP	GTP
0	0.24	100	0.69	5.3
38	0.13	55	0.32	3.9
76	0.06	23	0.28	2.6

* 2×10^7 exponentially growing cells were incubated for 180 min with 0, 38, and 76 μM Virazole. Subsequently the pools were determined as described under Methods.

sine nucleotides. The assumption is verified by our observation that in the presence of Virazole the intracellular pool sizes of dGTP and GTP decrease. Therefore at the present state of knowledge the inhibition of nucleic acid syntheses must be attributed to the depletion of the treated cell with regard to dGTP and GTP.

Our coincubation experiments with Virazole in combination with natural nucleosides using L5178y cells (not infected with virus) showed that adenosine had no influence on the Virazole-caused cytostasis, however guanosine, xanthosine and inosine abolished the inhibitory potency of Virazole. A similar finding was described for Vero cell cultures, infected with measles virus [30]; in these experiments the anti-virus activity of Virazole was also reduced by xanthosine and guanosine. From that it seems to be clear, that *first*, the biosynthesis of dGTP and GTP is blocked by Virazole at the step of IMP dehydrogenase or earlier; this dehydrogenase catalyzes the conversion of IMP to XMP [31]. *Second*, the step GMP-synthetase (conversion of XMP to GMP; [32]) is not affected.

The experiments of Streeter *et al.* [30] indicate that the monophosphate of Virazole is synthesized in intact eukaryotic cells. Nevertheless we tested Virazole 5′-triphosphates in different DNA- and RNA polymerizing enzyme systems and found no effect on eukaryotic DNA polymerase α and β, eukaryotic RNA polymerase I and II and eukaryotic poly(A) polymerase.

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