TOXICOLOGIC PATHOLOGY ISSN:0192-6233
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Vol. 13, No. 1, 1985
Printed in U.S.A.

Inhibition of DNA and RNA Synthesis in Rat Liver Nuclei by Oncogenic and Non-oncogenic β-Blockers*

LAURA RIBONI, MARCO PRESTA, SILVIA ZILIANI, CECILIA MAZZOCCHI, GIOVANNI BRAGA, GIOVANNA MAZZOLENI, AND GIOVANNI RAGNOTTI

Laboratory of General Pathology, Department of Biomedical Sciences, Faculty of Medicine, University of Brescia, 25124 Brescia, Italy

ABSTRACT

The β-blocker DL-1-(2-nitro-3-methylphenoxy)-3-*tert*-butylaminopropan-2-ol (ZAMI 1305), hepatocarcinogenic to the female rat, and the non-oncogenic β-blockers DL-1-(2-nitro-5-methylphenoxy)-3-*tert*-butylaminopropan-2-ol (ZAMI 1327), DL-propranolol, and DL-atenolol were studied for their capacity to interfere with hepatic DNA and RNA synthesis. These moieties inhibit DNA and RNA synthesis, in a dose-dependent fashion, when added *in vitro* to nuclei isolated from the liver of male or female rats. The inhibition is due to a decrease of the initial rate of synthesis and of the total amount of labeled precursor incorporated into the growing chains. When administered *in vivo* both the oncogenic ZAMI 1305 and its non-oncogenic isomer ZAMI 1327 inhibit hepatic DNA and RNA synthesis in female rats, as evaluated by the determination of nucleic acids synthesis in liver nuclei isolated from female rats 5 and 15 min after the injection of the drug. No influence on hepatic DNA and RNA synthesis is observed when the molecules are administered to male rats. The *in vivo* administration of DL-propranolol causes an increase of hepatic DNA and RNA synthesis in male rats, while it is uneffective in female rats.

INTRODUCTION

Tumor-promoting (5) and oncogenic (8, 9, 20) activities have been reported for some β-adrenoreceptor blocking agents. As for other chemicals, the oncogenic activity of β-blockers may depend on an interaction of the molecule with the template primer DNA (1, 11, 12, 17, 29) and/or with the DNA and RNA polymerases (7, 10, 11, 13). However, this capacity is common to other non-oncogenic molecules (18, 26, 27, 33).

Results obtained in our laboratory have demonstrated that the hepatocarcinogenic β-blocker DL-1-(2-nitro-3-methylphenoxy)-3-*tert*-butylaminopropan-2-ol (ZAMI 1305) (25) and its non-oncogenic isomer DL-1-(2-nitro-5-methylphenoxy)-3-*tert*-butylaminopropan-2-ol (ZAMI 1327) (25), neither of which was ever used in therapy, both inhibit the *in vitro* activities of purified yeast DNA polymerase I (24) and DNA polymerases α and β isolated from rat liver (22). Although confirming the capacity of a carcinogen to interfere with nucleic acids synthesis, our results do not clarify whether a cause-effect relationship exists between the capacity of a molecule to affect nucleic acids synthesis and its oncogenicity or not; possibly, this is because purified DNA polymerases represent a too simplified biologic system with respect to intact rat liver.

The effect of ZAMI 1305 and ZAMI 1327 on DNA and RNA synthesis in more complex biologic systems was then evaluated. In the *in vitro* experiments DNA and RNA synthesis was measured in nuclei isolated from livers of untreated rats of both sexes and added β-blockers, while in the *in vivo* experiments

* Requests for reprints should be addressed to Dr. Ragnotti at: Laboratorio di Patologia Generale, Facoltà di Medicina e Chirurgia, via Valsabbina 19, 25124 Brescia, Italy.

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these parameters were evaluated in liver nuclei isolated from male and female rats treated with the β-blocker.

This experimental schema showed the possible differences in the action of the hepatocarcinogen ZAMI 1305 with respect to the non-oncogenic ZAMI 1327 on hepatic nucleic acids synthesis and also, since ZAMI 1305 is oncogenic to the liver of female but not of male rat (25), the different effects on the two sexes.

Since two other non-oncogenic β-blockers, DL-propranolol and DL-atenolol, inhibit the *in vitro* activity of yeast DNA polymerase I (24) and of rat liver DNA polymerases α and β (22), the capacity of these molecules to affect DNA and RNA synthesis in isolated liver nuclei was also tested.

Materials and Methods

Chemicals

Nucleotides and deoxynucleotides were purchased from C. F. Boehringer und Soehne GmbH (Mannheim, West Germany). Other chemicals were of analytical reagent grade and were obtained from E. Merck A.G. (Darmstadt, West Germany) or from British Drug Houses (Poole, Dorset, United Kingdom). [^{3}H]dTTP and [^{14}C]UTP were purchased from Radiochemical Centre (Amersham, Bucks, United Kingdom). ZAMI 1305, ZAMI 1327, DL-propranolol, and DL-atenolol were gifts from Zambeletti S.p.A. (20021 Baranzate, Milan, Italy).

Animals

Male and female outbred Wistar rats (Nossan, Correzzana, Italy) weighing 130–160 g were used. Animals were maintained on a standard diet of laboratory chow (Piccioni, Brescia, Italy) and water *ad libitum* throughout the experimental period. Constant temperature (22–23°C) and alternating periods of 12-hr dark and 12-hr light were also maintained.

In Vitro Experiments

Isolation of Liver Nuclei. Nuclei were isolated from the liver of untreated male and female rats as previously described (31). Briefly, liver was passed through a tissue press made to the design of Porterfield (21) and homogenized in 2.0 volumes of a sucrose solution (0.32 M sucrose, 3 mM $MgCl_2$ adjusted to pH 7.4 with $NaHCO_3$). Aliquots (10 ml) of homogenate were layered on a 10-ml sucrose cushion (0.5 M, pH 7.4) and centrifuged at 700 × g for 10 min. Pellets were resuspended in 13 ml of 2.4 M sucrose, 1 mM $MgCl_2$, 0.2 mM spermidine and centrifuged at 100,000 × g for 60 min. The resulting nuclear fraction was resuspended (2×10^{8} nuclei/ml) in 50 mM Tris-HCl (pH 8.3), 5 mM $MgCl_2$, 5 mM dithiothreitol, 0.1 mM EDTA (sodium salt), and 25% glycerol for the determination of DNA and RNA synthesis.

DNA Synthesis. This was determined as previously described (6). Briefly, nuclear suspension was diluted to 2×10^{7} nuclei/ml with 0.32 M sucrose, 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes) (pH 7.5), and incubated at 4°C for 15 min with different concentrations (see Fig. 1) of β-blockers dissolved in Hepes buffer (pH 7.5). Control samples were incubated with equimolar concentrations of NaCl. Aliquots of the nuclear suspensions (1×10^{6} nuclei) were added to the assay medium containing (final concentrations): 10 mM ATP; 0.5 mM each of dATP, dCTP, and dGTP; 1.2 μM [^{3}H]dTTP (958 mCi/mmol); 10 mM $MgCl_2$; 50 mM Hepes buffer (pH 7.5). After a 30-min incubation at 37°C, DNA synthesis was stopped by the addition of cold 10% trichloroacetic acid, and the acid-precipitable material was collected on GF/C discs (2.5-cm diameter, Whatman Ltd., England). Discs, after washing and drying, were added with 10 ml of a scintillation solution (5.5 g Permablend I/700 ml toluol/300 ml ethylene glycol monomethyl ether) and counted for radioactivity in a Beckman LS 7500 β-counter with an error of 5% or better. In some experiments nuclei concentration or time of incubation was varied as specified in Figures 2A and 3A.

RNA Synthesis. This was determined as previously described (31). Briefly, nuclear suspension was diluted to 2×10^{7} nuclei/ml with 50 mM Tris-HCl (pH 8.3), 5 mM $MgCl_2$, 5 mM dithiothreitol, 0.1 mM EDTA, and incubated at 4°C for 5 min with different concentrations of the β-blocker tested, dissolved in Tris-HCl (pH 8.3). Control samples were incubated with equimolar concentrations of NaCl. Aliquots of the nuclear suspension (1×10^{6} nuclei) were added to the assay medium containing (final concentrations): 50 mM Tris-HCl (pH 8.3); 0.5 mM each of ATP, CTP, and GTP; 0.037 mM [^{14}C]UTP (2 Ci/mmol); 1 mM $MnCl_2$; 2.5 mM dithiothreitol; 0.05 mM EDTA; 0.2 mg/ml bovine serum albumin. After a 10-min incubation at 37°C the reaction was stopped by the addition of cold

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10% trichloroacetic acid. The radioactivity incorporated into the acid-precipitable material was determined as described for DNA synthesis. In some experiments, nuclei concentration or the time of incubation was varied as specified in Figures 2B and 3B.

In Vivo Experiments

Because atenolol showed such a small effect in the *in vitro* experiments (Figs. 1–3), it was omitted from the *in vivo* experiments. A total of 90 male and 90 female Wistar rats was used. Control animals were given an i.p. injection of saline; experimental animals were treated i.p. with ZAMI 1305 (100 mg/kg body weight) or ZAMI 1327 (100 mg/kg body weight or DL-propranolol (75 mg/kg body weight) dissolved in saline. This route of exposure was chosen to obtain a rapid and reproducible absorption of the drugs into the liver. At 5 or 15 min after the injection, the animals were killed by cervical dislocation. Each experimental group consisted, for each sex, of 10 control animals, 10 animals killed 5 min, and 10 animals killed 15 min after the injection of the β-blocker. In each experiment, livers from 2 or 3 animals were quickly removed and pooled together, nuclei were isolated, and DNA and RNA synthesis were determined as described for the *in vitro* experiments, with the difference that 2×10^6 and 2×10^7 nuclei/incubation were utilized for the determination of DNA and RNA synthesis, respectively. In each experiment nuclei isolated from livers of 2–3 male and 2–3 female rats treated with saline were used as controls; the amount of DNA and RNA synthesis in treated animals is expressed as percentage of the controls.

RESULTS

In preliminary experiments performed to determine the contribution of the different DNA and RNA polymerases to DNA and RNA synthesis in isolated liver nuclei, nuclei isolated from both male and female rat liver were incubated with 100 μg/ml heparin or 4 mM p-hydroxymercuribenzoate (inhibitors of DNA polymerase α activity (6)) or 5 μg/ml α-amanitin (inhibitor of RNA polymerase II activity (15)). Heparin or *p*-hydroxymercuribenzoate reduce the amount of DNA synthesis to 2–3% of the control, indicating that under our experimental conditions DNA synthesis is due exclusively to the activity of DNA polymerase α. α-Amanitin reduces the amount of RNA synthesis to 70% of the control, indicating that 70% of RNA synthesis is due to RNA polymerase I activity and 30% to RNA polymerase II activity. Under our experimental conditions, RNA polymerase III activity is considered to be negligible (15).

In Vitro Experiments. ZAMI 1305, ZAMI 1327, DL-propranolol, and DL-atenolol, when added to liver nuclei isolated from both male and female rat liver, inhibit DNA and RNA synthesis in a dose-dependent fashion with DL-propranolol being the most effective (Fig. 1, A and B). All of the β-blockers tested, except DL-atenolol, affect DNA synthesis more than RNA synthesis. For a given concentration of the β-blocker tested, an increase of nuclei concentration in the assay mixture causes a decrease of the degree of inhibition of DNA and RNA synthesis (Fig. 2, A and B). The analysis of the kinetics of the incorporation of [³H]dTTP and [¹⁴C]UTP (Fig. 3, A and B) shows that the β-blockers decrease the initial rate of synthesis and the total amount of the labeled precursor incorporated into the growing chains.

To evaluate the possibility of a different susceptibility of RNA polymerases I and II to β-blocker inhibition, nuclei were incubated with the β-blocker and RNA synthesis was measured in the absence and in the presence

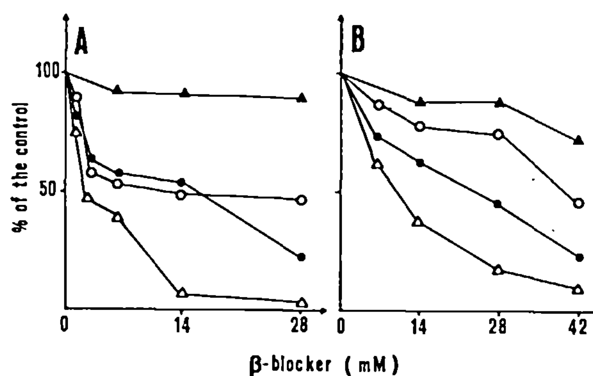

FIG. 1—*In vitro* inhibition of DNA (A) and RNA (B) synthesis by β-blockers in nuclei isolated from the liver of female rat. For the details of nuclei isolation, incubation with β-blockers, and evaluation of DNA and RNA synthesis see "Materials and Methods." Each point is the mean of 3–4 experiments in triplicate. DNA and RNA synthesis in control nuclei (taken as 100%) was equal to 1 pmol [³H]dTTP incorporated/hr/10^6 nuclei and 6 pmol [¹⁴C]UTP incorporated/hr/10^6 nuclei, respectively. Similar results are obtained when nuclei isolated from the liver of male rats are used. ▲, DL-atenolol; O, ZAMI 1327; ●, ZAMI 1305; △, DL-propranolol.

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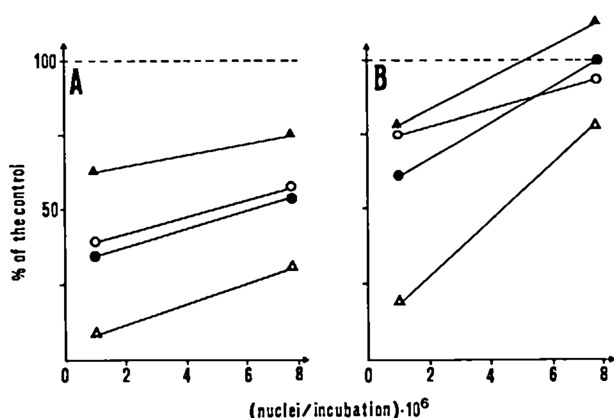

FIG. 2—Effect of different concentrations of nuclei on the *in vitro* inhibition of DNA (A) and RNA (B) synthesis by β-blockers. For the details of nuclei isolation, incubation with β-blockers, and evaluation of DNA and RNA synthesis see "Materials and Methods." β-Blockers were at the final concentration of 10 mM (A) or 14 mM (B). Each point is the mean of 3 experiments in triplicate. ▲, DL-atenolol; ○, ZAMI 1327; ●, ZAMI 1305; △, DL-propranolol.

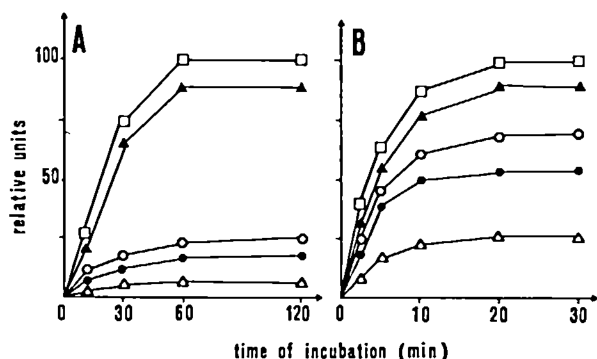

FIG. 3—Effect of β-blockers on the kinetics of incorporation *in vitro* of [^{3}H]dTTP in DNA (A) and [^{14}C]UTP in RNA (B) in nuclei isolated from the liver of female rat. For the details of nuclei isolation, incubation with β-blockers, and evaluation of DNA and RNA synthesis see "Materials and Methods." Each β-blocker was 28 mM. Each point is the mean of 2 experiments in triplicate. □, control; ▲, DL-atenolol; ○, ZAMI 1327; ●, ZAMI 1305; △, DL-propranolol.

of 5 μg/ml α-amanitin. The results (Fig. 4) demonstrate that the four β-blockers inhibit RNA polymerases I and II to a similar extent.

In Vivo Experiments. Since the *in vitro* experiments were unable to show any difference on DNA and RNA synthesis between the effect of the sex-dependent oncogenic ZAMI 1305 and that of the other nononcogenic β-blockers, the capacity of ZAMI 1305, its non-oncogenic isomer ZAMI 1327, and the non-oncogenic DL-propranolol to affect hepatic DNA and RNA synthesis was measured in nuclei isolated from the liver of animals treated *in vivo* with the β-blocker. DNA and RNA synthesis in isolated nuclei, depending on the *in vitro* elongation of the DNA and RNA chains initiated *in vivo* (6, 21, 31) represents in fact a parameter well related to the amount of the nucleic acids synthesis occurring *in vivo* at the time of death of the animal.

At 5 or 15 min after the administration of ZAMI 1305 or ZAMI 1327, no modification, in respect to the controls, of the amount of the hepatic DNA and RNA synthesis is detectable in male rats (Fig. 5); but ZAMI 1305 or ZAMI 1327 administration to female rats does affect both DNA and RNA synthesis (Fig. 5). In fact both molecules inhibit DNA and RNA synthesis in respect to the controls at 5 min; the inhibition is overcome at 15 min. DL-Propranolol administration causes an increase of DNA and RNA synthesis both at 5 and 15 min in male rats, but it is uneffective in female rats (Fig. 5).

The different sex response to β-blockers

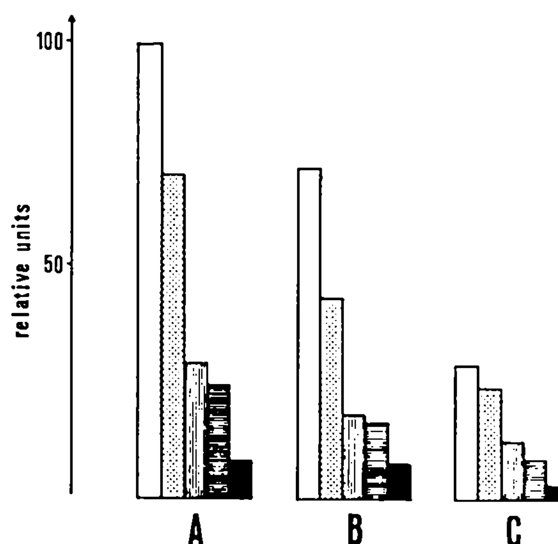

FIG. 4—Effect of β-blockers on RNA polymerase I and II activities in nuclei isolated from the liver of female rat. For the details of nuclei isolation, incubation with β-blockers, and evaluation of RNA synthesis see "Materials and Methods." Each β-blocker was 42 mM. Each value is the mean of 2 experiments in triplicate. A, RNA polymerase I and II activity (incubation without α-amanitin); B, RNA polymerase I activity (incubation with 5 μg/ml α-amanitin); C, RNA polymerase II activity (calculated by subtracting from each value shown in A the corresponding value shown in B). □, control; □, atenolol; □, ZAMI 1327; ⊟, ZAMI 1305; ■, propranolol.

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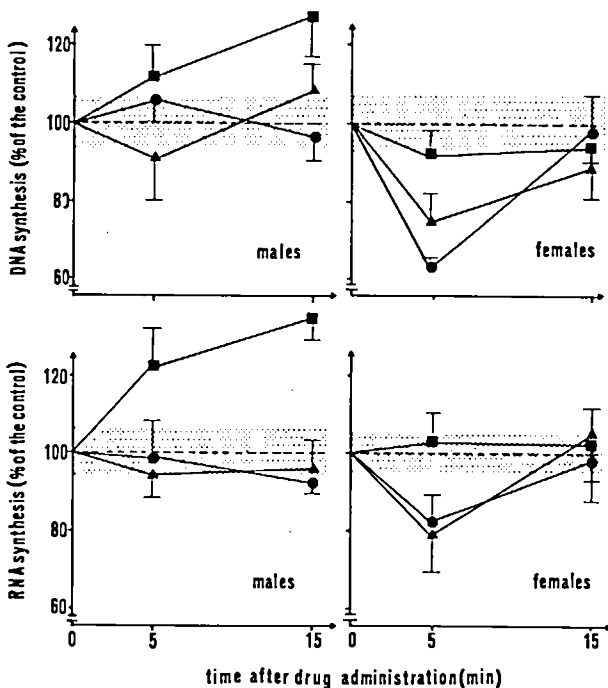


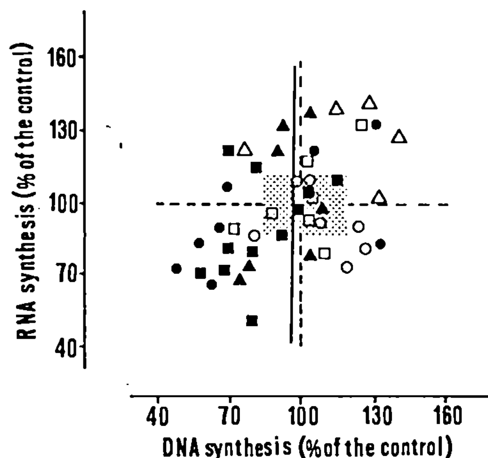
FIG. 5—DNA and RNA synthesis in liver nuclei isolated from male and female rats treated with ZAMI 1305 or ZAMI 1327 (100 mg/kg body weight) or DL-propranolol (75 mg/kg body weight). The drugs were injected i.p. and the animals were killed 5 or 15 min thereafter. For nuclei isolation and evaluation of DNA and RNA synthesis see "Materials and Methods." Each point is the mean ± S.E.M. of 3–5 experiments in triplicate. Shaded area represents the variability of the control. ●, ZAMI 1305; ▲, ZAMI 1327; ■, DL-propranolol.

administration is particularly evident when, for all of the individual experiments, the variation of DNA synthesis is plotted versus that of RNA synthesis (Fig. 6). The linear discriminant function analysis (2) performed on the above parameters independently of the time of treatment and of the drug administered does in fact demonstrate that male and female rats constitute two separate populations, which are different in their sensitivity to β-blockers. This discrimination is limited to the effect exerted by these molecules on DNA synthesis, which is not evident for RNA synthesis. From Fig. 6 it also appears that within the same sex a rather high variability of response to β-blocker administration is present. Since in control animals, for a given experiment, the value of the S.E.M. for the parameters tested is low, the variability does not appear to depend on experimental errors, but rather to reflect a physiological situation.

DISCUSSION

The oncogenic β-blocker ZAMI 1305 and the non-oncogenic β-blockers ZAMI 1327, DL-propranolol, and DL-atenolol inhibit DNA and RNA synthesis in a dose-dependent fashion when added *in vitro* to nuclei isolated from the liver of both male and female rats.

The mechanism of inhibition of nucleic acids synthesis by β-blockers could reside, as



Experimental group	Assigned group[a,b] males	females
males	15	4
females	9	16

FIG. 6—Hepatic DNA and RNA synthesis after β-blockers administration: linear discriminant function analysis of the data obtained in male *versus* female rats. The analysis was performed by comparing the two sexes independently of the time of treatment and of the β-blocker administered. The discriminant function is: $z = x - 0.0034\, y$, where x and y are the percentage of modification, in respect to the controls, of DNA and RNA synthesis, respectively. The data shown are those utilized in Fig. 5. Each point represents one experiment. Shaded area represents the variability (± S.E.M.) of the controls. ○, △, □, males; ●, ▲, ■, females; ○ and ●, ZAMI 1305; △ and ▲, ZAMI 1327; □ and ■, DL-propranolol. [a]By the discriminant function analysis the points are assigned to the group of males for $z > 96.86$ and to the group of females for $z < 96.86$. [b]Significance ($p < 0.01$) of the difference of assignment of male *versus* female rats was assessed by X^2 test. The significance of the test indicates the efficacy of the linear discriminant function analysis to separate the two sexes.

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for others chemicals (1, 7, 10–13, 17, 29), in an interaction of these molecules with the template primer DNA and/or with the DNA and RNA polymerases. An interaction with DNA is indirectly supported by the consideration that all of the β-blockers tested except DL-atenolol cause DNA fragmentation when added *in vitro* to isolated liver nuclei (23). On the other hand, this does not seem to be the main mechanism involved since: (a) DL-atenolol, which inhibits to some extent DNA and RNA synthesis, does not affect DNA integrity (23); (b) the four β-blockers tested in this work inhibit the activity of purified yeast DNA polymerase I (24) and of isolated mammalian DNA polymerases α and β (22) by interacting with the enzyme and not with the activated DNA.

It seems possible to hypothesize that the interaction of the β-blockers with DNA and RNA polymerases represents the main mechanism of inhibition of DNA and RNA synthesis in isolated liver nuclei. Even though the nature of the interaction is at present unknown, it is worth noting that for all biosynthetic processes studied in our laboratory (DNA and RNA synthesis in isolated liver nuclei, *in vitro* activity of yeast DNA polymerase I (24), and *in vitro* activities of hepatic DNA polymerases α and β (22)), the inhibitory capacity of β-blockers increases in this order: DL-atenolol < ZAMI 1327 ≤ ZAMI 1305 < DL-propranolol. Since these molecules differ essentially for the structure of their aromatic moiety, the observation suggests that this portion of the β-blocker molecule plays an important role in the inhibitory activity of these drugs and that the inhibition is caused by a mechanism common to all the biologic systems studied.

The *in vitro* experiments performed on isolated liver nuclei, as well those on yeast DNA polymerase I (24) and on isolated rat liver DNA polymerases α and β (22), failed to demonstrate any difference between the effects exerted by oncogenic and non-oncogenic β-blockers. Therefore, we have investigated if these molecules, according to their different oncogenic potential, exerted a different influence on hepatic nucleic acids synthesis *in vivo*. Accordingly, we have compared the effect of ZAMI 1305 administration in female rats (the sex susceptible to the oncogenic activity of this molecule) to that observed in male rats and the effect of this oncogen to that of the non-oncogenic β-blockers ZAMI 1327 and DL-propranolol in rats of both sexes.

In female rats inhibition of both DNA and RNA synthesis is observed for all of the β-blockers tested in most of the experiments, even though in some experiments, especially after DL-propranolol administration, activation of DNA and/or RNA synthesis is also observed (see Fig. 6). In male rats DL-propranolol induces an increase of nucleic acids synthesis; the rate of synthesis is unaffected by the administration of both ZAMIs. Thus, the β-blockers tested, independently of their different oncogenicity, affect hepatic nucleic acids synthesis in a different manner in female and in male rats.

On this basis the following points are worth discussion: (a) the different effect of β-blockers administration on DNA and RNA synthesis in female *versus* male rat liver; (b) the different influence exerted by the two ZAMIs *versus* DL-propranolol in the two sexes. Different hypotheses can be made to explain the sex-dependent effect of β-blockers on DNA and RNA synthesis. A different bioavailability of these molecules to the nucleus of the hepatocyte in the two sexes could depend on a different permeability of the cell membrane and/or on a different transport through the cytoplasm to the nucleus. It must be pointed out however that the transport of DL-propranolol through the cell membrane of the hepatocyte is a passive, not saturable process (19), and therefore remarkable differences in the two sexes in this respect seem to be unlikely. Different hepatic metabolism of β-blockers in the two sexes is well known. In fact the activity of the hepatic microsomal mixed function oxidase system is lower in female than in male rat (14). This could cause a reduced rate of inactivation of β-blockers by female rat liver, with a consequent higher inhibitory effect by these molecules in this sex. Preliminary experiments (not shown) indicate that three metabolites of ZAMI 1305 (2-nitro-3-methylphenoxypropan-1,2-diol; 2-nitro-3-methylphenoxyethylaminopropan-2-ol; 2-amino-3-methylphenoxy-*tert*-butylaminopropan-2-ol) present in the urine of male and female treated animals (25) do not affect *in vitro* nucleic acids synthesis. Quantitatively different pharmacologic activity of β-blockers in the two sexes. β-Blockade, with a consequent decrease of intracellular levels of cAMP (4, 28), could enhance cellular multiplication (30), hence increasing nucleic acids synthesis. Estrogens are demonstrated to affect the number of β-receptors and their affinity for sympathicomimetic amines (3, 16,

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32). This could cause, for a given β-blocker, a different modulation of β-receptors in the two sexes, with a consequent different influence on the intracellular levels of cAMP.

At present no experimental data are available to prove any of these hypotheses; further studies are therefore necessary to clarify the sex-dependent response of nucleic acid synthesis in rat liver to β-blocker administration.

The molecules tested influence hepatic nucleic acids synthesis independently of their different oncogenic capacity. Similar results are in fact obtained after administration of the oncogenic ZAMI 1305 and of the non-oncogenic ZAMI 1327, while the non-oncogenic DL-propranolol affects DNA and RNA synthesis in a different manner in respect to the two ZAMIs. The different effect exerted by the β-blockers tested could depend on the same mechanisms previously hypothesized to explain the different response observed in the two sexes. For both ZAMIs, molecules strictly related by chemical structure, one or more of these mechanisms could be relevant in determining the *in vivo* alterations of nucleic acids synthesis in respect to DL-propranolol molecule with a quite different chemical structure; in this latter case the pharmacologic β-blockade could exert, with the cAMP-mediated mechanism previously proposed, a predominant role.

Acknowledgments

This work was partially supported by Grant 82.02818.04 from the Consiglio Nazionale delle Ricerche and from the Ministero della Pubblica Istruzione (40% National Project of Experimental Oncology). We wish to thank E. Dimito for her skilled technical assistance.

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