DNA intercalators include aromatic heterocyclic compounds of various chemical classes with profound biological activities. Intercalators are the most important group of compounds that interact reversibly with DNA double helix. The flat molecules of these ligands intercalate between pairs of DNA helix, lengthening and unwinding this structure at the intercalation sites. Intercalating agents share common structural features such as the presence of planar polyaromatic systems which bind by insertion between DNA base-pairs, with a marked preference for 5’-pyrimidine–purine 3’ steps and possess a polyunsaturated flat chromophore with a marked electron deficiency, and basic chains linked to the chromophores (1). Nowadays, it is well accepted that antitumor activity of intercalators may be nearly related to the ability of these compounds to stabilize the DNA-intercalator-topoisomerase II ternary complex.

Interest in DNA-intercalating ligands as anti-cancer drugs has greatly developed since the clinical success of doxorubicin. However, despite a great deal of „rational design” of synthetical DNA-intercalators only a few such compounds have proved clinically useful. Some of them are valuable drugs currently used in the treatment of ovarian and breast cancers and acute leukemias, while many others are in different phases of clinical trials (2). Condensed cinnoline and quinoline derivatives are of great interest due to their biological properties. In particular, their antibacterial (3, 4, 5) and antitumor activity (6, 7) makes these systems attractive for further modification. Many authors report the antitumor activity of condensed cinnoline systems as intercalating agents (8) and topoisomerase I-targeting activity (9).

Because the relationships between structure and the ability to inhibit topoisomerases are still not well defined, in our search for new therapeutics, we have synthesized some pyrimido[5,4-c]cinnoline [VI f-h] and pyrimido[5,4-c]quinoline [VI a-e, IV, VII] derivatives to evaluate their cytotoxic activity on two leukemia cell lines. The chemical structure of synthesized tricyclic N-substituted may suggest their activity as potential intercalators. They have a planar polyaromatic system and ability to form hydrogen bond with DNA base (Figure 1). A basic terminal group in the side chain, linked to the planar ring system, may also play an important role in the activity of these compounds. The tested compounds exhibit large polarizability as well and can be good electron acceptors to DNA base pair.

SYNTHESIS AND CYTOTOXICITY OF NEW POTENTIAL INTERCALATORS BASED ON TRICYCLIC SYSTEMS OF SOME PYRIMIDO[5,4-c]CINNOLINE AND PYRIMIDO[5,4-c]QUINOLINE DERIVATIVES. PART I

WIESŁAWA LEWGOWD1, ANDRZEJ STAŃCZAK1, ZBIIGNIEW OCHOCKI1, URSZULA KRAJEWSKA1 and MAREK RÓZALSKI2

1 Department of Pharmaceutical Chemistry and Drug Analysis, 2Department of Pharmaceutical Biochemistry, Medical University of Łódź, I Muszyńskiego Str., 90-151 Łódź, Poland

Abstract: Pyrimido[5,4-c]cinnoline and pyrimido[5,4-c]quinoline derivatives have been tested as potential intercalators. All of them embody structural properties alike to afford intercalating activity. Their cytotoxicity was determined on the two human leukemia cell lines, the promyelocytic HL-60 and the lymphoblastic NALM-6. The viability of cells exposed continuously to tested compounds was estimated by the trypan-blue exclusion assay. IC50 data for the NALM-6 cell line are lower than for the HL-60 cell line, what suggested that the HL-60 leukemia cells are more resistant to toxic action of tested compounds. All compounds exerted moderate cytotoxic activity. The compounds were analyzed with the HyperChem/ChemPlus software trying to find basic structure-activity relationships.

Keywords: pyrimido[5,4-c]cinnoline; pyrimido[5,4-c]quinoline; cinnoline; quinoline; intercalators
EXPERIMENTAL

Chemistry

The synthesis of 4-amino-3-cinnolinecarboxamides or 4-amino-3-quinolinecarboxamides [II] was based on the Friedel-Crafts cyclocyclocondensation of (phenylhydrazono)(cyano)acetamides or (phenylamino-methylene)(cyano)acetamides [I] in dry chlorobenzene in the presence of anhydrous aluminium chloride and was conducted according to the procedure shown in Scheme (10, 11). The carboxamides [II] were also hydrolyzed to the corresponding 4-amino-3-cinnolinecarboxylic acids and 4-amino-3-quinolinecarboxylic acids [III] and subsequently transformed with acetic anhydride to 2-methyl-1,3-oxazino[5,4-c]cinnolin-4(3H)-ones or 2-methyl-1,3-oxazino [5,4-c]quinolin-4(3H)-ones [V]. The final step to the N-3 substituted 2-methylpyrimido[5,4-c]cinnolin-4(3H)-ones or 2-methylpyrimido[5,4-c]quinolin-4(3H)-ones [VI] were accomplished by the reaction of 2-methyl-1,3-oxazines [V] with various amines. A different pathway was employed to prepare 1,2,3,4-tetrahydropryrimido[5,4-c]cinnolin-2,4-diones and 1,2,3,4-tetrahydropryrimido[5,4-c]quinolin-2,4-diones [IV]. The compounds [IV] were obtained by cyclization of amides [II] with diethylcarbonate (12).

The ethyl 1-(3-chlorobenzyl)-4-imino-6-methyl-1,4-dihydro-3-quinolinecarboxylate [VII] was prepared from ethyl 4-amino-3-quinolinecarboxylate, which was formed by the reaction of 4-amino-3-quinolinecarboxylic acid [III] with ethyl iodide and DBU as a catalyst (13). Alkylation of quinoline ester was performed with 3-chlorobenzylchloride in the presence of K2CO3.

The structure of all compounds in Table 1 was earlier confirmed by IR, ‘H NMR spectra, the elemental analysis, and published elsewhere (10–12).

Biological test in vitro

Cell culture

Acute and chronic leukemias are widespread human cancers. Taking this into account it was decided to use two leukemia cell lines: myeloblastic (HL-60) and lymphoblastic (NALM-6) in cytotoxicity screening assay of synthesized compounds. The HL-60 cell line was obtained from the Institute of Immunology and Experimental Therapy (Wroclaw, Poland) and the NALM-6 cell line was purchased from the German Collection of Microorganisms and Cell Cultures (14). The cells were grown in RPMI 1640 medium (Cambrex, Verviers, Belgium), supplemented with L-glutamine (2 mM), gentamycin (5 µg/mL) and 10% heat-inactivated fetal calf serum, at 37°C under 5% CO2, 95% air atmosphere.

Cytotoxicity assay

Exponentially growing HL-60 and NALM-6 cells were seeded at 0.4 × 10⁶ per each well of a 24-well plate (Nunc, Roskilde, Denmark) and cells were then exposed for 48 h to the compounds indicated in the experimental section. Stock solutions of the test compounds were prepared freshly in DMSO and were used for serial dilutions in complete cul-
ture medium. The final concentration of DMSO in medium was 0.2%. The number of viable cells was counted in a Bürker haemocytometer using a trypan-blue exclusion assay, and dose-response curves were determined. Values of IC50 (the concentration of tested compounds required to reduce leukemia cell survival fraction to 50% of control) were used as a measure of cellular sensitivity to a given treatment.

Statistical analysis of the data
The statistical analysis of cytotoxicity data of all compounds were performed with the STATISTICA v. 6.0 software (15). The cytotoxicity coefficient (IC50) was expressed as means ± standard error of mean (SEM). If not statistically significant differences between variations were found, the differences between means were assessed by applying Student test. Otherwise, Cochran-Cox test was used. The value p<0.05 was considered as significant.

RESULTS AND DISCUSSION
Chemistry
SAR analysis

For tested pyrimido[5,4-c]quinoline derivatives [VI a-e, IV, VII] and pyrimido[5,4-c]cinnoline derivatives [VI f-h], a comparative computer analysis was done. All the structures of the studied

<table>
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<td>CH3</td>
<td>CH</td>
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<tr>
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<td>CH</td>
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<tr>
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<td>H</td>
<td>CH</td>
</tr>
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<td>CH3</td>
<td>H</td>
<td>CH</td>
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<td>CH3</td>
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</table>

** as hydrochloride , ** as potassium salt

*Table 1. Chemical structure of pyrimido[5,4-c]quinoline [VI a-e, IV, VII] and pyrimido[5,4-c]cinnoline derivatives [VI f-h]*
compounds were geometrically optimized by the use of a semiempirical method INDO [algorithm Polak-Ribiere, RMS grad = 0.01 kcal/(GC5 mole), in vacuo, HyperChem 5.1] (16). The systematic conformational analysis was not used. The physicochemical parameters of the compounds were received from ChemPlus modules of HyperChem and summarized in Table 2. Geometric optimization of the studied compounds revealed a flat character of the basic core pyrimido[5,4-c]quinoline and pyrimido[5,4-c]cinnoline derivatives. C2, C8 and C9 substituents are located in the same plane, while morpholinoethyl- or diethylaminoethyl- group in position N3 are placed above the plane of the tricyclic system. Studied compounds were characterized by a similar vector of polarization. The negative pole of polarized molecules of all derivatives is located in the central area of the pyrimidine ring. It seems that electronegative atoms, built in the heterocyclic ring of cinnoline, quinoline and pyrimidine or the structure of substituents in C3 and C4 positions, may be responsible for the interaction with biological targets, because the formation of hydrogen bond with nucleic acids of DNA may only be possible for electronegative atoms in the molecules. The more cytotoxic compounds [VI a, d, h, VII] possess Van der Waals surface of molecule (GRID) above 300 \( \text{E}^2 \) and similar volume of molecule, above 300 \( \text{E}^3 \) as well. Thus, their capacity are like other small organic molecules, applied as intercalating agents, such as naphtalimides. They show also higher values of refractivity and polarizability, which can influence flexibility of these derivatives while interacting with biological target. However, molecules of pyrimido[5,4-c]quinoline [VI b, c, e, IV] and of pyrimido[5,4-c]cinnoline derivatives [VI f, g] with moderate cytotoxic activity are smaller and have lower values of polarizability and refractivity, except [VI f]. This pyrimido [5,4-c]cinnoline derivative though possesses similar values of physicochemical parameters as more active biologically group [VI a, d, h, VII], demonstrates low cytotoxic effect. This is likely to be related with lower values of charge on N6 atom in pyrimido[5,4-c]cinnoline molecule.

It is rather difficult to say that there is a clear relationship between calculated physicochemical parameters and cytotoxic activity of compounds, but our study was only an initial screening. Further studies will be extended not only to the synthesis of new compounds, but examination of their affinity to form the DNA-intercalator-topoisomerase II ternary complex as well.

**Biological test in vitro**

Cytotoxicity

The cytotoxicity of the pyrimido[5,4-c]quinoline derivatives [VI a-e, IV, VII] and pyrimido[5,4-c]cinnoline derivatives [VI f-h] was determined on the two human leukemia cell lines, the promyelocytic HL-60 and the lymphoblastic NALM-6. The viability of cells exposed continuously to tested compounds was estimated by the trypan-blue exclusion assay. Cytotoxicity towards both cell lines was determined in a broad concentration range, between \( 10^{-6} \) and \( 10^{-3} \) M. The values of IC\(_{50}\) (the concentration of test compounds required to reduce the cell survival fraction to 50% of that observed in the control cells) were calculated from dose-response curves.
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shown in Figures 2a, 2b and Figures 3a, 3b. The IC50 values are presented in Table 3.

IC50 data for the NALM-6 cell line are lower than for the HL-60 cell line. The only exception was compound \[ \text{VI c} \]. Such results indicate that HL-60 leukemia cells are more resistant to toxic action of tested compounds. In the case of more sensitive NALM-6 cell line two groups of compounds can be arbitrary distinguished. The first group with a moderate cytotoxic activity (IC50 = 439-575 µM) contains four pyrimido[5,4-c]quinoline derivatives [VI a-e, IV, VII]. Each point represents the mean of 4 independent determinations. SEM are excluded for clarity and do not exceed 20% of the mean value for each point.

Figure 2 A, B. Survival curves for HL-60 (A) and NALM-6 (B) leukemia cells exposed to pyrimido[5,4-c]quinoline derivatives VI a-e, IV, VII. Each point represents the mean of 4 independent determinations. SEM are excluded for clarity and do not exceed 20% of the mean value for each point.

Figure 3 A, B. Survival curves for HL-60 (A) and NALM-6 (B) leukemia cells exposed to pyrimido[5,4-c]cinnoline derivatives VI f-h. Each point represents the mean of 4 independent determinations. SEM are excluded for clarity and do not exceed 20% of the mean value for each point.

rate cytotoxic activity (IC50 = 439-575 µM) contains four pyrimido[5,4-c]quinoline derivatives [VI b, c, e, IV] and two pyrimido[5,4-c]cinnoline derivatives [VI f, g]. The second group, include more cytotoxic compounds [VI a, d, h, VII] with IC50 in the range of 188-373 µM. The presence of the morpholino–group, bound to pyrimidine moiety by two-carbon spacer arm is characteristic of the most strongly acting compound [VI d] (IC50 = 188 µM). Biological effectiveness of this derivative was statistically significantly higher in comparison to all compounds...
from the first group. The terminal basic function of the morpholino group and flexibility of the ethyl chain allows to connect the compound with the element of DNA as an acceptor of protons in hydrogen bond.

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