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Potential antineoplastic activity of keto-C-glycosides —a new family of cytostatic agents

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We have examined the biological activity of keto-C-glycosides (KCGs), a new family of drugs displaying antiproliferative and cytotoxic properties on tumor cells. KCG1, the most powerful drug tested on epithelial derived neoplastic cells, was 25–125 times more cytostatic on epithelial cells than on lymphoma. By contrast, KCG10 proved to be more cytostatic on lymphoma than on epithelial cells. Correlations were found between the cytostaticity of KCGs and their lipophilicity, and are discussed within the framework of the structure–activity and the structure–selectivity relationships.

Key words: Antiproliferative effects, keto-C-glycosides, selectivity, structure–activity relationship, tumoral cells.

Introduction

The keto-C-glycosides (KCGs)[1–3] constitute a new family of synthetic compounds with chemical structures based on natural product models. KCGs are molecules containing a conjugated enone system in the sugar moiety, an analogous structure to that found in numerous natural polycyclic molecules displaying cytotoxic properties (quassinoides, trichothecanes and sesquiterpenes). We report herein the structure–activity relationships, established *in vitro* on hepatoma (LFCL2A) and lymphoma cells (RAJI), of 22 newly synthesized C-glycosides (CGs) and KCGs, as well as the study of the relationships between the physiochemical properties of the drugs, some biological features of the cell lines used and the antitumoral activities obtained. The most powerful and selective drugs were tested on other human and rodent tumoral cell lines [i.e. the hepatoma HepG2, the colon carcinoma HT29, the epidermoid carcinoma KB, the lymphoma Daudi and the Chinese hamster ovary (CHO) cells LR73]. These studies allow us to select compounds KCG1 and KCG10 to further investigate their cytostatic and cytotoxic potential.

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Materials and methods

Cells and media

LFCL2A is an established cell line derived from an hepatocarcinoma induced in the Commentry rat by 4-dimethyl-aminoazobenzene in our laboratory.[4,5] These cells were grown in Eagle's minimum essential medium (EMEM; Eurobio, France) supplemented with 10% new born calf serum (Flow Laboratories). They were usually seeded at 25 000 cells/ml medium and subcultured twice a week. HepG2 (human hepatoma cell line), HT29 (human colon carcinoma cell line) and KB (human epidermoid carcinoma cell line) were grown in Dulbecco's modified EMEM (Eurobio) containing 4.5 g/l glucose and supplemented with 10% fetal calf serum (FCS; Flow laboratories for HepG2 and HT29 cells, Gibco BRL for KB cells). They were usually seeded at 100 000 cells/ml medium and weekly subcultured. Raji and Daudi cells (human

cells derived from Burkitt lymphoma, expressing Epstein–Barr antigens) were grown in RPMI 1640 (Gibco BRL) supplemented with 10% FCS (Bayer Diagnostics, France) inactivated by heating for 30 min at 56°C. They were seeded at 150 000 cells/ml and subcultured twice a week. LR73 cells (CHO cells) were grown in MEM alpha medium with ribonucleosides and deoxyribonucleosides (Gibco BRL) supplemented with 10% FCS (Gibco BRL).

All media were supplemented with 2 mM glutamine and antibiotics (Eurobio; 100 IU penicillin/ml and 100 μg streptomycin/ml) and the cells incubated at 37°C in a humidified incubator with an atmosphere of air/CO_2 (95/5).

Drugs

The KCGs presented in this work (Figure 1) were synthesized by RM, JH and KA. General methods for their synthesis and full NMR data have previously been published elsewhere.[1–3]

Proliferation and cytostaticity assays

For all the tests, drugs were kept frozen at −20°C and freshly diluted in dimethyl sulfoxide (DMSO; Prolabo) at a final concentration of 0.5% in culture medium. Cell suspensions (200 μl) were dispensed in wells of microtest plates. Cells were incubated with the cytostatics for 72 h for epithelial cells, and 48 h for Raji and Daudi cells. The antiproliferative effects of the drugs were measured by the use of the [^{3}H]thymidine uptake test: 5 h before the cells were harvested, 1 μCi [^{3}H]thymidine (Amersham) was added to each well. Cultures were washed and collected with an automated sample harvester (Skatron) on glass fiber filters (Whatman). The filters were dried and the radioactivity was counted in omnifluor in a liquid scintillation spectrometer. The cytotoxicity was evaluated by light microscopic numeration using the trypan blue exclusion test. Each experiment was conducted in triplicate and the data presented are the mean of at least three independent experiments. The results obtained were processed with the computerized program *inhib v* 1.0 conceived in our laboratory,[6] and the drug concentration that inhibits the [^{3}H]thymidine incorporation by half or that kills 50% of the cell population (IC_{50}) as compared with the control (cells treated with 0.5% DMSO) was calculated by the same program.

Results

Structure–activity relationships

The nomenclature and classification used in this work are summarized in Figure 1. The cytostatic activities displayed by CGs and KCGs on LFCL2A

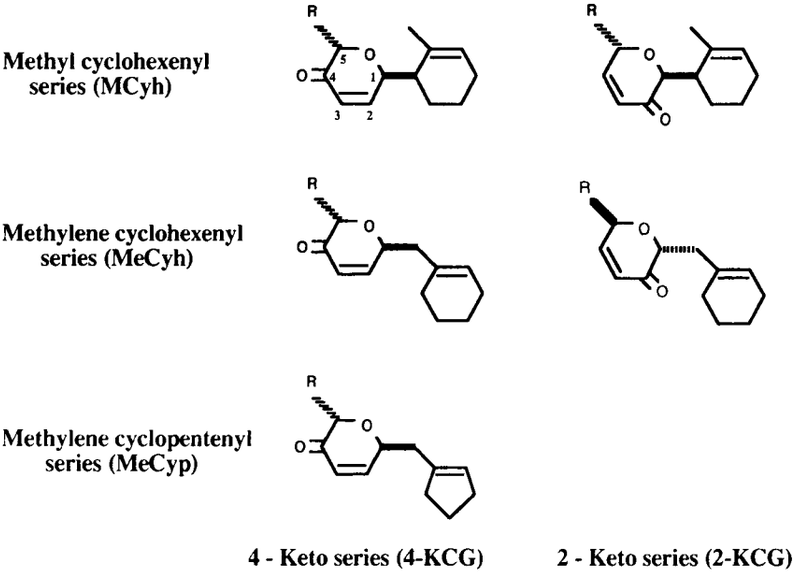


Figure 1. Nomenclature and subclasses of KCGs related to the nature of the aglycone moiety and to the keto position.

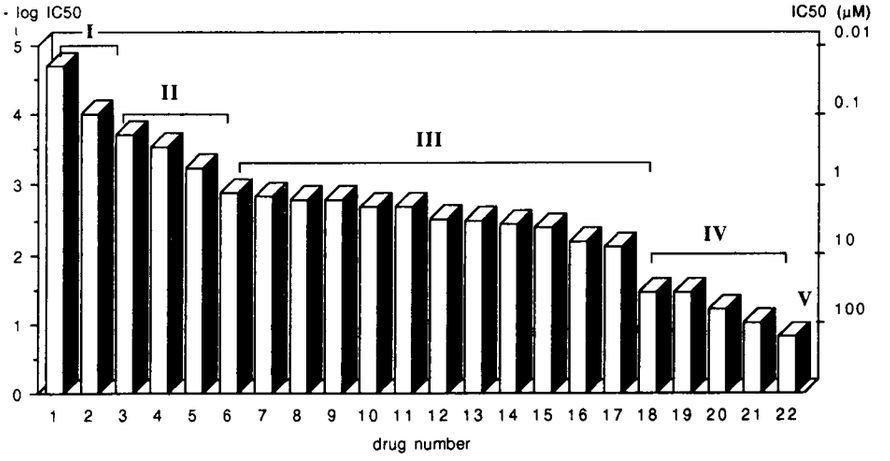


Figure 2. Logarithmic representation of IC_{50} values of KCGs on LFCL2A cells. Group I: $0.01 < IC_{50} < 0.1$ μM, group II: $0.1 < IC_{50} < 1$ μM, group III: $1 < IC_{50} < 10$ μM, group IV: $10 < IC_{50} < 100$ μM, group V: $IC_{50} > 100$ μM.

cells ranged from 152 to 0.02 μM. To facilitate comparison and discussion of results, we used the logarithmic scale advocated by Venugopal and Luckery.[7] The negative logarithm of IC_{50} expressed in mM ($-\log IC_{50}$) was calculated and we classified the 22 compounds into five groups (I–V in order of decreasing cytostaticity; Figure 2). We showed that:

(i) The conjugated enone system was absolutely required for the biological activity of KCGs (Table 1). For instance, the acetylation of the 4-keto group in compounds **10** and **3** led to the two CGs (molecules lacking keto group) **22** and **18** which were, respectively, 75 and 170 times less cytostatic.

(ii) KCGs with an acyclic aglycone (**19–21**) possessed low cytostaticities ($IC_{50} > 34$ μM) showing that in addition to the presence of a conjugated enone, a cyclic aglycone bound to the anomeric carbon of a pyrane structure was also decisive for the activity. The lack of one or other of these conditions dramatically depressed the cytostaticity (Figure 2, groups IV and V).

(iii) When these two conditions were required, any changes at R_1 modulated the bioactivity: the R_1-unsubstituted drugs, as well as the R_1-CH_2OH and the most part of R_1-CH_2OAc substituted compounds, exhibited moderate cytostatic effects with IC_{50} values of 1–10 μM. These drugs were classified into group III.

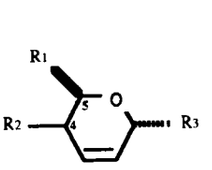
(iv) The other compounds were shared out among groups II and I, the four most powerful drugs were all methylated at R_1. C_5-CH_3 appeared to be strongly implicated in the cytostaticity developed on hepatocarcinoma cells. Table 2 summarizes the importance of the substitutions at C_5 among the three classes of KCGs. It appears that the modulation of the cytostaticity by R_1 substitutions was more important in the MCyh series than in the MeCyh and MeCyp series.

(v) Group III also includes the most part of 2-KCGs and the keto position also appeared to be important in the case of methylated drugs (Table 3). As a matter of fact, the 4-KCGs were more potent than their 2-keto isomers on all the epithelial cell lines studied, whereas an inverse phenomenon was observed on Raji cells. In the case of compounds with a CH_2OAc at the R_1, the 4-keto isomer was more potent on lymphoma cells than the 2-keto analog, although with less pronounced cytostaticity differences.

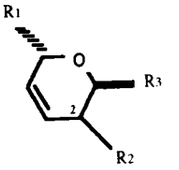
Hydrophobicity–activity relationships

The lipophilicity of drugs was determined from the partition coefficient *P*, which represents the ratio of amounts of drug shared out among the two phases of a resting water–solvent mixture (usually water–octanol). It can also be calculated by several methods with generally good approximations. We estimated the *P* coefficient as calculated by Rekker *et al.*[8] *P* values are listed in Table 1, values of $P_{calc} > 1$ indicate that the drugs are lipophilics. Some correlations between the hydrophobicity and the activity were observed; the four most active drugs proved to be among the most lipophilic and the less cytostatic proved to be hydrophilic. Other correlations were found for the fairly cytostatic molecules. Some drugs displaying about similar

Table 1. Chemical structure, cytotoxicity and hydrophobicity of CGs and KCGs



CG and 4-KCG



2-KCG

Group	Drug	R_1	R_2	R_3	IC_{50} (μM)	*P*
I	1	CH_3	=O	MCyh	0.02 ± 0.011	245
	2	CH_3	=O	MeCyp	0.1 ± 0.06	74
II	3	CH_3	=O	MeCyh	0.2 ± 0.07	245
	4*	CH_3	=O	MCyh	0.3 ± 0.06	245
	5	CH_2OAc	=O	MeCyp	0.6 ± 0.15	1
III	6	CH_2OH	=O	MeCyp	1.3 ± 0.50	7
	7	H	=O	MeCyp	1.5 ± 0.05	22
	8	H	=O	MeCyh	1.6 ± 0.22	74
	9*	CH_2OAc	=O	MeCyh	1.6 ± 0.24	3
	10	CH_2OAc	=O	MeCyh	2 ± 0.4	3
	11*	CH_2OAc	=O	MCyh	2 ± 0.12	3
	12	CH_2OAc	=O	MCyh	3 ± 0.5	3
	13	CH_2OH	=O	MeCyh	3.2 ± 0.33	23
	14	H	=O	MCyh	3.7 ± 0.21	74
	15*	CH_2OH	=O	MCyh	4 ± 0.3	23
	16	CH_2OH	=O	MCyh	6.4 ± 0.17	23
	17*	CH_2OH	=O	MeCyh	7.7 ± 0.89	23
IV	18°	CH_3	CH_2OAc	MeCyh	34 ± 4.4	132
	19*	CH_3	=O	$CH_2C(Cl)(CH_3)(CH_2)_2OAc$	34 ± 1.8	0.3
	20	H	=O	$CH_2CH(CH_3)CHO$	61 ± 15.5	0.1
	21	CH_3	=O	$CH_2CH(CH_3)CHO$	99 ± 27.1	0.4
V	22°	CH_2OAc	CH_2OAc	MeCyh	152 ± 3.3	1.5

IC_{50} ± SD were obtained on LFCL2A cells. Each experiment was conducted in triplicate and the data are the mean of at least three independent experiments. The coefficient of partition *P* was calculated by the Rekker method.[8] Asterisks indicate that the molecule is a 2-KCG and open circles that the molecule is a CG. MCyh, MeCyh and MeCyp are, respectively, methyl cyclohexenyl, methylene cyclohexenyl and methylene cyclopentenyl subclasses of CGs and KCGs. Ac: acetate.

cytostatic potential exhibited similar *P* values (compounds **3** and **4**, $P = 245$; compounds **9–12**, $P = 3$; compounds **13**, **15–17**, $P = 23$; compounds **19–21**, $0.1 < P < 0.4$). Table 4 indicates other correlations: the C_6-hydroxylated compounds **13** and **16** exhibited similar IC_{50} ratios (unsubstituted/substituted drug) against the tested hepatoma and lymphoma. Furthermore, they were moderately cytostatic on all cell lines studied and the IC_{50} ratios appeared to be very close to those of P_{calc}.

By contrast, no correlation was found between the hydrophobicity and the activity with the C_6-acetylated compounds **10** and **12**: in spite of their low lipophilicity, compared with the unsubstituted

Table 2. Ratio of cytotoxicities of C_5-unsubstituted, C_6-hydroxylated and C_6-acetylated compounds to the C_5-methylated drugs on LFCL2A cells: evidence for the importance of the C_5-methyl on the bioactivity

IC_{50} ratio	MCyh	MeCyh	MeCyp
C_5-H/C_5-Me	185	8	15
C_5-CH_2OH/C_5-Me	320	16	13
C_5-CH_2OAc/C_5-Me	150	10	6

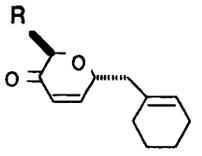
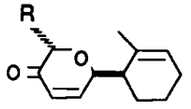
C_5: carbon 5 of the ketosugar moiety, Ac: acetate, Me: methyl. The given ratios represent the ratio of IC_{50} expressed in μM.

Table 3. Cytotoxicity ratio of 2-keto and 4-keto analogs of the MCyh subclass of KCGs on several epithelial and lymphoblastoid tumoral cells

Drugs	LFCL2A	HepG2	HT29	KB	LR73	RAJI
4/1	15	25	15	16	16	0.2
11/12	0.7	0.5	0.3	ND	ND	2

Drug **1** is a 4-KCG methylated at C_5 and compound **4** its 2-keto analog. Drugs **11** and **12** are acetylated at C_6 and are, respectively, 2-keto and 4-keto analogs. The given data are the IC_{50} ratios. ND: not done.

Table 4. Cytotoxicity and hydrophobicity ratios of unsubstituted and C_5-substituted 4-KCGs

Subclass	Drug	Substituent R	LFCL2A	Raji	P_{calc} ratio
 A	**8**	H	1	1	1
	13	CH_2OH	0.5	0.5	0.3
	10	CH_2OAc	0.8	13	0.04
 B	**14**	H	1	1	1
	16	CH_2OH	0.6	0.3	0.3
	12	CH_2OAc	1.2	14	0.04
	1	CH_3	185	1.3	3.3

(A) Methylene cyclohexene and (B) methyl cyclohexene subclasses of 4-KCGs. The given data are the ratio of IC_{50} of the substituted drug to the unsubstituted drug (R = H). P_{calc} ratio: ratio of coefficients of partition *P* as calculated according to Rekker *et al.*[8]

drugs **8** and **14** used as references, they were more than 10 times more cytostatic on lymphoma cells. We noticed that the ratios were very similar between the two subclasses (MCyh and MeCyh) of KCGs. Concerning drug **1**, no correlation was found between the P_{calc} ratio and the IC_{50} ratio on either Raji or LFCL2A cells. Indeed, KCG1 proved to be more than 180 times more cytostatic on LFCL2A cells than compound **14**, and as cytostatic as the latter on Raji cells.

Structure–selectivity relationships

Figure 3 shows the IC_{50} obtained on Raji cells versus the IC_{50} obtained on LFCL2A cells of groups I, II and III. Some drugs exhibited a significant selective cytostaticity. Thus compounds **7**, **8**, **10**–**13** and **17** displayed some preferential cytostaticity on Raji cells. KCG1 and KCG10 were studied more extensively (Table 5): the comparison of their IC_{50} on LFCL2A, HepG2, HT29, Raji and Daudi cells showed real discriminating effects of KCG1 and KCG10 depending on the epithelial or lymphoblastoid nature of the neoplastic cells.

Discussion

KCGs are molecules containing a pyrane structure similar to that found in some natural cytotoxic products such as the terpenoid agents, quassinoides and sesquiterpenes,[9] or other cytotoxics such as trichothecanes.[10] The pyrane unit has been used in the synthesis of ketonucleosides and Antonakis *et al.* have already demonstrated that the cytotoxicity of this family of compounds is strongly linked to the presence of a conjugated enone group in the sugar moiety.[11] Unfortunately, many natural and modified nucleosides used in chemotherapy are promptly inactived *in vivo* by deamination, which subsequently reduces their clinical use.[12,13] Furthermore, the puric or pyrimidic nature of the nucleotide had a limited influence on the activity of the ketonucleosides studied, whereas any chemical changes in the ketosugar moiety strongly affected the cytotoxicity of the drug.[11,14] These considerations led to the design of KCGs as molecules possessing an aglycone moiety bound to the anomeric

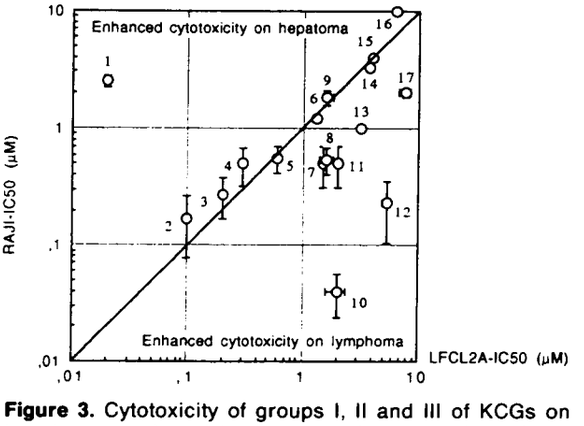


Figure 3. Cytotoxicity of groups I, II and III of KCGs on lymphoma and hepatoma cells: demonstration of the selective cytotoxic potential of some drugs. Each point is the mean of at least three independent experiments. Horizontal and vertical bars represent the standard deviation of IC_{50}. The diagonal corresponds to a ratio of IC_{50} (LFCL2A/Raji) of 1.

Table 5. Comparative cytotoxicities of KCG1 and KCG10 on epithelial and lymphoblastoid tumoral cells: evidence for differential cytotoxic effects

Cell line	Drugs	
	KCG1	KCG10
LFCL2A	20 ± 11 (6)	2000 ± 400 (4)
HepG2	60 ± 12 (3)	500 ± 300 (3)
HT29	90 ± 26 (3)	1000 ± 200 (2)
KB	50 ± 6 (3)	ND
LR73	50 ± 9 (3)	ND
Raji	2500 ± 260 (5)	40 ± 16 (5)
Daudi	2320 ± 132 (2)	44 ± 13 (2)

The given data are IC_{50} ± SD (in nM: $p < 0.05$). Numbers in parentheses represent the number of independent experiments; each experiment was conducted in triplicate. ND: not done.

carbon of a pyrane-containing enone structure and previous reports showed that KCGs exerted *in vitro* cytotoxic effects on rat hepatoma cells.[5]

The purpose of the work presented here was to investigate some biological properties of KCGs and to look for their antiproliferative potential against neoplastic cells. Thus, the cytostaticity of 22 new CGs and KCGs was studied in several tumoral epithelial and lymphoblastoid cell lines. We used the hepatocarcinoma cell line LFCL2A to screen the cytostatic drugs and to set up the structure–activity relationships. For this, we studied the effect of the aglycone on the cytostaticity by testing 2- and 4-keto α,β unsaturated CGs with linear or cyclic aglycones. We also investigated the significance of substitutions at the C_5 as well as the importance of the keto group position. Our data show that all the KCGs possessing cyclic aglycones were significantly cytostatic against all tumoral cell lines tested with IC_{50} lower than 10 μM. Some drugs, like the C_5-methylated KCG and the C_6-acetylated KCG, displayed cytostaticities at the nanomolar level on hepatoma and lymphoma, respectively. The study of the hydrophobicity of KCGs highlighted some outstanding features of this family of drugs. Some interesting correlations were observed between the cytostaticity displayed by the drugs and their lipophilicity as expressed by the coefficient of partition *P*. The most lipophilic drugs proved to be among the most potent and the less lipophilic among the ineffective compounds. The hydrophobicity–cytostaticity relationships were more significant when only KCGs are taken into account, thus CGs **18** and **22** should not be considered in this study. However, KCG1 and KCG10 showed interesting properties in regard to their high cytostaticity and selectivity on some specific tumoral types (see Table 4). By contrast, compounds **13** and **16** were moderately cytostatic; their reduced cytostaticity compared with the reference compounds **8** and **14** (see Table 3) was probably due to the decreased hydrophobicity and to the consequently reduced cellular uptake. Our findings concerning KCG1 and KCG10 suggest that certain cellular phenomena are implicated, like metabolism- or receptor-mediated cytotoxicity, and partly rule out the possibility of passive drug uptake and therefore a simple hydrophobicity-associated cytotoxicity. The mechanisms by which chemical substitutions at C_5 induce the diversity of the cytostaticity and hydrophobicity ratios, as well as the reason(s) for the occurrence of the selective effects of KCG1 and KCG10, are not yet understood.

In conclusion, the assessment of the antiproliferative potential of some newly synthesized KCGs and the study of their structure–activity relationships established *in vitro* on hepatocarcinoma and lymphoma cells has allowed us to select compounds KCG1 and KCG10 as two powerful antiproliferative drugs (Table 5). These two drugs proved to be selectively cytostatic on epithelial and lymphoid tumoral cells, respectively. The selectivity represents an important criterion required for cytostatics susceptible to be used in human therapy. Thus, KCG1, which was highly active on epithelial neoplastic cells and ineffective on lymphoma, is likely to lack or even be devoid of immunosuppressive effects. Immunosuppression is the most common side effect encountered in cancer chemotherapy and limits the potency of such treatments. KCG10, which displayed an inverse pattern of cytostaticity and was moderately cytostatic on all epithelial cell lines tested, is likely to be an efficient antineoplastic for lymphoma chemotherapy without inducing strong hepatotoxicity. We are currently carrying out other *in vitro* and *in vivo* experiments to allow us to better grasp the biological features and antineoplastic potential of KCG1 and KCG10.

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