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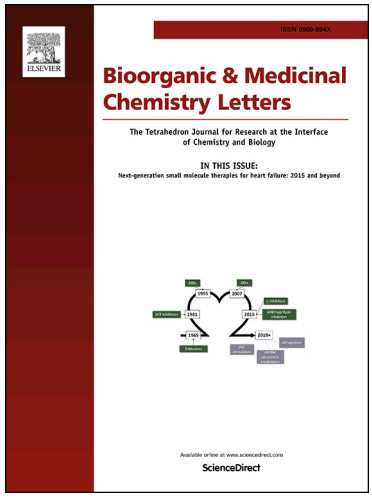

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PII: S0960-894X(18)30997-1
DOI: https://doi.org/10.1016/j.bmcl.2018.12.050
Reference: BMCL 26219

To appear in: *Bioorganic & Medicinal Chemistry Letters*

Received Date: 4 October 2018
Revised Date: 29 November 2018
Accepted Date: 3 December 2018

Please cite this article as: Sengmany, S., Sitter, M., Léonel, E., Le Gall, E., Loirand, G., Martens, T., Dubreuil, D., Dilasser, F., Rousselle, M., Sauzeau, V., Lebreton, J., Pipelier, M., Le Guével, R., Synthesis and biological evaluation of 3-amino-, 3-alkoxy- and 3-aryloxy-6-(hetero)arylpyridazines as potent antitumor agents, *Bioorganic & Medicinal Chemistry Letters* (2018), doi: https://doi.org/10.1016/j.bmcl.2018.12.050

Synthesis and biological evaluation of 3-amino-, 3-alkoxy- and 3-aryloxy-6-(hetero)arylpyridazines as potent antitumor agents

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Keywords: Electrosynthesis; Nickel catalysis; Arylpyridazines; Biological evaluation; Cytotoxic activity

ABSTRACT: Various 3-amino-, 3-aryloxy- and alkoxy-6-arylpyridazines have been synthesized by an electrochemical reductive cross-coupling between 3-amino-, 3-aryloxy- or 3-alkoxy-6-chloropyridazines and aryl or heteroaryl halides. *In vitro* antiproliferative activity of these products was evaluated against a representative panel of cancer cell lines (HuH7, CaCo-2, MDA-MB-231, HCT116, PC3, NCI-H727, HaCaT) and oncogenicity prevention of the more efficient derivatives was highlighted on human breast cancer cell line MDA-MB 468-Luc prior establishing their interaction with p44/42 and Akt-dependent signaling pathways.

The pyridazine ring system can currently be considered as a privileged scaffold in medicinal chemistry due to its widespread distribution in active compounds displaying significant and varied biological properties.[1] Pyridazines also represent interesting phenyl surrogates due to possible secondary electrostatic interactions with proteins' receptors and exhibit modified ADME profile. Among these, very important subclasses are 3-amino-6-arylpyridazines, and to a lesser extent, 3-aryloxy- and 3-alkoxy-6-arylpyridazines, which prove to display diverse biological activities including analgesic,[2] anti-inflammatory,[3-6] anti-hypertensive,[7-10] and anti-depressant activities.[11-13] They have also found applications in the treatment for obesity,[14-17] neurodegenerative diseases,[18-24] and were additionally used as GABA-A receptor antagonists.[25-27] Interestingly, whereas fused pyridazinic rings are also privileged in numerous potential anticancer drugs acting either as DNA chelators, angiogenesis inhibitors, or protein kinase inhibitors,[28] only few examples of simple 3,6-disubstituted pyridazine derivatives exhibiting cytotoxic properties have been documented yet (Fig. 1). Therefore, in 2002, general screening using the rat aortic ring assay (*in vitro* functional model of angiogenesis) led to the discovery of a thiadiazole pyridazinic derivative (R 90324) with nanomolar *in vitro* anti-angiogenic potency.[29] A few years ago, George described the pyrazoline derivatives bearing phenyl pyridazine core as new apoptosis inducers.[30] IC_{50} up to 1.67 µM are reported against HepG-2, MCF-7 and CaCo-2 cancer cell lines. Although limited, these activities have to be balanced with a remarkable selectivity, normal cell lines remaining safe until 300 µM. In 2013, Ahmed described the synthesis and biological evaluation of a series of novel 3,6-disubstituted pyridazines based on vatalanib's (PTK787) structure.[31] One of the tested compounds proved five times more active on HT-29 cancer cell lines than the reference drug. More recently, Ahmed reported the synthesis of new pyridazinic analogues of imatinib targeting VEGFR kinase.[32] Biological assays revealed that one compound shows improved cytotoxic activity compared to the reference drug imatinib on colon cancer cell line (HCT-116). It was also demonstrated that the antitumor activity of some compounds is greater *in vivo* than that of imatinib against Ehrlich's ascites carcinoma (EAC) solid tumors in mice.

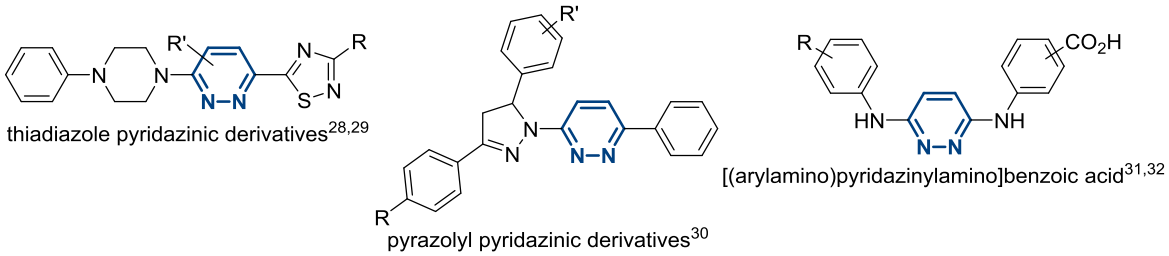
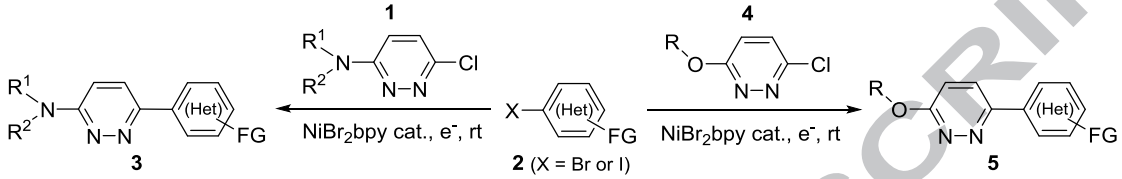


Fig. 1. Aminopyridazine derivatives described as potential antitumor agents.

In the last years, our group focused on electroreductive cross-couplings of diazinic rings with aryl halides using a sacrificial anode process.[33,34] As such experimental conditions are well mastered in the laboratory, we desired to highlight the efficiency of the electrochemical process for the synthesis of a wide range of compounds embedding a 3,6-subtituted pyridazinic ring. In addition, as this subunit is a common feature of some scaffolds exhibiting promising anti-cancer activities, we were prompted to undertake the biological assessment of this series of compounds against various cancer cell lines. Therefore, we report herein the easy access to various 3-amino-, 3-aryloxy- and 3-alkoxy-6-arylpyridazines and their biological evaluation as potent antitumor agents.

The synthesis of 3-amino-, 3-aryloxy- and 3-alkoxy-6-(hetero)arylpyridazines **3** and **5** was undertaken from the corresponding chloropyridazines **1** and **4**, in the presence of aryl or heteroaryl halide partners **2** following the general and effective electrochemical reductive cross-coupling process developed in our laboratory (Scheme 1).[33]

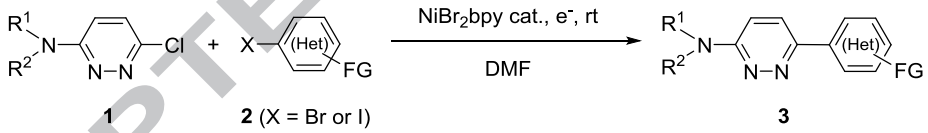


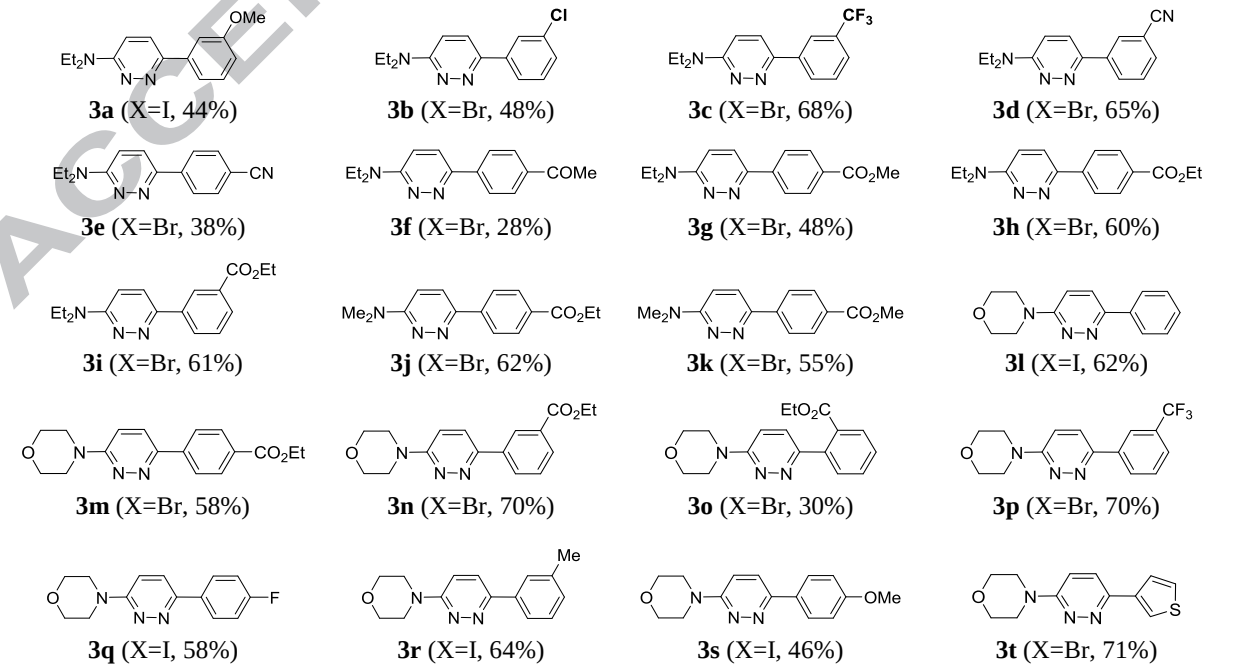
Scheme 1. Overview of the electrochemical couplings.

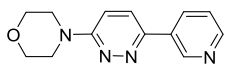
Electrolyses were performed in an undivided cell, under galvanostatic mode, using an iron/nickel (64/36) anode and a nickel foam cathode, until consumption of the starting materials. Thus, after a short optimization of the general reaction parameters, it was found that under a constant current intensity of 0.2 A, the cross-coupling between chloropyridazines **1** or **4** (1 equiv.) proceeded better with (hetero)aryl halides **2** (2 equiv.) in *N*,*N*-dimethylformamide (DMF) at room temperature, in the presence of $NiBr_2bpy$ (10 mol%) as a pre-catalyst and tetrabutylammonium bromide as supporting electrolyte.

The synthesis of 3-amino-6-(hetero)arylpyridazines **3** from 3-amino-6-chloropyridazines **1** was examined first.[35] Results are reported in Table 1.

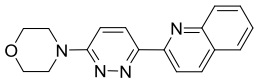
Table 1
Synthesis of 3-amino-6-(hetero)arylpyridazines **3**[a,b].



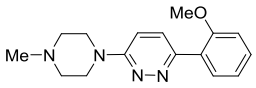




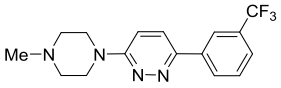
3u (X=Br, 31%)



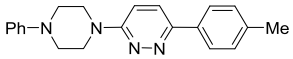
3v (X=Br, 26%)



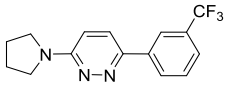
3w (X=I, 67%)



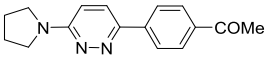
3x (X=Br, 66%)



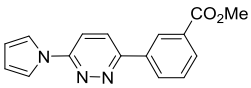
3y (X=I, 72%)



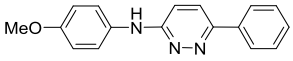
3z (X=Br, 67%)



3aa (X=Br, 58%)



3ab (X=Br, 36%)

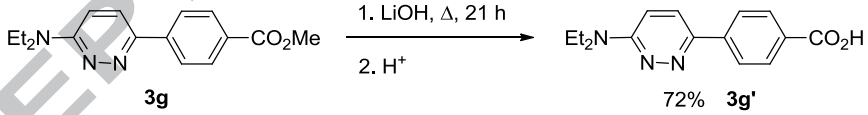


3ac (X=I, 52%)

[a] Typical reaction conditions: iron/nickel (64/36) rod anode, nickel foam cathode, DMF (50 mL), tetrabutylammonium bromide (1.2 mmol), 1,2-dibromoethane (2.5 mmol), $NiBr_2$bpy complex (10 mol%), chloropyridazine **1** (4 mmol), aryl or heteroaryl halide **2** (8 mmol), I = 0.2 A. [b] Isolated yields.

Coupling products were prepared using either aryl bromides or iodides[36] in limited to good yields (26 to 72%), depending on the substitution pattern of the organic halide. It was also observed that substituted phenyl bromides are more efficient as partners for the coupling reaction when the substituent is electron-withdrawing whereas iodides react better in the presence of electron-donating groups (compounds **3a**, **3l**, **3q-s**, **3w**, **3y** and **3ac**). The position of the substituent has a non-negligible influence on the coupling efficiency as variable yields are obtained with *ortho*, *meta*, and *para*-substituted aryl halides (compounds **3m**, **3n** and **3o**). Both electronic effects and steric hindrance can explain low yields with aryl bromides or heteroaromatic partners substituted at the *ortho*-position of an electron-withdrawing group like CO_2Et (compound **3o**).[37] The nature of the amino group at the *para*-position shows notable influence on the coupling yield, as a pyrrolyl group (compound **3ab**) leads to worst results than aliphatic amino groups like diethylamino or morpholino groups (compounds **3i** and **3n**). However, amino groups like piperazines are well tolerated, with some yields close to 70% (compounds **3w**, **3x** and **3y**).

Encouraging biological results obtained with *para*-amino esters **3g** and **3h** (see biological part below) prompted us to prepare the amino acid mimic **3g'** derived from **3g** (Scheme 2). Indeed, the hydrolysis of the ester group prior to biological assessment would likely anticipate potential hydrolysis that often occurs in physiological medium. It was thus considered relevant to compare the *in vitro* cytotoxic activity of a genuine amino ester to that of the corresponding hydrolysed species.

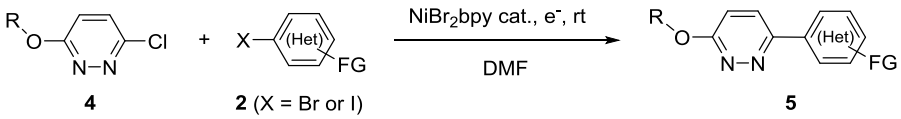


3g **3g'**

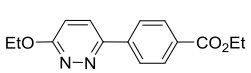
Scheme 2. Synthesis of amino acid **3g'**.

Going further through this work, the synthesis of the oxygenated counterparts 3-alkoxy- and 3-aryloxy-6-(hetero)arylpyridazines **5** from 3-alkoxy- and 3-aryloxy-6-chloropyridazines **4** was undertaken. Results are reported in Table 2.

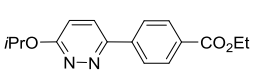
Table 2
Synthesis of 3-aryloxy- and 3-alkoxy-6-(hetero)arylpyridazines **5**[a,b].



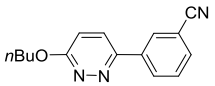
4 **2** (X = Br or I) **5**



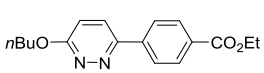
5a (X=Br, 55%)



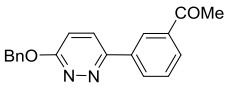
5b (X=Br, 58%)



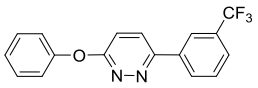
5c (X=Br, 63%)



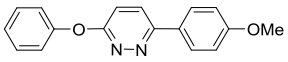
5d (X=Br, 86%)



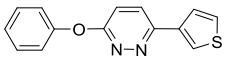
5e (X=Br, 51%)



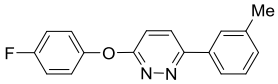
5f (X=Br, 51%)



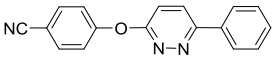
5g (X=Br, 36%)



5h (X=Br, 79%)



5i (X=I, 57%)



5j (X=I, 52%)

[a] Typical reaction conditions: iron/nickel (64/36) rod anode, nickel foam cathode, DMF (50 mL), tetrabutylammonium bromide (1.2 mmol), 1,2-dibromoethane (2.5 mmol), $NiBr_2$bpy complex (10 mol%), chloropyridazine **4** (4 mmol), aryl or heteroaryl halide **2** (8 mmol), I = 0.2 A. [b] Isolated yields.

Electrosyntheses worked fairly well and afforded cross-couplings products in 36 to 86% yields. In a general manner, results of cross-couplings in oxygenated series are comparable to those obtained from aminated pyridazinic substrates, whatever the nature of the substituents on the phenyl ring. It can be noted that the coupling is also efficient with heteroaryl halide such as 3-bromothiophene (compound **5h**). Due to the possible presence of an aryloxy substituent linked to the pyridazinic moiety, it was envisaged to additionally assess to effect of substituents connected to the *O*-Ar moiety through the preparation of compounds **5i** and **5j** embedding *para*-fluoro and -cyano substituent, respectively.

The antiproliferative activity of these 3-substituted-6-(hetero)aryl-pyridazine derivatives **3** and **5** were then evaluated *in vitro* in seven human cancer cell lines HuH7 (hepatocellular carcinoma), Caco-2 (colorectal adenocarcinoma), MDA-MB-231 (breast carcinoma), HCT116 (colorectal carcinoma), PC3 (prostate carcinoma), NCI-H727 (lung carcinoid) and HaCaT (skin carcinoma) with roscovitine (cyclin-dependent kinase inhibitor), doxorubicin (DNA topoisomerase inhibitors), Taxol (mitotic inhibitor) and Pi103 (dual PI3Kα/mTOR inhibitor) as the reference compounds (see 2.1. and 2.2. of supplementary data). IC_{50}, reported in Table 3, was defined as the drug concentration causing 50% inhibition of cell proliferation (values between parentheses indicate the maximum cell death percentage induced by the pyridazine derivatives).

Table 3
IC_{50} for compounds **3** and **5** on cancer cell lines.

Compound	**Cell lines**							
	HuH7	**CaCo-2**	**MDA-MB-231**	**HCT116**	**PC3**	**NCI-H727**	**HaCaT**	**Fibroblast**
DMSO	>25	>25	>25	>25	>25	>25	>25	>25
Rosco (µM)	20 (90%)	10 (90%)	12 (80%)	9 (100%)	12 (8%)	20 (40%)	12 (80%)	12 (40%)
Doxo (µM)	0.03 (40%)	0.09 (60%)	0.012 (50%)	0.05 (80%)	0.08 (80%)	0.03 (60%)	0.03 (70%)	0.005 (40%)
Taxol (µM)	0.012 (50%)	0.02 (60%)	0.06 (60%)	<0.001 (90%)	<0.001 (60%)	n.d.	0.002 (70%)	<0.001 (30%)
Pi103 (µM)	6 (40%)	0.8 (60%)	8 (40%)	3 (80%)	1.2 (60%)	>25	2 (70%)	>25
3a-3e (µM)	>25	>25	>25	>25	>25	>25	>25	>25
3f (µM)	25 (80%)	15 (80%)	5 (60%)	6 (100%)	>25	25 (80%)	20 (80%)	>25
3g (µM)	**0.09 (60%)**	**0.25 (80%)**	**0.14 (60%)**	**0.06 (100%)**	**0.09 (72%)**	**0.09 (60%)**	**0.08 (85%)**	**0.10 (40%)**
3g' (µM)	>25	>25	>25	>25	>25	>25	>25	>25
3h (µM)	9 (60%)	4 (80%)	1.5 (70%)	0.5 (90%)	1 (80%)	1.5 (60%)	0.5 (80%)	3 (40%)
3i (µM)	>25	>25	>25	>25	>25	30 (40%)	>25	>25
3j (µM)	>25	>25	>25	>25	>25	>25	-	>25
3k (µM)	>25	>25	>25	>25	>25	>25	-	>25
3l-3v (µM)	>25	>25	>25	>25	>25	>25	>25	>25
3w-3ac (µM)	30 (75%)	50 (80%)	>25	>25	>25	>25	>25	>25
5a-5j (µM)	>25	>25	>25	>25	>25	>25	>25	>25

Among all evaluated 1-amino-6-(hetero)arylpyridazines in series **3**, three derivatives **3f**, **3g** and **3h** showed a noticeable proliferation inhibition on selected cancer cells lines with a lower impact on healthy cells (i.e. fibroblast) which shown almost 60% of survival cells, albeit with a similar IC_{50}. While 3-*N*,*N*-diethylamino-6-*para*-acetophenone-pyridazine (**3f**) showed a modest antiproliferative activity with IC_{50} ranging between 5 and 25 µM, the two alkyl 4-(6-(diethylamino)pyridazin-3-yl)benzoate derivatives **3g** and **3h** exhibited significant IC_{50} with mean ranges of 0.1 µM for **3g** and 3 µM for **3h**. Antiproliferative activity of **3g**, in which a methyl ester group substitutes the aryl moiety in *para* position, appeared to be the most efficient with ten-fold increase on CaCo-2, MDA-MB231, HCT116, and HaCaT cell lines, and hundred-fold on HuH7 and PC3, compared to the ethyl ester homologue **3h**.

Increasing the electron-withdrawing effect by the introduction of a cyano group (compound **3e**) resulted in a total loss of antiproliferative activity (IC_{50} > 25 µM). The presence of either electron-donating or electron-withdrawing groups at *meta*-position of the phenyl did not restore the activity (compounds **3a-3d** and **3i**, IC_{50} > 25 µM). A similar result was observed with a cyclic amino group at 3-position of pyridazines instead of the 3-*N*,*N*-dialkylamino residues, as shown with the morpholino derivatives **3l-3v**. None of other modifications performed at 3- and 6-positions of pyridazines **3w-3ac**, using various combinations of cyclic amino residues (substituted piperazine, pyrrolidine) and aryl or hetetoaryl moieties, respectively, were able to restore proliferation inhibition activity.

These data seem to highlight a surprising selective behavior of the alkyl 4-(6-(dialkylamino)pyridazin-3-yl)benzoate scaffold that would be ascribed, with caution, to a possible electronic delocalization, occurring from the electron-donating dialkylamino residue to the electron-withdrawing aromatic ring, through the pyridazine moiety. This subtle contribution of a polarized form in the activity of such pyridazine backbone is widened by the antiproliferative inefficiency of all other analogues including compounds in series **5** designed to establish this hypothesis further. In this latter series **5a-5j**, the *N*,*N*-dialkylamino group at 3-position of the pyridazine derivatives has been replaced by less electron-donating ether linkages or electron-withdrawing groups, giving IC_{50} values over 25 µM on all tested cancer cell lines.

More intriguing, ethyl 4-(6-(dimethylamino)pyridazin-3-yl)benzoate **3j** and its methyl ester homologue **3k**, also appeared inactive under 25 µM in all cancer cell lines. These two analogues **3j** and **3k** only differ from pyridazines **3h** and **3g** by *N*,*N*-dialkylamino residue, which could counterbalance the electronic hypothesis made previously as the only or main cause of their biological antiproliferative activity. As predictable, the amino acid **3g'** did not bring more information due to a probable inability to cross cell membrane at physiological pH.

Thus, these previous results demonstrated that methyl 4-(6-(diethylamino)pyridazin-3-yl)benzoate **3g** is able to decrease cancer cells proliferation, albeit with low selectivity between lines. However, the cancer cells, regardless of their origin, appear more sensitive to this new compound than healthy cells (i.e. fibroblasts). The results strongly suggest a cytostatic activity on cell cycle rather than a cytotoxic effect from **3g** that increase the interest of such novel series of small molecules. Currently, the molecular mechanism allowing this activity is not identified inasmuch as a slight change of substituent undergo a drastic decrease of their cancer cells antiproliferative effect.

The capacity of the selected molecules **3f**, **3j**, **3k**, **3h** and **3g** to disrupt the phosphorylation level of p44/42 and Akt, two enzymes involve in the cell cycle process, was then examined. However, instead of focusing the investigation on widely studied HCT-116 tumor cells, the ability of these particular short aminoarylpyridazines to inhibit these key enzymes on human triple-negative breast cancer cell line, one of the most aggressive cancers highly associated with metastasis formation, was assessed. Only, few therapeutic solutions are currently available to prevent oncogenicity of these particular cells and the discovery of simple molecules limiting their proliferation is off great interest to design new therapeutic drugs. The breast cancer MDA-MB-468 cell line is characterized by the ability to hyperactivate p44/42 and Akt-dependent signaling pathways promoting cell proliferation and linked to tumor progression in triple-negative breast cancer.[38,39] The capacity of the selected molecules **3f**, **3j**, **3k**, **3h** and **3g** to disrupt the phosphorylation level of p44/42 and Akt at a concentration of 10^{-5} M was examined first (Fig. 1A). By western blot analysis (see 2.3. of supplementary data), it was observed that EGF-stimulated MDA-MB-468 cells show potent decreased of P-p44/42 abundance (almost 70% of inhibition) after both **3h** and **3g** treatments, whereas **3f** exhibits less potency (50%) (Fig. 1B). However, **3g** seems to maintain a potent effect on P-Akt inhibition (over 70%) compared to **3h** and, as observed in proliferation assays, no significant inhibitory effect was recorded with compounds **3j** and **3k** upon both enzyme abundances.

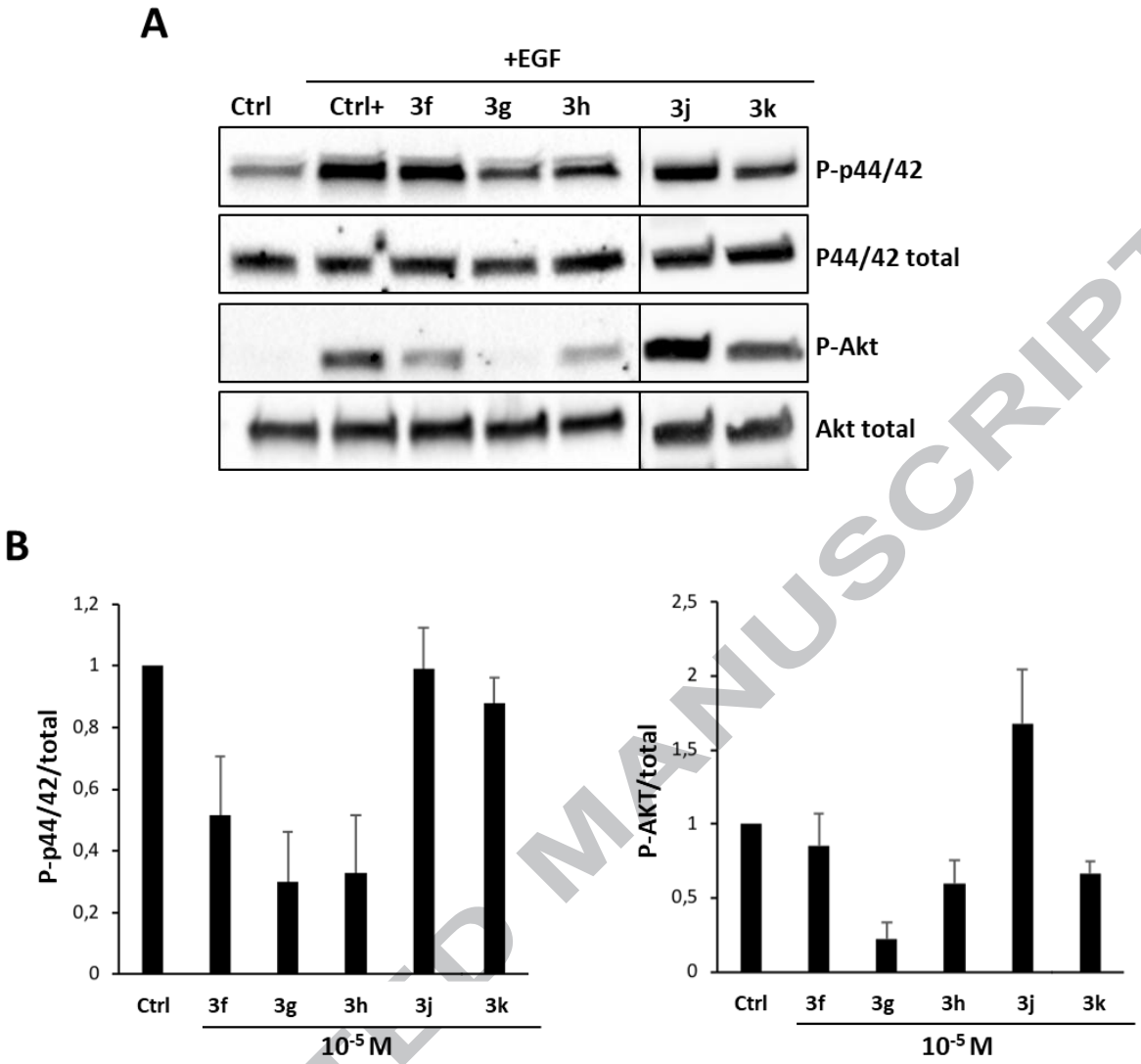


Fig. 1. (A) Immunoblot analysis of P44/42 and Akt phosphorylation in MDA-MB-468-Luc cells treated with indicated molecules. (B) Corresponding quantification of P44/42 and Akt phosphorylation.

Inhibition of clonogenic outgrowth of human breast cancer cells was then evaluated, showing a very good correlation with their antiproliferative activity. MDA-MB 468-Luc cell line was cultured for 15 days in the presence or not of molecules **3f**, **3g**, **3h**, **3j** and **3k**. Then, cell cultures were stained with Giemsa to visualize and count the foci of transformed cells (see 2.4. of supplementary data). As expected the two selected compounds **3g** and **3h** exhibited markedly, whereas **3f, 3j** and **3k** displayed very poor or no significant effects. Increasing concentrations allowed to establish IC_{50} (Fig. 2) and to confirm the higher activity of alkyl 4-(6-(diethylamino)pyridazin-3-yl)benzoate analogues **3g** and **3h** (IC_{50} = 0.015 and 0.12 µM, respectively) compared to their dimethylamino counterparts **3j** and **3k,** respectively (IC_{50} = 26 for **3j** and 3.5 µM for **3k**). In this experiment, pyridazine **3f** lacking ester function also exhibited an unexpected activity with an IC_{50} of 0.65 µM.

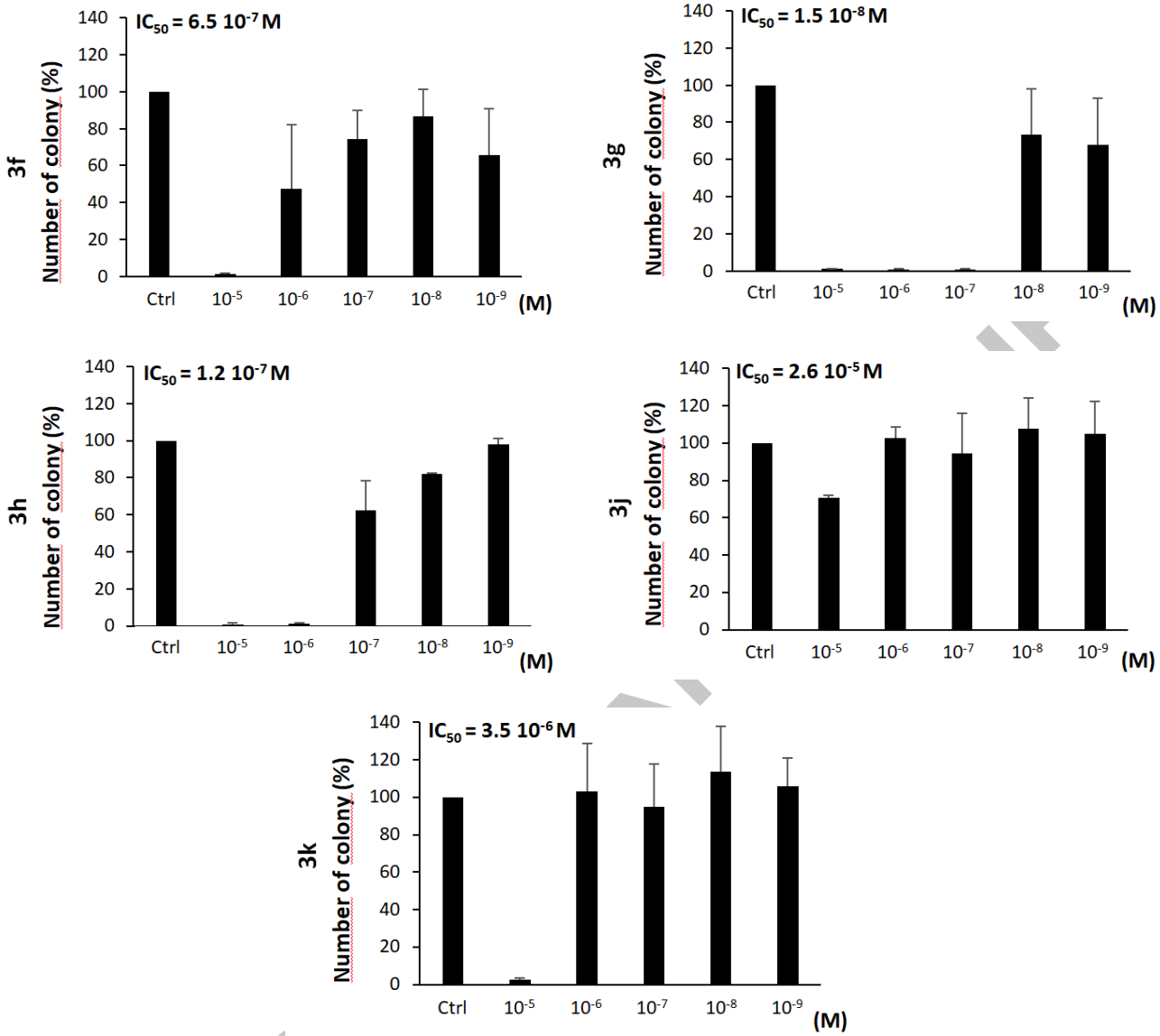


Fig. 2. Focus formation assay. The human breast cancer cell line MDA-MB-468-Luc was treated with indicated molecules (**3f, 3g, 3h, 3j** and **3k**). Foci were photographed and counted after 2 weeks**.**

The toxicity of the most active molecules **3g** and **3h** was further evaluated *in vitro* on human hepatocytes, HepaRG (see 4. of supplementary data) and *in vivo* on zebrafish assays (see 5. of supplementary data). Therefore, HepaRG cells were incubated with 2.5 µM solutions of compounds **3g** and **3h** for 48 h (n = 3) without shown toxicity. Furthermore, zebrafish embryos, incubated for 72 h with both molecules at two different concentrations (0.8 and 2.5 µM), expressed toxicity only at a concentration of 2.5 µM, not observed at 0.8 µM. At this concentration, the embryos remained alive, mobile, and their development was similar to the DMSO reference without visible deformation. However, a reduced size of eyes observed in the presence of **3g** at upper concentration of 2.5 µM seemed to indicate a possible effect on this organ, undetectable at 0.8 µM.

In conclusion, several 3-amino-, 3-aryloxy- and alkoxy- 6-(hetero)arylpyridazines (series **3** and **5**) have been synthesized by an efficient electrochemical reductive cross-coupling process. All the prepared compounds were tested for their *in vitro* antiproliferative activity against seven cancer cell lines and showed a very sensitive structural effect depending of the nature of amino substituent and aryl ester group at positions 3 and 6 of the pyridazine anchor, respectively. Only *N,N*-dialkylamino functions, with a preference for *N,N*-diethylamino *vs N,N*-dimethylamino, and their exclusive association with a *para*-benzoate group at position 6, provided noticeable activity. Consequently, the highest activity was found for compound **3g**, exhibiting IC_{50} values at 10^{-1} µM range on several cancer cell lines (MDA-MB231, HCT116, PC3, HaCaT, HuH7 and CaCo-2), albeit with a lack of selectivity. Regarding its ability to inhibit key hyperactivated p44/42 and Akt-dependent signaling pathways, pyridazine **3g** proved to be very interesting with a potential tumor-selective mechanism of action. This compound also showed a potent ability to inhibit

clonogenicity of human breast cancer cell line, one of the most aggressive cancer, without inducing toxicity under 2.5 µM neither on human hepatocytes *in vitro* and *in vivo* on zebrafish thus paving the route for the design of novel short pyridazine derivatives with therapeutic interest.

Acknowledgements

The authors are grateful to the CNRS and the Université Paris-Est Créteil for financial support on this work.

A. Supplementary data

Supplementary data associated to this article can be found, in the online version, at https://dx.doi.org/xxxx.

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Graphical Abstract