

Squalene-Adenosine Nanoparticles: Ligands of Adenosine Receptors or Adenosine Prodrug?[§]

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ABSTRACT

Adenosine receptors (ARs) represent key drug targets in many human pathologies, including cardiovascular, neurologic, and inflammatory diseases. To overcome the very rapid metabolism of adenosine, metabolically stable AR agonists and antagonists were developed. However, few of these molecules have reached the market due to efficacy and safety issues. Conjugation of adenosine to squalene to form squalene-adenosine (SQAd) nanoparticles (NPs) dramatically improved the pharmacological efficacy of adenosine, especially for neuroprotection in stroke and spinal cord injury. However, the mechanism by which SQAd NPs displayed therapeutic activity remained totally unknown. In the present study, two hypotheses were discussed: 1) SQAd bioconjugates, which constitute the NP building blocks, act directly as AR ligands; or 2) adenosine, once released from intracellularly processed SQAd NPs, interacts with these

receptors. The first hypothesis was rejected, using radioligand displacement assays, as no binding to human ARs was detected, up to 100 μ M SQAd, in the presence of plasma. Hence, the second hypothesis was examined. SQAd NPs uptake by HepG2 cells, which was followed using radioactive and fluorescence tagging, was found to be independent of equilibrative nucleoside transporters but rather mediated by low-density lipoprotein receptors. Interestingly, it was observed that after cell internalization, SQAd NPs operated as an intracellular reservoir of adenosine, followed by a sustained release of the nucleoside in the extracellular medium. This resulted in a final paracrine-like activation of the AR pathway, evidenced by fluctuations of the second messenger cAMP. This deeper understanding of the SQAd NPs mechanism of action provides a strong rationale for extending the pharmaceutical use of this nanoformulation.

Introduction

Adenosine is an endogenous purine acting on four different G protein-coupled receptors (GPCRs) identified as A₁, A_{2A}, A_{2B}, and A₃. The wide distribution of these adenosine receptors (ARs) is associated with a great diversity of pathological effects, including regulation of cardiovascular, nervous, and immune systems (Fredholm et al., 2011). Although considerable efforts have been devoted to identifying new compounds interacting with ARs (Borea et al., 2018), very few molecules have reached the clinic due to activity and/or toxicity issues. On the other hand, because of a very short half-life in the blood circulation ($t_{1/2}$ = ~10 seconds), adenosine

must be administered at high doses, which results in severe side effects, thus limiting the clinical use of this molecule. Therefore, innovative pharmaceutical strategies are still needed to fully exploit adenosine's tremendous therapeutic potential.

Currently, the so-called "squalenylation" approach may represent a promising technological platform for delivering hydrophilic compounds with stability issues or off-target toxicity (Couvreur et al., 2006). This methodology consists of the conjugation of a fragile and quickly metabolized drug molecule to the squalene, a natural and biocompatible lipid. Interestingly, the resulting drug-squalene bioconjugates have the unique ability to self-assemble in water as nanoparticles (NPs). It was found that these squalene-based NPs favorably modified the in vivo pharmacokinetics and biodistribution of the conjugated drug molecules (Reddy et al., 2007). In particular, the delivery of adenosine

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ABBREVIATIONS: AR, adenosine receptor; CHO, Chinese hamster ovary; CholEsteryl BODIPY FL C₁₂, CholEsteryl 4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-dodecanoate; DMSO, dimethylsulfoxide; [³H]DPCPX, 1,3-[³H]-dipropyl-8-cyclopentylxanthine; EHNA, erythro-9-(2-hydroxy-3-nonyl)-adenine; ENT, equilibrative nucleoside transporter; FBS, fetal bovine serum; GPCR, G protein-coupled receptor; HPLC, high-performance liquid chromatography; LDL, low-density lipoprotein; LDLR, low-density lipoprotein receptor; LPDS, lipoprotein-deficient serum; NP, nanoparticle; PBS, Dulbecco's phosphate-buffered saline; Pdl, polydispersity index; [³H]PSB11, [³H]8-ethyl-4-methyl-2-phenyl-(8R)-4,5,7,8-tetrahydro-1H-imidazo[2,1-i]-purin-5-one; [³H]PSB603, [³H]8-(4-(4-(4-chlorophenyl)piperazine-1-sulfonyl)phenyl)-1-propylxanthine; Ro 20-1724, 4-(3-butoxy-4-methoxybenzyl)imidazolidone; SQAd, squalene-adenosine; ZM241385, 4-(-2-[7-amino-2-(2-furyl)]{1,2,4}triazolo{2,3-a}{1,3,5}triazin-5-yl-amino]ethyl)phenol; [³H]ZM241385, [2-³H]-4-(2-[7-amino-2-(2-furyl)]{1,2,4}triazolo{2,3-a}{1,3,5}triazin-5-yl amino]ethyl)phenol.

via squalene-adenosine (SQAd) NPs showed an impressive neuroprotective efficacy in brain ischemia in mice and in spinal cord injury in rats (Gaudin et al., 2014). This effect was explained by a prolonged circulation of SQAd NPs in the bloodstream compared with free adenosine (Gaudin et al., 2015) and appeared to originate from the interaction of SQAd NPs with the brain endothelial cells. However, the nature of the interaction between SQAd NPs and cells still remains totally unknown.

Thus, the current study aimed at elucidating the mechanism of action of SQAd NPs *in vitro*. Two hypotheses were tested: 1) the SQAd NPs or SQAd bioconjugates would act as direct AR agonists (or antagonists) by binding them via their adenosine moiety; or 2) they would function as prodrugs by slowly releasing adenosine intracellularly and into the extracellular space.

The first hypothesis was checked by radioligand displacement assays on membrane fractions of cells overexpressing human ARs. The second hypothesis was tested on the HepG2 cell line, a well characterized *in vitro* human hepatocyte model. Liver cells were chosen for this study, as it has been previously shown that SQAd NPs accumulate in high quantities in the liver after their intravenous injection in mice (Gaudin et al., 2015). The cell capture of SQAd NPs was followed by radiolabeling and fluorescent labeling, while adenosine release was determined by high-performance liquid chromatography (HPLC). Finally, the activation of the adenosine receptors was also evaluated.

Materials and Methods

Materials. SQAd bioconjugate originated from Holochem (Val-de-Reuil, France). D-(+)-glucose was obtained from Sigma (St. Quentin Fallavier, France). Absolute ethanol and methanol came from VWR Chemicals (Strasbourg, France), while dimethylsulfoxide (DMSO) was from Carlo Erba Reagents (Val-de-Reuil, France). MilliQ water (resistivity 18.2 M Ω ·cm) was purified on a system from Millipore (Molsheim, France). CholEsteryl 4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-dodecanoate (CholEsteryl BODIPY FL C₁₂) and wheat germ agglutinin Alexa Fluor 555 conjugate were purchased from Thermo Fisher Scientific (Karlsruhe, Germany). [2,8-³H]-Adenosine (specific activity 20.3 Ci/mmol) was obtained from Moravek Biochemicals (Brea, CA), while SQ-[³H]-adenosine (SQ-[³H]-Ad) (specific activity 2.8 Ci/mmol) was synthesized as previously reported (Gaudin et al., 2014). Soluene-350 and Hionic Fluor were provided by PerkinElmer (Courtaboeuf, France). Radioligands 1,3-[³H]-dipropyl-8-cyclopentylxanthine ([³H]DPCPX, specific activity 120 Ci/mmol) and [2-³H]-4-(2-[7-amino-2-(2-furyl)](1,2,4)triazolo[2,3-a]{1,3,5}triazin-5-yl amino)ethyl)phenol ([³H]ZM241385, specific activity 50 Ci/mmol) were purchased from ARC Inc. (St. Louis, MO). [³H]8-(4-(4-(4-Chlorophenyl)piperazine-1-sulfonyl)phenyl)-1-propylxanthine ([³H]PSB603, specific activity 79 Ci/mmol) and [³H]8-ethyl-4-methyl-2-phenyl-(8R)-4,5,7,8-tetrahydro-1H-imidazo[2,1-i]-purin-5-one ([³H]PSB11, specific activity 56 Ci/mmol) were kindly provided by C. E. Müller (University of Bonn, Germany). 5'-N-ethylcarboxamidoadenosine was purchased from Sigma-Aldrich (Steinheim, Germany). N⁶-cyclopentyladenosine was purchased from Abcam (Cambridge, UK). Unlabeled 4-(2-[7-amino-2-(2-furyl)](1,2,4)triazolo[2,3-a]{1,3,5}triazin-5-yl-amino)ethyl)phenol (ZM241385) was a gift from Dr. S. M. Poucher (Astra Zeneca, Macclesfield, UK). Adenosine deaminase was purchased from Boehringer Mannheim (Mannheim, Germany). Bicinchoninic acid and bicinchoninic acid protein assay reagent were obtained from Pierce Chemical Company (Rockford, IL). Chinese hamster ovary (CHO) cells stably expressing the human A₁, A_{2B}, or A₃ ARs were provided by

S. J. Hill (University of Nottingham, UK), Dr. S. Rees (AstraZeneca), and Dr. K.-N. Klotz (University of Würzburg, Germany), respectively. HEK293 cells stably expressing the human A_{2A} AR were kindly provided by Dr. J. Wang (Biogen/IDEC, Cambridge, MA). The hCMEC/D3 cell line was a gift from Dr. Couraud (Hôpital Cochin, Paris, France). The HepG2 cell line, Dulbecco's modified Eagle's medium high glucose, Dulbecco's phosphate-buffered saline (PBS), lipoprotein deficient serum (LPDS) from fetal calf, trypsin and penicillin-streptomycin, HEPES, hydrocortisone, basic fibroblast growth factor, ascorbic acid, adenosine, cathepsin B from human liver, theophylline, dipyridamole, erythro-9-(2-hydroxy-3-nonyl)-adenine (EHNA) hydrochloride, paraformaldehyde, isobutyl-1-methylxanthine, and 4-(3-butoxy-4-methoxybenzyl)imidazolidone (Ro 20-1724) were obtained from Sigma. Endothelial basal medium was purchased from Lonza (Basel, Switzerland). Gibco fetal bovine serum (FBS) was obtained from Life Technologies (Saint-Aubin, France).

All other chemicals were of analytical grade and were obtained from standard commercial sources.

SQAd NPs Preparation. SQAd NPs were prepared as previously described (Gaudin et al., 2014). In brief, 333 μ l of an ethanolic solution of 6 mg/ml SQAd was added dropwise into 1 ml of a solution of 5 wt. % dextrose under stirring. Ethanol was then removed by evaporation using a Rotavapor (Buchi, Rungis, France) to obtain a final concentration of 2 mg/ml SQAd NPs. Radiolabeled NPs were obtained following the same procedure, with the addition of SQ-[³H]-Ad to the ethanolic SQAd solution (final radioactivity 10 Ci/mol). Similarly, fluorescently labeled SQAd NPs were prepared by adding 40 μ l of CholEsteryl BODIPY FL C₁₂ to the 333- μ l ethanolic SQAd solution (the final concentration of fluorescent probe was 1 wt. %). Excitation and emission spectra were recorded using an LS 50B Luminescence Spectrometer (PerkinElmer) (Supplemental Fig. 1).

The mean NP size and polydispersity index (PdI) were checked from 1-mg/ml suspensions by dynamic light scattering using a Zetasizer Nano ZS (173° scattering angle, 25°C; Malvern, UK).

Radioligand Displacement Assay. CHO or HEK293 cells stably expressing the human A₁, A_{2A}, A_{2B} or A₃ ARs were cultured, and membranes were prepared as previously described (Heitman et al., 2009; Yang et al., 2017). Buffer compositions, membrane concentrations, radioligands, and nonspecific binding compounds used for each receptor type, as well as their concentrations, are listed in Supplemental Table 1.

For radioligand displacement assays, membrane aliquots at given concentrations (Supplemental Table 1) were incubated in a total volume of 100 μ l of assay buffer (Supplemental Table 1) at 25°C for 60 minutes (A₁) to 120 minutes (other receptors). The DMSO stock solution of SQAd as single bioconjugate molecules or the SQAd NPs preparation were diluted in assay buffer or FBS (DMSO final concentration <1%). Samples with serum were incubated for 4 hours at 37°C on an Eppendorf Thermomixer before the experiment. Displacement experiments were performed in the presence of specific radioligands at given concentrations (Supplemental Table 1), while nonspecific binding was determined in the presence of nonspecific binding compounds at given concentrations (Supplemental Table 1). Incubations were terminated by dilution with ice-cold assay buffer, immediately followed by separation of bound from free radioligand by rapid filtration through 96-well GF/B filter plates using a PerkinElmer FilterMate Harvester (PerkinElmer, Groningen, Netherlands). Filters were subsequently washed three times with 2 ml of ice-cold washing buffer (Supplemental Table 1). The filter-bound radioactivity was determined by scintillation spectrometry using a P-E 1450 Microbeta Wallac Trilux scintillation counter (PerkinElmer). IC₅₀ values were obtained by fitting the curves with a one-site binding model using GraphPad Prism 7.00 software. K_d values were deduced using the Cheng-Prusoff equation (Cheng and Prusoff, 1973).

Cell Culture. HepG2 cells were cultured in Dulbecco's modified Eagle's medium high-glucose medium supplemented with 10%

FBS, penicillin (50 U/ml), and streptomycin (50 μ g/ml). If not stated otherwise, cells were plated on 24-well plates at a density of 200,000 cells per well and left to adhere overnight before any further procedure.

hCMC/D3 cells were cultured in endothelial basal medium supplemented with 5% FBS; penicillin (50 U/ml) and streptomycin (50 μ g/ml); 1.4 μ M hydrocortisone; 5 μ g/ml ascorbic acid; 1% chemically defined lipid concentrate; 10 mM HEPES; and 1 ng/ml basic fibroblast growth factor. Cells were used between passages 4 and 8. Cells were plated on 24-well plates at a density of 100,000 cells per well and left to adhere overnight before any further procedure.

All cells were maintained at 37°C, 5% CO₂, 100% humidity. Cells were passaged twice a week.

Cell Uptake of Radiolabeled SQAd NPs and Free Adenosine. HepG2 cells were treated with radiolabeled SQAd NPs or radiolabeled free adenosine at concentrations of 10 μ M and 0.1 μ Ci/ml. At different time points, cells were washed twice with PBS and detached with trypsin before being pelleted and resuspended in PBS. Sample radioactivity was measured using a β -scintillation counter (LS6500; Beckman Coulter) after overnight incubation with 1 ml of Soluene-350 at 50°C and addition of 10 ml of Hionic Fluor.

Cell Uptake of Fluorescently Labeled SQAd NPs. For confocal microscopy imaging, HepG2 cells were plated onto sterile glass coverslips placed in 24-well plates, at a density of 50,000 cells per well, and left to adhere for 48 hours. Cells were incubated with fluorescently labeled SQAd NPs (10 μ g/ml) for 2 hours at 37°C. After several washing steps with PBS, membranes were stained for 20 minutes with 10 μ g/ml of wheat germ agglutinin Alexa Fluor 555. Cells were then washed again and fixed with 4% paraformaldehyde for 15 minutes. After final washing, coverslips were mounted on slides using Vectashield mounting medium with 4',6-diamidino-2-phenylindole (Vector Labs) and sealed with nail polish. Images were acquired using a Leica TCS SP8 system, with a Leica HC Plan Apochromat 63 \times 1.4NO oil CS2 objective lens.

For studying the role of adenosine transporters, HepG2 cells were treated with fluorescently labeled SQAd NPs (10 μ g/ml) and dipyrindamole (20 μ M). For studying the role of low-density lipoprotein receptors (LDLRs) in SQAd NPs cell uptake, 150,000 cells were plated by well. Cells were washed with PBS, and medium was replaced with normal medium or medium supplemented with LPDS instead of FBS. Cells were then incubated for 24 hours to enhance LDLR expression, as proven by Western blot [protocol from Sobot et al. (2017a); Supplementary Fig. 2)], and subsequently treated with fluorescently labeled SQAd NPs (10 μ g/ml). In both cases, after 30 minutes or 2 hours, cells were finally washed with PBS, detached with trypsin, and resuspended in PBS. Cell fluorescence was evaluated using a BD Accuri C6 flow cytometer (BD Biosciences) with an excitation wavelength of 488 nm. Emission was recorded with the FL1 filter (533/30 nm).

SQAd Degradation in Acidic Conditions. A solution of 1 μ l of SQAd (2.5 mg/ml dissolved in DMSO) was incubated at 37°C under shaking in 100 μ l of 0.3 M sodium acetate buffer (pH 4.8) containing cathepsin B (10 U/ml). Degradation was stopped at different time points by the addition of 233 μ l of methanol. Five microliters of gemcitabine-squalene (0.2 mg/ml dissolved in DMSO) was added as internal standard. Samples were centrifuged (14,000 rpm, 10 minutes), and resulting supernatants were evaporated to dryness at 37°C under airflow. The dried samples were then dissolved in 100 μ l of mobile phase [methanol for SQAd detection; methanol/KH₂PO₄ (10 mM, pH 4), 20/80 v/v, for adenosine detection] prior to HPLC analysis.

Adenosine Extracellular Release. HepG2 or hCMC/D3 cells were treated with SQAd NPs (100 μ M), free adenosine dissolved in 5 wt. % dextrose (100 μ M), or 5 wt. % dextrose. After 5 minutes to 4 hours, extracellular medium was replaced by 1 ml of simplified medium (composition is given in Supplemental Table 2) supplemented with 50 μ M EHNA (to avoid adenosine deamination) and 2 μ M internal standard (theophylline). After 5 minutes to 4 hours, the extracellular medium was collected and extracted with 2.3 ml of

methanol. Samples were centrifuged at 17,000g for 10 minutes to remove insoluble materials, and supernatants were filtered and evaporated to dryness at 37°C under airflow. The dried samples were dissolved in 100 μ l of mobile phase [methanol/KH₂PO₄ (10 mM pH 4), 20/80 v/v] and filtered before HPLC analysis. The detection limit of the HPLC technique was 17 nM for the adenosine, while the quantification limit was 55 nM. This method exhibited linearity ($R^2 = 0.99$) over the assayed concentration range (100–1000 nM) and demonstrated good precision, with the relative S.D. being less than 5%.

HPLC Conditions. A Waters 717 plus autosampler was used, equipped with a Waters 1525 binary HPLC pump, a Waters 2487 dual λ absorbance detector, and a Waters Breeze software. The analysis was performed using a Halo C18 column (4.6 \times 250 mm, 5 μ m; Interchim, France). The injection volume was 40–50 μ l, and the elution was carried out at 0.8 ml/min isocratically for 10 minutes with the column temperature maintained at 30°C. Detection wavelengths were 273 nm for SQAd and 260 nm for adenosine.

Intracellular cAMP Levels. The day before the experiment, two plates were seeded with HepG2 cells: a 24-well plate with 200,000 cells per well, and a 96-well plate with 5000 cells per well.

On the day of the experiment, cells in the 24-well plate were treated with SQAd NPs, free adenosine, or dextrose as previously described. After 2 hours, extracellular medium was replaced with simplified medium containing 50 μ M EHNA and broad-range phosphatase inhibitors (500 μ M isobutyl-1-methylxanthine and 100 μ M Ro 20-1724). After 1 hour, 10 μ l of this extracellular medium was used to stimulate cells plated in the 96-well plate for 15 minutes. cAMP intracellular levels were then determined using the cAMP-Glo assay from Promega. In brief, cells were lysed with 10 μ l of cAMP-Glo Lysis Buffer for 15 minutes with shaking at room temperature. cAMP-Glo Detection Solution and Kinase-Glo Reagent were then added to all wells according to the manufacturer's protocol. Luminescence was detected using an EnSpire Alpha 2390 plate reader (PerkinElmer).

Results

SQAd NPs. Prepared by nanoprecipitation, SQAd NPs suspensions were monodisperse, displaying a hydrodynamic diameter of 71 ± 15 nm, a PdI of 0.08 ± 0.02 , and a drug loading of 37%.

SQAd Interaction with Human Adenosine Receptors. The direct interaction of SQAd with human ARs was checked by radioligand displacement assays. Specific radioligands were used for each adenosine receptor, and their displacement was measured in the presence of increasing concentrations of SQAd bioconjugate or NPs. Results are shown in Fig. 1.

Up to 1 μ M, SQAd showed no interaction with any of the ARs in the assay buffer, whether as a bioconjugate or assembled as NPs (Fig. 1, A and B, respectively). At and above 10 μ M, some displacement became detectable, with both SQAd bioconjugates and NPs showing a slight preference for the adenosine A₃ receptor (Fig. 1, A and B). Using the Cheng-Prusoff equation, the magnitude of SQAd (bioconjugate or NP) affinity for each of these receptors was determined. Results are given in Table 1. All ligand binding affinities (K_i) were in the micromolar to millimolar range.

To be as close as possible to the *in vitro* and *in vivo* conditions, SQAd bioconjugates and SQAd NPs were also preincubated for 4 hours at 37°C with FBS prior to the radioligand displacement assay. It should be noted that the interaction with the adenosine A_{2B} receptor could not be evaluated under these conditions due to unspecific interactions between the membranes and the FBS. Regarding the three other receptors, an interaction with SQAd bioconjugates or NPs could no longer

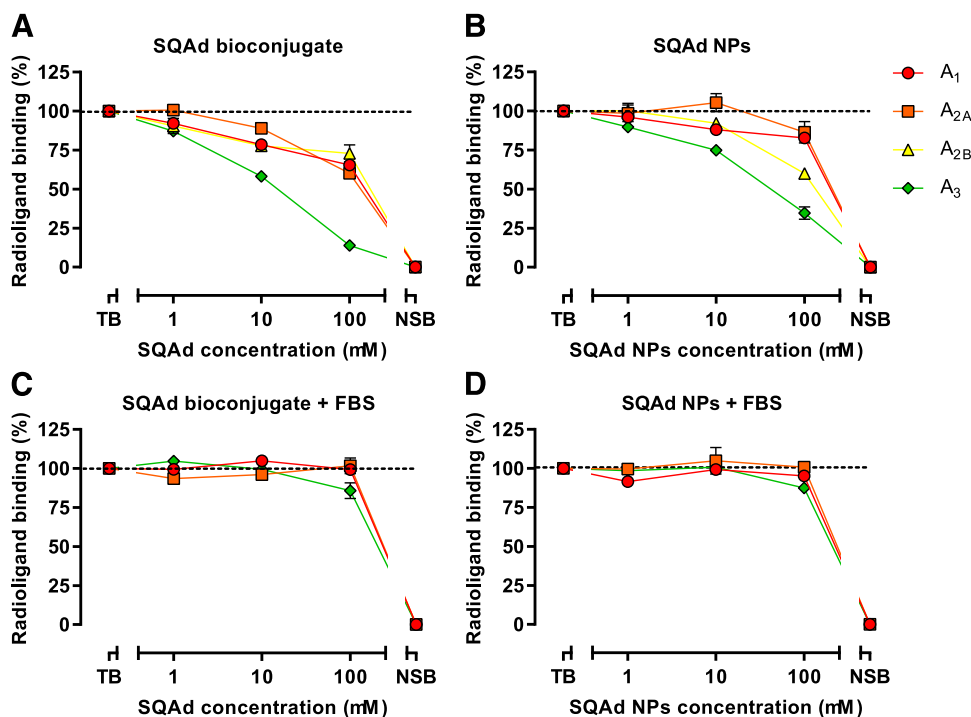


Fig. 1. Displacement of specific radioligand binding from the human adenosine receptors A_1 (red circle), A_{2A} (orange square), A_{2B} (yellow triangle), and A_3 (green diamond) by increasing concentrations of SQAd bioconjugate (A and C) or SQAd NPs (B and D) diluted in assay buffer (A and B) or preincubated for 4 hours at 37°C in FBS (C and D). Displacement experiments were performed at 25°C. NSB, nonspecific binding; TB, total binding. Data represent mean values \pm S.D. of three independent experiments.

be detected, even at the highest concentration (Fig. 1, C and D). Hence, it was concluded that the preincubation with serum prevented interaction of SQAd with adenosine receptors.

SQAd NPs Internalization. To follow the cell uptake of SQAd NPs, radiolabeled NPs were prepared by co-nanoprecipitation of SQAd and SQ- ^3H -Ad (for more information, see *Materials and Methods*), with no influence of the radiolabeled compound on the NP hydrodynamic diameter (56 ± 4 nm) or PdI (0.10 ± 0.04). HepG2 cells were then incubated at 37°C for different times with either radiolabeled SQAd NPs or ^3H -adenosine. The resulting intracellular radioactivity is presented in Fig. 2.

Both adenosine and SQAd NPs were internalized by HepG2 cells. However, the cell uptake of SQAd NPs occurred slower and to a lower extent than free adenosine. Indeed, after 30-minute incubation, the intracellular content in adenosine (and its tritiated metabolites) was 13 times higher than with SQAd (and its metabolites). After 24 hours, however, the adenosine-over-SQAd ratio was reduced to a value of only 3.

For further experiments, fluorescently labeled SQAd NPs (diameter of 60.8 ± 0.4 nm, PdI of 0.13 ± 0.01) were prepared by co-nanoprecipitation of SQAd with a fluorescent BODIPY-cholesteryl ester dye. The fluorescent nanoparticle maximum excitation and emission wavelengths were 484 and 520 nm,

TABLE 1

SQAd bioconjugate and SQAd NP pK_i magnitude for each adenosine receptor

The pK_i values were determined from the radioligand displacement curves using the Cheng-Prusoff equation.

	pK_i			
	A_1	A_{2A}	A_{2B}	A_3
SQAd bioconjugate	≤ 4	< 5	≤ 4	< 6
SQAd NP	< 4	< 4	< 5	< 5

respectively (Supplemental Fig. 1). NP uptake by HepG2 cells was visualized by confocal microscopy and measured by flow cytometry under various conditions (Fig. 3). The fluorescently labeled SQAd NPs were taken up by HepG2 cells (2-hour incubation) (Fig. 3A), whereas no fluorescence was visible when the cells were incubated with the fluorescent probe alone (Fig. 3B). Hence, the intracellular fluorescence was considered to correlate well with SQAd NPs uptake.

Then, considering that adenosine enters the cells via equilibrative nucleoside transporters (ENTs) (Baldwin et al., 2004), the cellular mechanism of uptake of SQAd NPs was also investigated using HepG2 cells treated with dipyridamole, an ENT inhibitor. After 30 minutes or 2 hours of incubation with fluorescently labeled NPs, no difference in intracellular fluorescence was observed between cells preincubated with or without dipyridamole (Fig. 3C), which clearly indicated that SQAd NPs did not enter the cells via ENTs.

The role of LDLRs was then evaluated by tuning their level of expression on HepG2 cells. Cells were preincubated for 24 hours with FBS-supplemented medium (containing a normal level of lipoproteins) and LPDS-supplemented medium (containing low levels of lipoproteins). Cell starvation using LPDS induced an overexpression of LDLRs, as evident from Western blot (Supplemental Fig. 2). It is noteworthy that in LPDS-supplemented medium, cells were found to internalize higher levels of fluorescently labeled SQAd NPs (Fig. 3D), thus demonstrating an influence of LDLR expression level on SQAd NPs uptake.

SQAd Degradation in Acidic Conditions. Assuming that, once in the cell, SQAd would undergo hydrolysis in acidic endo-lysosomal compartments, SQAd degradation was then evaluated over 24 hours in acidic conditions (pH 4.8) in the presence or absence of cathepsin B, a typical lysosomal enzyme able to cleave amide bonds. The percentage of intact prodrug under these conditions is shown in Fig. 4A.

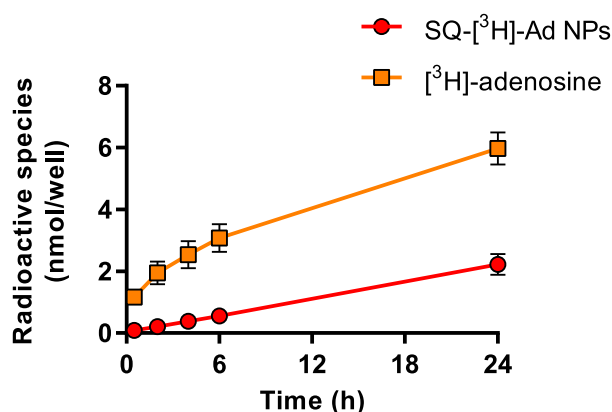


Fig. 2. Uptake of radiolabeled SQ-[³H]-adenosine (SQ-[³H]-Ad NPs (red circle) or [³H]-adenosine (orange square) by HepG2 cells (37°C over 24 hours). Data represent mean values ± S.D. of two independent experiments.

Although almost no bioconversion of the SQAd bioconjugate could be observed until 6 hours, up to 20% of SQAd was degraded after 24 hours of incubation in acidic conditions. The presence of cathepsin B did not influence the degradation profile over 24 hours. Interestingly, the degradation of SQAd led to the release of free adenosine (retention time 3.9 minutes), which was characterized by HPLC analysis (Fig. 4B).

Hence, SQAd intracellular bioconversion into adenosine should increase the adenosine intracellular content. And, as a result of the equilibrium between the intracellular and the extracellular adenosine levels, it was hypothesized that some adenosine could be further released in the extracellular compartment.

Adenosine Extracellular Release. Thus, the concentration of adenosine released in the cell culture medium was then determined by HPLC in the presence of EHNA, an inhibitor of adenosine deaminase. After incubation of HepG2 (or hCMEC/D3) cells with SQAd NPs, free adenosine, or 5% dextrose solution, the extracellular medium was replaced by 1 ml of a new simplified medium (composition is given in Supplemental Table 2) to remove adenosine (eventually resulting from the

release from SQAd NPs before their capture by the cells). After a further 5-minute to 4-hour incubation, the extracellular medium was finally collected to determine the quantity of adenosine released from the cells after exposure to SQAd NPs or adenosine (Fig. 5A).

After 5-minute incubation, cells treated with free adenosine released higher concentrations of adenosine (around 600 nM) than cells treated with dextrose or SQAd NPs (100–200 nM). This clearly highlighted the almost instantaneous ability of the cells to uptake free adenosine, whereas SQAd NP uptake and cleavage (which results in adenosine metabolite release) required more time (Fig. 5A). Interestingly, after a longer incubation time with free adenosine, a decrease in adenosine release was observed, which likely resulted from a consumption of the overall pool of adenosine by the cells. On the contrary, after a longer incubation time with SQAd NPs, higher levels of released adenosine were observed, which correlated well with an increased intracellular content of SQAd over time, hence producing a reservoir effect. Of note, very close results were obtained with hCMEC/D3 cells, a brain endothelial cell line derived from human temporal lobe microvessels (Supplemental Fig. 3).

To further study the intracellular reservoir effect of adenosine produced after incubation with SQAd NPs, cells were incubated for 2 hours with free adenosine, SQAd NPs, or dextrose, and the kinetics of adenosine release from the cells into the extracellular medium was determined. Results are shown in Fig. 5B. Interestingly, concentrations of released adenosine after incubation of the cells with dextrose or free adenosine did not increase over time, thus revealing that equilibrium was reached during the first minutes post incubation. On the contrary, when the cells were incubated with SQAd NPs, quantities of adenosine released by the cells increased over time. This clearly confirmed the reservoir effect resulting from the intracellular accumulation of SQAd NPs followed by the progressive cleavage of the SQAd bioconjugate, allowing a progressive release of free adenosine in the extracellular medium.

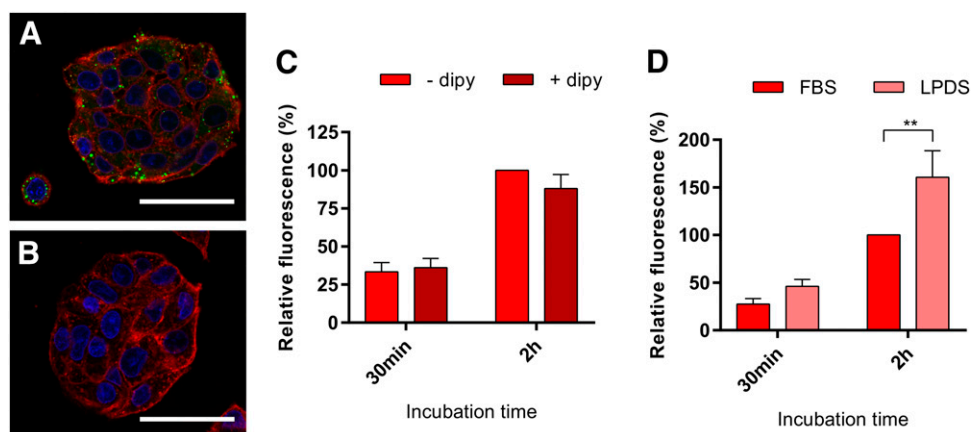


Fig. 3. Cell uptake of fluorescently labeled SQAd NPs by HepG2 cells. (A and B) Confocal microscopy images of HepG2 cells incubated for 2 hours with fluorescently labeled SQAd NPs (A) or the fluorescent tag BODIPY-cholesteryl ester dye as a control (B), prepared under the same conditions as NPs. Cell membranes were stained with wheat germ agglutinin Alexa Fluor 555 (red), whereas 4',6-diamidino-2-phenylindole was used to visualize nuclei (blue). Scale bar, 50 μ m. (C and D) Relative fluorescence measured by flow cytometry from HepG2 cells incubated for 30 minutes or 2 hours with fluorescently labeled SQAd NPs. (C) Cells treated with NPs were incubated in the absence (bright-red bars) or presence (dark-red bars) of dipyr (dipy). (D) Cells were preincubated for 24 hours with FBS-supplemented (bright-red bars) or LPDS-supplemented Dulbecco's modified Eagle's medium (pink bars). Cell autofluorescence was subtracted from each data set. Data represent mean values ± S.D. of three independent experiments. ** $P < 0.01$, two-way analysis of variance with Tukey's post hoc test.

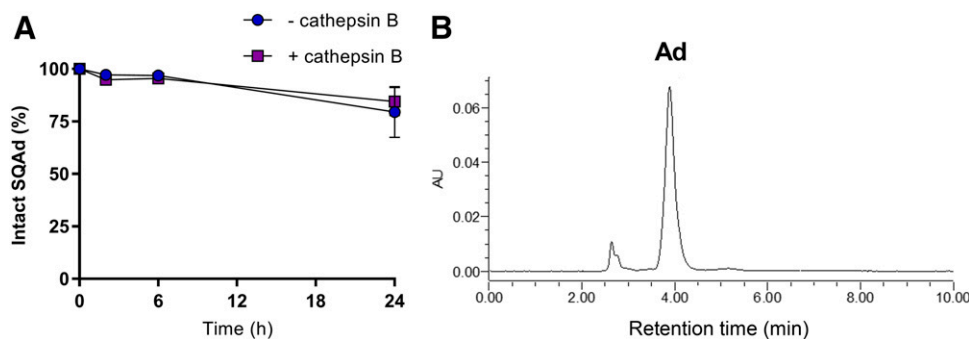


Fig. 4. (A) Percentage of intact SQAd after incubation until 24 hours, with or without cathepsin B (pH 4.8, 37°C). Data represent mean values \pm S.D. of two independent experiments. (B) Representative HPLC chromatogram of partial activation of SQAd into adenosine (Ad) (24-hour incubation, pH 4.8, 37°C, in the presence of cathepsin B). Of note, the peak corresponding to SQAd is not visible in the elution conditions used for adenosine detection. AU, Arbitrary Unit.

A remaining question was whether the released adenosine in the extracellular medium was capable of interaction with adenosine receptors on neighboring cells.

Adenosine Receptors Activation. Thus, adenosine receptor activation was evaluated by measuring the levels of intracellular cAMP. Indeed, adenosine receptors are GPCRs known to activate or inhibit adenylate cyclase, hence tuning intracellular levels of cAMP. As in the previous set of experiments, HepG2 cells were incubated with dextrose, free adenosine, or SQAd NPs for 2 hours and then allowed to release adenosine during 1 hour in their extracellular medium. This medium was then used as an adenosine pool to investigate the interaction, through 15-minute incubation, with other naïve cells by measuring their intracellular cAMP levels. As can be seen in Fig. 6, the extracellular medium originating from cells treated with control vehicle (5 wt. % dextrose) or free adenosine did not induce major changes in intracellular cAMP levels. However, extracellular medium arising from cells treated with SQAd NPs induced a significant increase in cAMP, comparatively to vehicle. Interestingly, adenosine levels in extracellular medium originating from cells treated with SQAd NPs or free adenosine were very close under the conditions used (2 hours of treatment and 1 hour of release, cf. Fig. 5A). Hence, adenosine might not be the only factor in the extracellular medium interacting with GPCRs.

Discussion

This study aimed at clarifying the mechanism of action of SQAd NPs in vitro. The main objective was to determine

whether SQAd acted directly on ARs as a specific ligand or indirectly as a prodrug-releasing active adenosine.

By using radioligand displacement experiments, it was found that SQAd as a bioconjugate or as NPs displayed a rather weak affinity for human ARs, showing K_i superior to 10^{-6} M for A_3 and 10^{-5} M for all other ARs. As a matter of comparison, the native ligand adenosine has an affinity of around 10^{-7} M for A_1 , A_{2A} , and A_3 and 10^{-6} M for A_{2B} (Yan et al., 2003).

More importantly, when SQAd bioconjugate or NPs were preincubated in serum to mimic the in vivo conditions, no more binding onto ARs could be detected. This was attributed to the interaction of SQAd with plasma proteins, especially with cholesterol-rich lipoproteins, i.e., low-density lipoproteins (LDLs) in humans and high-density lipoproteins in rodents (Sobot et al., 2017b). In silico simulations have confirmed that the interaction of the squalene-based bioconjugates with the LDL was driven by the squalene moiety, allowing the hydrophilic adenosine to insert deeply into the LDL hydrophobic core (Yesylevskyy et al., 2018). Thus, LDLs may hinder the possible interaction of SQAd with ARs. As a result, the hypothesis that SQAd may act directly on ARs as a ligand could be rejected.

To assess the second hypothesis related to the progressive release of adenosine from SQAd NPs after cell capture, cultured hepatocytes were used. The HepG2 cell line was chosen because SQAd NPs are concentrated into the liver tissue after intravenous administration (Gaudin et al., 2015).

It was observed that the cell capture of SQAd NPs was independent from the ENT adenosine transporters. And as

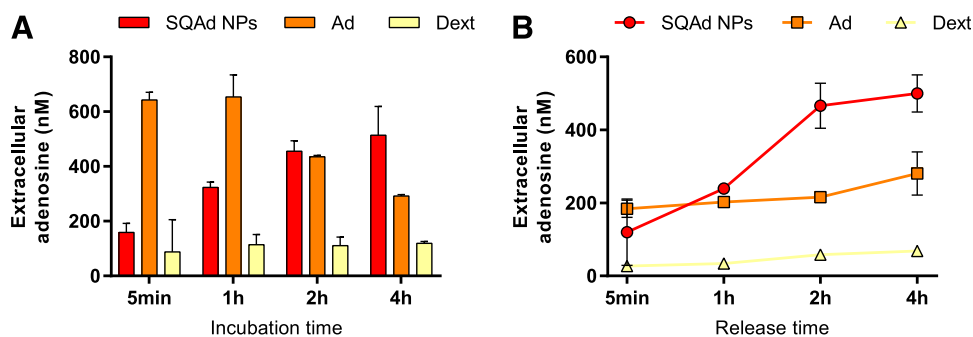


Fig. 5. Concentrations of adenosine released in the extracellular medium from HepG2 cells exposed to various treatments. (A) Cells were incubated for indicated times with 100 μ M SQAd NPs (red bars), adenosine (Ad) (orange bars), or 5 wt. % dextrose (Dext) (yellow bars), and released adenosine concentrations were measured 1 hour following cell culture medium renewal. (B) Cells were incubated for 2 hours with 100 μ M SQAd NPs (red circles), adenosine (orange squares), or 5 wt. % dextrose (yellow triangles), and released adenosine concentrations were measured at different times. Data represent mean values \pm S.D. of three (A) or two (B) independent experiments.

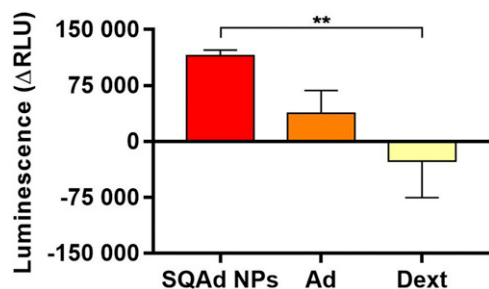


Fig. 6. Changes in intracellular cAMP levels of naïve cells incubated for 15 minutes with extracellular medium collected from other cells exposed to SQAd NPs, free adenosine (Ad), or 5 wt. % dextrose (Dext). Changes in relative luminescence units (Δ RLU) were calculated as the difference in light units between treated and untreated cells. Data represent mean values \pm S.D. of three independent experiments. $**P < 0.01$, one-way analysis of variance with Tukey's post hoc test.

ENTs selectively mediate the bidirectional transport of nucleosides, nucleobases, and nucleoside analogs (Boswell-Casteel and Hays, 2017), the presence of the squalene bulky moiety in SQAd was supposed to prevent the interaction of the adenosine fragment with ENTs. By contrast, the cell internalization of SQAd NPs was found to occur through the LDLRs.

As LDLRs are known to trigger the cell internalization via clathrin-dependent endocytosis (Goldstein et al., 1985), it was quite likely that SQAd followed the same pathway, thus ending up in endosomes and then lysosomes. This hypothesis is in perfect agreement with the confocal microscopy images obtained in this study (Fig. 3A). Once in the lysosomes, SQAd underwent a slow degradation under acidic conditions. Even though cathepsin B alone did not accelerate this proteolytic degradation under tested conditions, its combination with other enzymes such as cathepsin D (Couvreur et al., 2006) and proteases may accelerate this phenomenon in vitro and in vivo. In addition, it was observed that the intracellular cleavage of SQAd into free adenosine also resulted in an extracellular release of adenosine, which gradually increased over time. In other words, SQAd behaves as an intracellular reservoir, slowly releasing adenosine in the extracellular medium, probably via ENTs, since these

transporters could act bidirectionally. Interestingly, the human brain endothelial hCMEC/D3 cell line showed similar adenosine extracellular release after SQAd NPs internalization, which suggests that the interaction of SQAd NPs with HepG2 cells represents a more general trend also occurring in other cell types.

Finally, higher cAMP second messenger levels were observed in cells incubated with the extracellular medium originating from cells treated with SQAd NPs, which clearly indicated an increased activation of ARs. However, since adenosine levels in the extracellular medium originating from cells treated with SQAd NPs or free adenosine were very close under the conditions used, adenosine might not be the only factor in the extracellular medium interacting with GPCRs.

In a summary, this study led to a better understanding of the SQAd NPs mechanism of action, as illustrated in Fig. 7. Unlike currently proposed agonists and antagonists of ARs, SQAd NPs do not interact directly with the ARs but function in a very original way, as a prodrug delivering high quantities of adenosine into the cells. Hence, cells dispose of an important intracellular reservoir of adenosine, progressively releasing the nucleoside which, in turn, can act as an autocrine or paracrine factor. In conclusion, SQAd NPs provide an efficient system for prolonged adenosine delivery, resulting in ARs activation. This new delivery pathway may open interesting prospects in terms of clinical applications.

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Authorship Contributions

Participated in research design: Rouquette, Lepetre-Mouelhi, Couvreur.

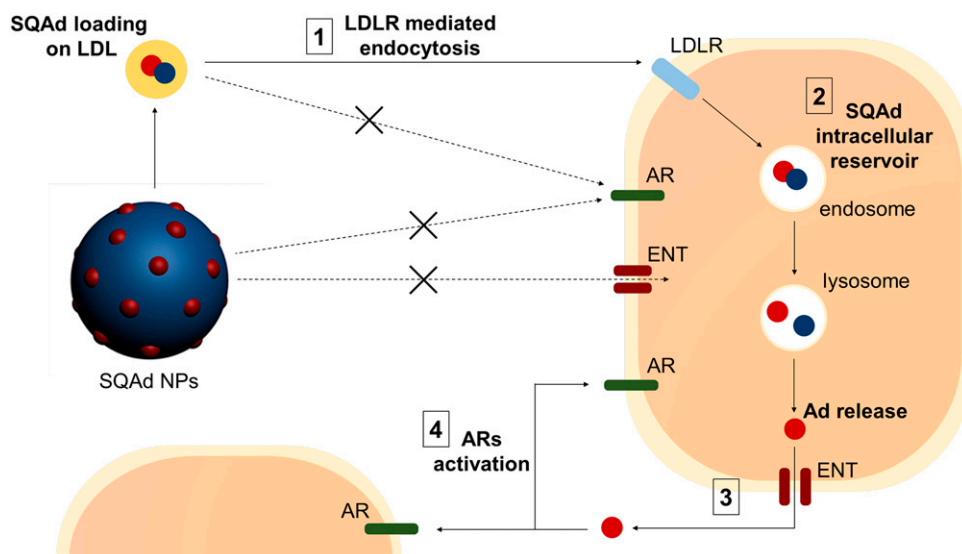


Fig. 7. Schema of the interaction of SQAd NPs with cells. 1) The SQAd NPs are taken up by the cells through the LDL receptors. 2) When located in the intracellular endo-lysosomal compartments, SQAd behaves as an intracellular reservoir, progressively releasing adenosine. 3) Adenosine is finally discharged outside of the cell and 4) interacts with ARs as an autocrine or paracrine signal. Ad, adenosine.

Conducted experiments: Rouquette, Dufranchais, Yang, Mougin.

Contributed new reagents or analytic tools: Pieters, Garcia-Argote.

Performed data analysis: Rouquette.

Wrote or contributed to the writing of the manuscript: Rouquette, Lepetre-Mouelhi, Yang, IJzerman, Couvreur.

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