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DIREITOS DE AUTOR E CONDIÇÕES DE UTILIZAÇÃO DO TRABALHO POR TERCEIROS

STATEMENT OF INTEGRITY

I hereby declare having conducted this academic work with integrity. I confirm that I have not used plagiarism or any form of undue use of information or falsification of results along the process leading to its elaboration. I further declare that I have fully acknowledged the Code of Ethical Conduct of the University of Minho.

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Abstract

Fluorescent nucleobases are critical for the understanding of the structural dynamics and functions of DNA and RNA. 2-aminopurine is a well-known fluorescent nucleobase, with many useful properties, like its high quantum yield, its ability to be introduced in DNA and be used as a guanine mimic, but with several restrictions, like its strong loss of fluorescence from quenching and disturbance it causes when introduced in DNA. Seven new 2-aminopurine derivatives with promising fluorescence properties were tested for toxicity and cellular integration in *Escherichia coli* and *Schizosaccharomyces pombe* (wild type and a mutant affected in *chk1*), with liquid cell cultures growing in minimal media in the presence of said derivatives. Several derivatives caused a decrease of culture growth, one in *E.coli* and four in *S.pombe*, which is correlated with the size of the substituents, with the larger ones having more effect. Accumulation of two of the smaller derivatives in *S.pombe* cells was observed by detecting their own fluorescence inside the cells' vacuoles, further showing the derivatives interactivity with the cells. The mechanisms of these derivatives integration and toxicity are not known, but due to their accumulation in the cells, coupled with their fluorescence, and the fact that they exhibit toxicity shows that there is sufficient potential in these compounds in applications in vacuole and lysosome studies.

Abbreviations

2AP	2-aminopurine
Etheno-A	Ethenediamine
DAP	2,6-diaminopurine
m^5K	5-methylpyrimidine-2-one
tC	1,3-diaza-2-oxophenothiazine
3-MI	3-methyl isoxanthopterin
6-MI	6-methyl isoxanthopterin
6MAP	4-amino-6-methyl-pteridone
DMAP	4-amino-2,6-dimethyl-pteridone
8-AzaG	8-azaguanine
8vdA	8-vinyl-deoxiadenosine
BPP	Benzopyridopyrimidine
thieno-dU	thienouridine
BgQ	Benzo[g]quinazoline-2,4(1H,3H)-dione
NER	nucleotide excision repair
FRET	fluorescence resonance energy transfer
DAPI	4',6-diamidino-2-phenylindole
AO	Acridine orange
PI	Propidium iodide
EMM	Edinburgh minimum medium
REMM	Rich Edinburgh minimum medium

Resumo

As nucleobases fluorescentes são críticas para a compreensão das dinâmicas da estrutura e funções do DNA e RNA. A 2-aminopurina é uma nucleobase fluorescente bem conhecida, com muitas propriedades úteis, como o seu alto rendimento quântico, a sua capacidade de ser introduzida no DNA e de ser usada como mimetizador de guanina, mas possuí várias restrições, como a sua forte perda de fluorescência por "quenching" e perturbações que causa quando introduzida em DNA. Sete novos derivados de 2-aminopurina com propriedades de fluorescência promissoras foram testados quanto à sua toxicidade e integração celular em *Escherichia coli* e *Schizosaccharomyces pombe* (estirpe selvagem e a mutante afetada em *chk1*-), com culturas de células líquidas crescendo em meio mínimo na presença dos derivados. Vários derivados causaram uma diminuição nas taxas de crescimento de culturas expostas a eles, um em *E. coli* e quatro em *S.pombe*, estando correlacionada com o tamanho dos radicais, com efeito maior com o seu tamanho. A acumulação de dois dos derivados menores nas células de *S. pombe* foi observado pela detecção da sua própria fluorescência dentro dos vacúolos das células, mostrando ainda que os derivados interagem com as células. Os mecanismos de integração e toxicidade desses derivados não são conhecidos, mas devido à sua acumulação nas células, à sua fluorescência, e o facto de apresentarem toxicidade mostra que há potencial suficiente nestes compostos em aplicações em estudos de vacúolos e lisossomas.

1 - Introduction

1.1. Fluorescence spectroscopy

Fluorescence spectroscopy (also known as fluorimetry) is one of the techniques under electromagnetic spectroscopy, that analyzes fluorescence emitted from samples. It is an important optical method used to investigate biomolecules. In order to understand this methodology, it's required to understand fundamental concepts that are fundamentally rooted in quantum mechanics. However, much of this understanding can be obtained from simple principles. The discussion of the physical interaction between light and biomolecules or other optical markers is also required.

1.2. Light

Light is an electromagnetic wave, composed of oscillating electric and magnetic fields propagating through space. These components are in phase and perpendicular to each other and to the propagation direction. It is quantified in discreet units known as photons, that have a specific energy (E) expressed as follows (Equation 1).

$$\mathrm{E} = \mathrm{h}\nu = \frac{hc}{\lambda} \qquad (1)$$

where h is the Planck's constant, ν is the frequency of the electric and magnetic field oscillation, c is the speed of light and λ is the wavelength of the oscillation. According to this formula, the energy E is dependent of the wavelength of the light radiation. E is also linearly proportional to the frequency ν and inversely proportional to the wavelength λ. With these ratios, we can show light of different energies through their wavelengths (Figure 1) [1].

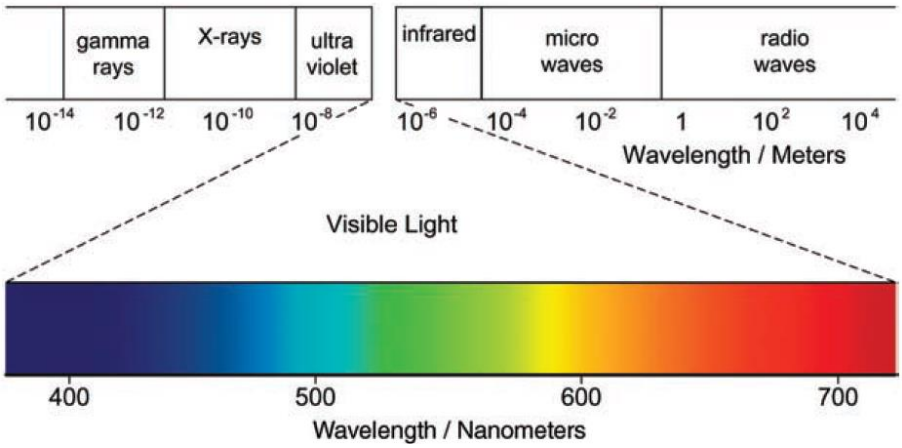

Figure 1 – Spectrum of electromagnetic radiation [1]

The size of typical molecules or biomolecules is in the order of nanometers, beyond the size of visible light waves, which have the size of a few hundreds of nanometers. When molecules are placed within light waves, the different charged components of the molecule, like electrons, are affected by the electric field component of the light. If the molecular structure is appropriate, it can absorb photons of specific wavelength, resulting in electronic structure rearrangements.

1.3. Electronic and vibrational energy levels and fluorescence

The energy levels of the ground (unexcited) and excited states in a molecule or atom can be well described in a Perrin-Jablonski diagram (Figure 2). Most molecules are in the lowest electronic state (S_0), and usually in the lowest vibrational level, at room temperature. In the event that a molecule absorbs a photon, which only occurs if the energy of the absorbed photon is exactly equal to the energy difference between the molecule's energy levels (conservation of energy), it is excited to a higher state than S_0 in less than 10^{-15} s. Depending on the energy distribution of the excitation light and the spatial intersection between the nuclei in the ground excited vibrational states, the initial excited vibrational level of the excited state will be different.

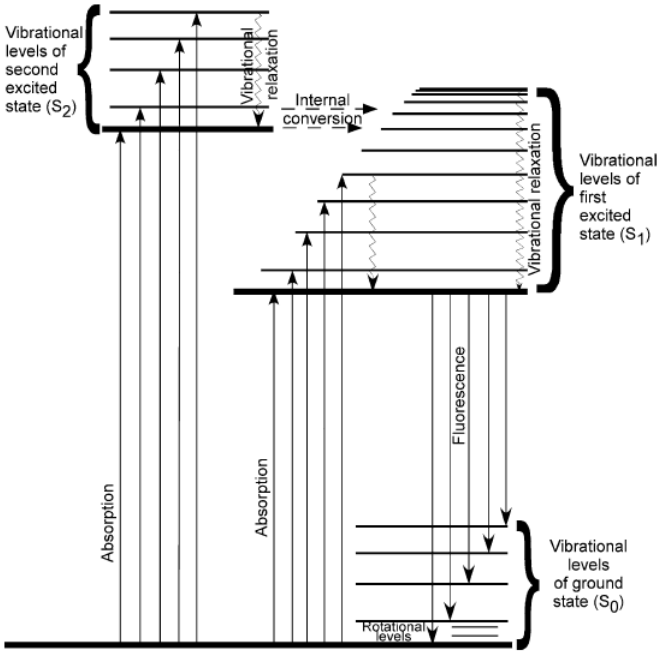

Figure 2 – Perrin-Jablonski diagram showing the energy levels and transitions involved in absorption and fluorescence. [2]

After excitation, the molecules lose this extra energy quickly (10^{-12} s) through collisions with the medium and internal vibrations. Depending on the initial excited state, the molecules will return to lower excited states in different ways. If a molecule is excited to the higher S_2 state, rapid vibrational relaxation occurs, leaving the molecule in the higher vibrational states of the S_1 state, which then passes to the lower vibrational states of S_1 in another 10^{-12} s. From this state, the molecule will leave this excited state through many other pathways.

One of these pathways is the emission of a photon, that can have a large spectrum of different wavelengths, because of the different transitions from the lowest vibrational state of the S_1 state to different vibrational levels of S_0. Because of this, the emission of photons has a lower energy than the excitation photons; this is called the Stokes shift. This is the main reason the emission spectrum is red-shifted in relation to the absorption spectrum. This shift is bigger in polar environments, because it lowers the energy level of S_1.

Other pathways from the S_1 state are depicted in Figure 3, regarding the transition between the excited singlet state S_1 to the excited triplet state T_1. This passage is called “intersystem crossing”. Depending on conditions and on the molecule, the rate at that each conversion occurs will be different, and can be quantified with rate constants. Each separate conversion has its own rate constant and variations of these can provide information of local environment conditions and neighboring molecules.

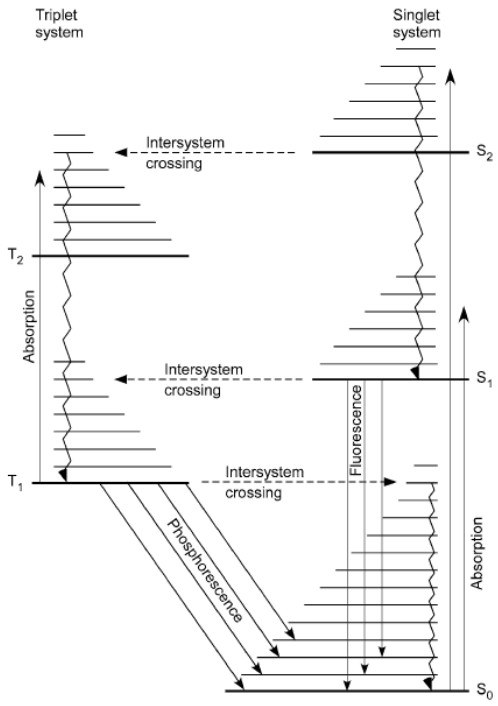

Figure 3 – Energy levels of singlet and triplet states, and their conversions and fates of the excited states. [2]

We can also see that a direct transition from a T_1 state to the ground state is also possible, known as phosphorescence. This, like fluorescence, also involves the emission of a photon, but of much larger wavelength than that of the photons emitted by fluorescence. It is also much slower to occur, because a triplet state is more long lived than a singlet state. However, this emission is usually quenched by the presence of ground state oxygen in the air, making it difficult to detect [2].

In a Perrin-Jablonski diagram, many important parameters can be derived, one of the most important ones being the fluorescence quantum efficiency (or yield; Φ_{FI}), which is defined as (Equation 2):

$$\Phi_{\mathrm{FI}} = \frac{\text{Number of photons emitted as fluorescence}}{\text{Number of absorbed photons}} \qquad (2)$$

This property should be high in fluorescence markers, to allow for increased sensitivity of these markers in biological contexts.

1.4. Biochemical applications

The concepts mentioned above find application in a variety of different techniques used in the investigation of biomolecular processes or biomolecules themselves. Most biomolecules are not intrinsically fluorescent in the range that is usable for their identification or characterization. Therefore, the labelling of the relevant biomolecules is required for many applications, so that the resulting biomolecule conjugate is fluorescent.

Several key requirements must be met so that a fluorescing molecule can be used as a marker for biomolecules. The absorption spectrum should be narrow, it should have a high extinction coefficient (that is, how strongly a molecule absorbs light of a specific wavelength) and a high fluorescence quantum yield, such that the marker is detectable in the presence of background effects.

Several labelling reactions were developed, for many different types of biomolecules and applications, in particular for proteins. The most common targets are primary amino groups and thiol groups. Other systems use specific protein-protein interactions such as avidin-biotin and his-tag techniques, biomolecule immobilization on solid surfaces, and the use of intrinsically fluorescent proteins, such as the green fluorescent protein (GFP) [1].

Proteins are not the only relevant biomolecules being studied. Nucleic acids, such as DNA and RNA, were also targeted for fluorescence-based studies [3]. But first, we need to understand their photochemical properties to create appropriate fluorescent markers.

1.5. Photochemical properties of DNA and analogues

DNA is composed of nucleotides and nitrogenous bases, the latter being organized in two types, purines and pyrimidines. DNA contains two major purine bases, adenine (A) and guanine (G), and two major pyrimidine bases, cytosine (C) and thymine (T or uracil (U), in the case of RNA). They are weak fluorescent molecules as they have a weak extinction coefficient (Figure 4) [4,5], making their utility in fluorescence-based assays limited. So, the development of fluorescent nucleobases has emerged as potent chemical and biological tools for the understanding of nucleic acid structures.

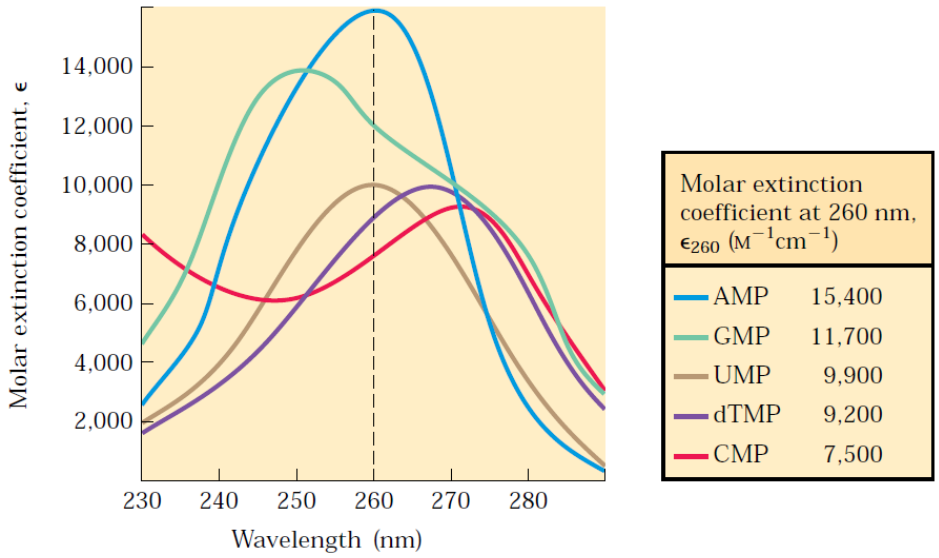

Figure 4 – Absorption spectra of naturally occurring nucleotides. Tabled molar extinction coefficients at pH 7. [4]

Fluorescent nucleobases are modified analogues of the natural DNA and RNA nucleobases that exhibit improved fluorescence properties and retain biologically relevant functions, such as stacking, base-pairing and enzyme interaction.

Different strategies were employed over time in the construction of fluorescent nucleobase analogues. These strategies are employed in two different ways: either preserving the Watson-Crick-like base pairing (that is, to keep the classic A-T and C-G pairing, also known as canonical pairing) or performing larger modifications to the nucleobase structure, that can create novel designs. These two strategies separate fluorescent nucleobases, respectively, into two distinct groups, “canonical” and “non-canonical”. These two groups of nucleobases are used in different ways, to collect different types of information regarding the structure, base-pairing, or protein interactions and recognition of specific sequences of DNA or RNA [3].

The fluorescent properties of DNA are dependent on the stacking of neighboring bases by the formation of double strands [6]. As such, these shifts can be used for analysis of local structures of nucleic acids [7].

1.5.1. Development of fluorescent nucleobases

The first fluorescent nucleobase discovered and studied was 2-aminopurine (2AP). The first reports on this fluorescent nucleobase were made by Ward and co-workers, in 1969, alongside with formacyn and 2,6-diaminopurine (DAP) [8]. 2AP is still today a widely employed reporter of the structure and dynamics of

nucleic acids. This is because of its ability to form pairs with thymine (or uracil), its selective excitation wavelengths and sensitivity in a structural context.

After this report, Stryier discovered another purine analogue, ethenediamine (Etheno-A), that cannot form base pairs [3]. In the 90s, pyrimidine-based analogues emerged, like 5-methylpyrimidine-2-one (m^5K) and pteridines [3]. Other development was also made in the attachment of pyrene and other hydrocarbons to the deoxyribose during this period. In the 2000s, base-discriminating fluorescent nucleobases and a thiol-appended cytidine analogue (tC) were developed, and conjugated linkers were used to modify the electronics of canonical nucleobases [3]. Many other fluorescent nucleobases were designed since then (Figure 5), all of them with different emission profiles and base stacking and pairing properties.

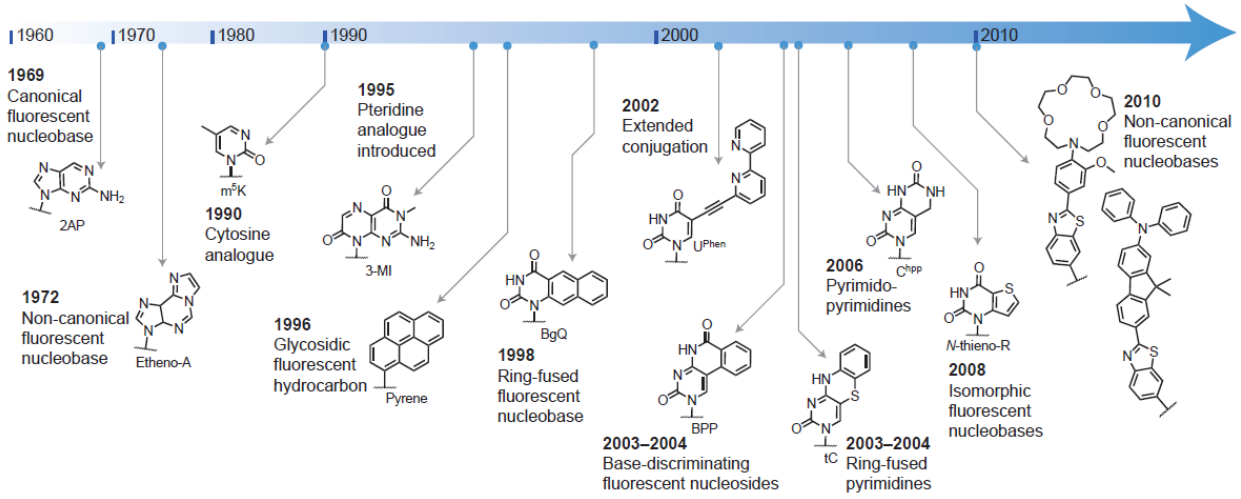

Figure 5 – Development of fluorescent nucleobases schedule [3].

1.5.2. Canonical fluorescent nucleobases

Canonical nucleobase analogues are defined as having a purine or a pyrimidine underlying architecture and by retaining at least two Watson-Crick hydrogen-bonding groups, such that they can form pairs with some complementary base. These conditions limit the number and type of modifications that can be applied, since only regions that do not block base pairing can be modified. The design must also consider the electronic arrangement of either the purine or pyrimidine skeleton.

For the applications of these canonical nucleobases *in vivo*, it is important to avoid background emissions from components present in the cell and cytotoxicity from ultraviolet irradiation. To do so, it is common to

modulate the excitation and emission wavelengths of fluorescent nucleobases to the red part of the spectrum. This can be done extending conjugate π-systems and adding heteroatoms, but disruption of base pairs and DNA helical structure can occur.

In purines, fluorescence-enhancing modifications are mostly made on the five-membered ring, namely on position 8 for adenine and positions 7 and 8 for guanine. (Figure 6a, 6b). Since base paring is required, in most cases, no modifications are made on positions 1, 2 or 6, where base pairing occurs. Despite this, adenine has seen several examples of extensions in the 2 position. Several different types of extension have been performed, which were separated into four categories: purine ring structure modifications; extended fluorescent scaffolds via conjugated linkers; purine substituent modifications; and purine ring fusions.

Modification of the purine ring can cause shifts of 100 nm into absorption and emission wavelengths and increase the quantum yield by several orders of magnitude. Examples of these include pteridines, like 3-methyl isoxanthopterin (3-MI), like 6-methyl isoxanthopterin (6-MI), 4-amino-6-methyl-pteridone (6MAP) and 4-amino-2,6-dimethyl-pteridone (DMAP) (Figure 6c). Conjugated scaffold extension has the potential of expanding the emission spectrum, as in the example of appending a pyrene fluorophore to an adenine or guanine, that led the nucleobase to be excited at ranges of 380 to 420 nm (long UV to short blue), with emissions of 450 to 480 nm (blue light) (Figure 6d). Purine substituent modification is the most prevalent category of fluorescent base design and it focuses on replacing the regular purine substituents with other groups. 2AP is part of this category, moving the 6-amino group of an adenine to the 2 position, while DAP simply adds an amino group to the 2 position in a regular adenine. Both of these show a redshift in relation to adenine of 50 nm and 30 nm to 2AP and DAP respectively. Other examples use the guanine skeleton, like 8-azaguanine (8-AzaG), where the carbon at position 8 is swapped with a nitrogen, or like 4-thieno[3,2-d]-R, that has sulfur instead of nitrogen on position 7. Both modified guanines emit at similar wavelengths (347 nm for 8-AzaG and 351 nm for 4-thieno-R), but they absorb different wavelengths (256 nm for 8-AzaG and 294 nm for 4-thieno-R). Another way to modulate the optical profile of purines is adding small functional groups. As an example, the addition of vinyl group into position 8 to either purine base causes the emission to redshift and increase in quantum yields (Figure 6e). Finally, ring fusion can be made on purine at positions 7 and 8 of the five membered ring. Various ring sizes were used in new nucleobase design, like benzene or naphthalene, creating a tricyclic structure. This structure expands aromatic conjugation causing strong redshifts and increases in the fluorescence intensity (Figure 6f) [3].

In contrast with purines, pyrimidine analogues have less potential modifications, because of its simpler structure. Most modifications are made on positions 4, 5 and 6, leaving positions 2 and 3 able to form base pairs. Three categories separate different strategies for modifications in pyrimidines: modified pyrimidine substituents; extended fluorescent scaffolds using conjugated linkers; and ring-fused pyrimidine ring systems.

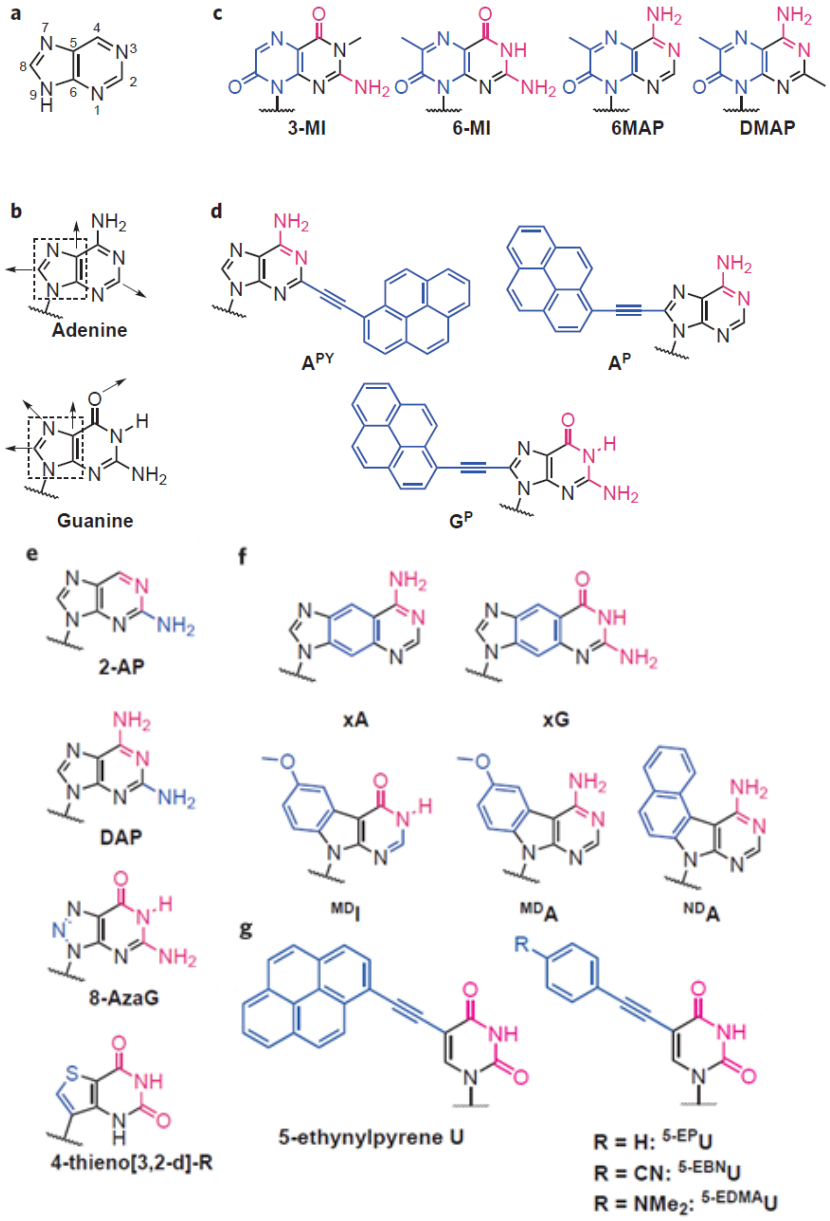

Figure 6 – Strategies for design of fluorescent purines and pyrimidines. Numbering of the purine structure (a). Potential modification sites of adenine and guanine, indicated by the arrows (b). Examples of purine ring modifications(c). Examples of extension of purine scaffold by conjugated linkers (d). Examples of purine substituent modifications (e). Examples of purine ring fusions (f). Extensions of pyrimidines via conjugated linkers (g). [3]

Modifications on substituents are the most prevalent design for new fluorescent pyrimidine nucleobases. One of the earliest examples is m^5K, that does not have the amino group at position 4 present in cytosine and has a methyl group on position 5. These modifications cause a slight redshift and a big increase in emission wavelength. Similarly to purines, extension of pyrimidine structure by conjugate linkers is another way to modify pyrimidines. Ethynyl and heterocyclic linkers allow for various nucleobases to be constructed, including pyrene, fluorenone or boron-dipyrromethane (BODIPY) dyes (figure 6g). Most extensions were made in position 5 of the pyrimidine ring, to avoid disturbing base pairing. When the extended fluorophore has different substituents, the electronic effects are different. If the para-position of the phenyl ring has an electron-donor, both the emission and absorbance redshifts were obtained in comparison with a phenyl ring without a substituent, while in the case of an electron-withdrawing group, it causes a blue shift. Another linker used is triazole, which has extended conjugation and can be attached to other fluorescent modules. Finally, ring fusion can also be made on pyrimidines. This ring addition can greatly improve the fluorescent properties of the natural nucleobases. Examples include thienouridine (thieno-dU) and Benzo[g]quinazoline-2,4(1H,3H)-dione (BgQ), the former having a thiophene ring fused to the positions 5 and 6 of uracil, while the latter has naphthalene is fused to thymine [3,9].

1.5.3. Non-canonical fluorescent nucleobases

Non-canonical fluorescent nucleobases do not have to maintain the capacity to form base pairs, or even need to maintain the skeleton of either a purine or a pyrimidine. By not having to follow these limitations, many more strategies can be employed, so that broader fluorescence properties can be obtained. These properties include the improvement of emission by redshift avoiding, by that way, ultra-violet toxicity. However, these bases tend to be too large to fit in a double-stranded helix, and DNA polymerase enzymes do not recognize them efficiently. As such, several strategies were developed for the incorporation of these bases into DNA.

This type of fluorescent compounds is becoming increasingly more popular, with a large variety of structures being built. They can be separated into two categories: nucleobases made from polycyclic hydrocarbons; and nucleobases made from planar heterocycles.

Even though these nucleobases have the potential limitation of not being able to form regular Watson-Crick base pairs, there has been research in overcoming this issue. One strategy involved the use of very

large molecules that occupy the entire space of a base pair, relying entirely on base stacking interactions to stabilize them [3].

1.5.4. Comparison of canonical and non-canonical bases

The major separation between the two types is the restriction of maintaining or not the structure of natural purines (adenine and guanine) and natural pyrimidines (thymine, cytosine and uracil). This is done on canonical nucleobases, in order to maintain the capacity of base pairing, and they are much easier to insert into a DNA helical backbone and maintain DNA-protein interactions. However, these restrictions limit the variety of fluorescence properties, such as absorption and emission wavelengths. Non-canonical have opposite advantages and disadvantages: wider variety of fluorescence properties but diminished DNA integration [3].

1.6. 2-aminopurine: properties and analogues

2-aminopurine is the most used fluorescent base analogue, as it is very fluorescent. It's an adenine (6AP) analogue that forms stable base pairs with thymine, uracil, and not very stable pairs with cytosine. It has an absorption band centered at 305 nm, which is outside the absorption range of regular nucleobases. So 2AP can be selectively excited in their presence, and its emission maximum is at 370 nm. 2AP has a fluorescence quantum yield of 0.68 while free in solution but it is decreased by a factor of 100 when it is incorporated into nucleic acids [10,11]. This quenching can occur even in dinucleotides, and guanine is the strongest quencher of 2AP from all other nucleobases, including inosine [12–14]. It is a result of π-stacking interactions, where charge transfer between bases can occur [13]. In principle, since adenine has several tautomeric forms (Figure 7), where the 9H tautomer is the most stable, 2AP could have these forms too.

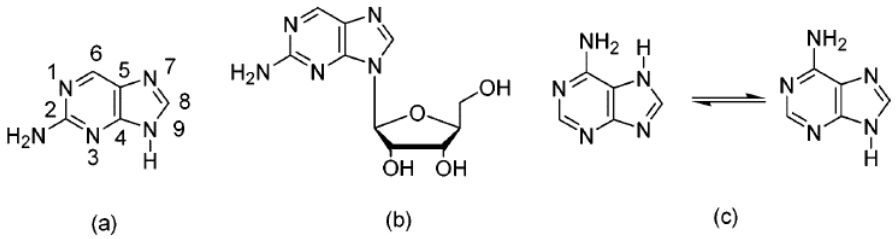

Figure 7 – Structures of 2AP (a), 2AP riboside (b) and the 7H and 9H tautomers of adenine (c). [14]

These tautomers have closely overlapping spectra, but they can be distinguished by their fluorescence lifetimes. Various analogues of adenine exist, mostly produced by substituent modification. Examples include 2,6-diaminopurine (DAP), 6MAP, DMAP and 8-vinyl-deoxiadenosine (8vdA; Figure 8).

2AP exhibits a 50 nm redshift in comparison with adenine, while DAP has a 30 nm redshift [3]. As mentioned before, 2AP suffers from a quenching effect when incorporated into DNA. 8vdA is a potential replacement, because it's hydrogen binding pattern is unchanged in relation to 2AP. It has higher quantum yield and extinction coefficient, but it loses quantum yield when inserted into DNA, although its quantum yield was still larger than 2AP [11,15]. It has an excitation maximum at 290 nm and, like 2AP, can be selectively excited, and it has an emission maximum at 382 nm, slightly redshifted from 2AP.

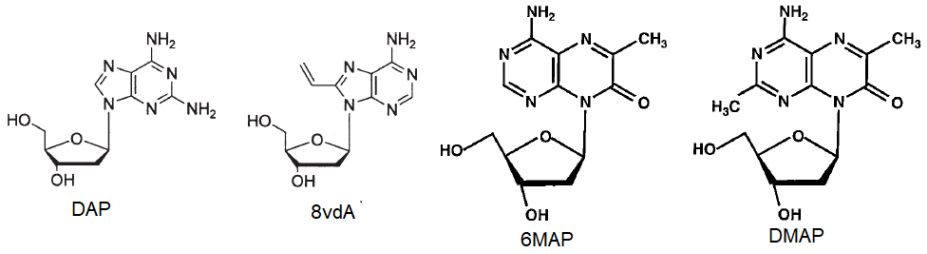

Figure 8 - Structures of adenine analogues: 2,6-diaminopurine (DAP), 8-vinyl-deoxiadenosine (8vdA), 4-amino-6-methyl-pteridone (6MAP) and 4-amino-2,6-dimethyl-pteridone (DMAP) [15,16]

6MAP and DMAP are two adenosine analogues that have properties similar to guanosine analogues. The excitation wavelength maximum for 6MAP is 320 nm and for DMAP is 310 nm, and both have an emission wavelength maximum of 430 nm. The quantum yields of 6MAP and DMAP monomers are 0.39 and 0.48 respectively. Similarly to 2AP and DAP, these analogues are quenched when incorporated into oligonucleotides, and this quenching was smaller when these analogues were surrounded by pyrimidines and larger when they were surrounded by purines [16]. However, this effect does not occur in all nucleobase analogues. For instance, the cytosine analogue, pyrrolocytosine, when introduced into dinucleotides and trinucleotides, has enhanced fluorescence emission [17]. Also, it is quenched when introduced in DNA-RNA duplexes, so it is expected that this analog could be used in RNA structural analysis [18].

Incorporation of these analogues into DNA is also a concern, since these base analogues can be unrecognized by DNA polymerases. As such, specialized methods for DNA synthesis with these bases may be required. One method for the preparation of oligonucleotides is done using automated solid phase DNA synthesis and a phospharamidite derivative of 2AP. Solid phase techniques were used before in DNA synthesis, using enzymes, but it suffered from low yield [19].

1.7. Applications in Biology

Fluorescent nucleobases have been employed in many different types of applications. Implementations can range from nucleotide polymorphism detection, structural measurements and all the way to enzyme activity testing, mostly of DNA polymerase. To accommodate all these different applications, a wide variety of different nucleotides were developed. For instance, in experiments where enzyme activity is to be reported, we require fluorescent nucleobases that interact either as a substrate or with the natural substrate of that enzyme, such that their fluorescence signal is interfered and therefore, could be used as a reporter of enzyme activity.

1.7.1. DNA repair mechanisms

Fluorescent nucleosides are used as reporters in structural studies and the elucidation of biological processes, such as DNA repair. Different mechanisms for DNA repair are present in cells, one of these is nucleotide excision repair (NER), a highly conserved DNA repair mechanism, which recognizes DNA lesions, including artificially made ones. In prokariotes, like *E.coli*, 3 enzymes (UvrA, UvrB and UvrC) are required to accomplish the recognition of DNA lesions and subsequent DNA incisions required for DNA. To elucidate this mechanism, the binding of UvrB to DNA fragments with cholesterol lesions can be detected by the insertion of a fluorescent nucleoside, like 2AP, immediately next to the lesion. The action of the enzyme causes perturbations in the base stacking, reducing the quenching suffered by 2AP, by partly reducing base stacking interactions. UvrA, on the other hand, does not cause significant base pair disruption, not allowing for 2AP fluorescence emission [20].

1.7.2. Study of DNA duplexes

For a fluorescent nucleobase to be used to study various DNA duplexes, its fluorescence properties should be invariant to the environment, and upon introduction into a DNA strand. 2AP has a high fluorescence quantum yield (68%), but it's greatly reduced when incorporated into oligonucleotides.

This sensitivity to its excited lifetimes to the environment makes it unreliable as a probe of molecular dynamics using fluorescence anisotropy measurements and energy transfer. A new fluorescent tricyclic

cytosine analogue, 1,3-diaza-2-oxophenothiazine (tC; Figure 9), shows that its quantum yield, while smaller than 2AP (20%), stays the same when it's incorporated in DNA duplexes. Furthermore, tC can be selectively excited and can be used as a fluorescence resonance energy transfer (FRET) donor, in conjunction with rhodamine as an acceptor [21].

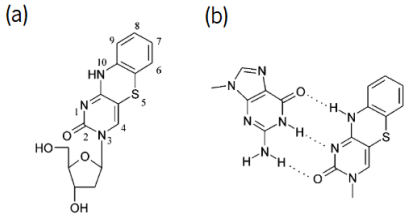

Figure 9 - Structures of tC nucleoside (a) and G-tC base pair (b). [21]

1.7.3. Study of DNA triplexes

Triplex helix formation requires specific features that not every double-stranded sequence adheres to; one is the occurrence of homopurine stretches. Another constraint is the weak binding interaction between the third strand and the double-stranded target. In order to increase the stability of triplexes, modified bases with extended aromatic domains were made, to increase the third strand binding through improved stacking interactions. Benzo[g]- and benzo[f]quinazoline-2,4-dione-(1H,3H)-dione were used as substituents of thymine the canonical TA*T triplet, and it was revealed that benzo[g]quinazoline has strong fluorescence emission properties that could be used to selectively monitor the formation of triple-helical structures [22].

1.7.4. Elucidation of enzyme specificity

Describing enzyme specificity for substrates and cofactors is possible by defining the dimensions of molecules that can fit in enzyme active sites. For enzymes like ATP and GTP kinases and DNA-dependent RNA polymerases, purine nucleotides with modified triphosphate groups were proven to be effective analogues for studying the activation and inhibition of these enzymes. With this in mind, new purine analogues were synthesized with more defined dimensional changes, by inserting a benzene ring in the center of the purine ring system: 8-aminoimidazo[4,5-g] quinazoline, 9-aminoimidazo[4,5-f] quinazoline, and 6-aminoimidazo[4,5-h] quinazoline (Figure 10). These "stretched-out" analogues have dimensions that set

limitations on the size and flexibility of the enzyme binding sites specific to purine substrates, and as such, can be used to elucidate the specificity of several enzymes, like adenosine deaminase and xanthine oxidase [23].

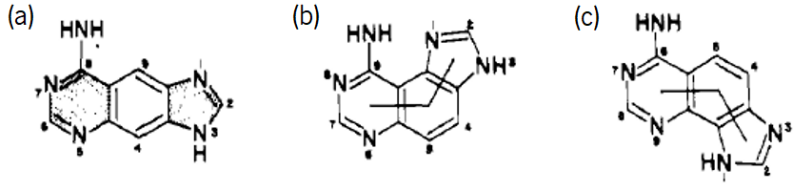

Figure 10 - Structures of 8-aminoimidazo[4,5-g] quinazoline (a), 9-aminoimidazo[4,5-f] quinazoline (b), and 6-aminoimidazo[4,5-h] quinazoline (c) [23].

1.7.5. Determination of changes in purine bases on complementary strands

Fluorescent nucleosides, like 2AP, are used as site-specific probes. These probes are sensitive to the local environment of DNA, so changes to this environment, like hybridization changes and conformational shifts, will cause a variation in their emission, allowing the detection of these changes. However, this method is unable to distinguish the type of base causing the changes. Benzopyridopyrimidine (BPP) has a base-pairing degeneracy, and it allows BPP to distinguish between purine bases on the complementary strand by a sharp fluorescence change. Oligonucleotides containing BPP are then effective probes for typing of the A/G single-nucleotide polymorphism [24].

1.7.6. Multiple dye interaction

The study on how multiple dyes interact has been an interesting development. This technique uses different fluorescent DNA nucleobases, in adjacent positions in a DNA strand. This arrangement of molecules allows them to interact closely, and several mechanisms are known to transfer energy or excitation from one dye to another. As an example, two pyrene nucleosides in adjacent positions form an excimer complex that emits green light, while a pyrene alone emits blue light. This change can be used to detect when two probes bind side-by-side. Many fluorosides, that is, nucleosides that replace the DNA base with fluorophores, are being studied for interactions between them, by creating oligomers with these bases. These oligomers show

different effects on color and intensity of emission, and a small set of fluorosides can generate a wide variety of probes with properties that single dyes don't have [9,25].

1.7.7. Studies of DNA base stacking

Base stacking of short RNA helices has its effects examined, with a good amount of experimental data [26]. However, much less data has been made on the topic of base stacking of DNA helices. To study this topic, polycyclic aromatic probes were synthesized as probes for electrostatics, van der Waals effects and hydrophobicity, which have a role in base stacking of DNA. The fluorescent properties of pyrene and phenanthrene nucleosides are also of interest. Pyrene has been incorporated into the ends of DNA strands by flexible chains in several studies. This flexibility allows for the pyrene incorporated to adopt different conformations, and allow for sensitive information about the structure of nucleic acid helices [27].

1.7.8. Detection of abasic sites in DNA and local viscosity

Abasic sites are locations in DNA where there is no purine or pyrimidine base. These locations occur spontaneously or due to genetic damage. To detect these locations, fluorescent nucleosides with segmental mobility can be used. Several nucleosides are composed of aromatic rings linked to a flexible bond. These nucleosides have low quantum yields due to the free internal rotation of the rings, however, when they are restricted, their quantum yield increases. As such, when these nucleosides are hybridized to an abasic site, the restriction caused by the presence of two neighboring Watson-Crick base pairs will increase fluorescence. Any form of restriction would also be able to be detected by these nucleosides, such as an increase of local viscosity or protein-DNA interactions [28].

1.8. Cellular effects and applications of 2AP

2-aminopurine is a known mutagen that causes transition mutations in prokaryotes. These mutagenic effects of 2AP are from several sources. Base-pairing ambiguities can occur, because of 2AP's ability to bind to thymine while in the amino state and to bind to cytosine while in the rarer imino state (Figure 11). This ability suggests that mutations could be induced as a result of pairing errors of the analog [29].

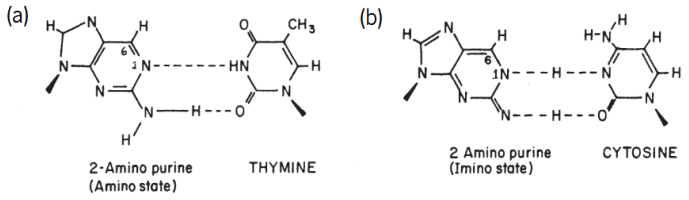

Figure 11 – Hydrogen bonding of 2AP and base pairing. "Normal"pairing of 2AP and thymine (a). Rare imine form of 2AP binding with cytosine (b). [29]

Another source is the disturbance of normal deoxyribonucleoside triphosphate pool sizes. This can happen because the 2AP metabolite, 2AP-deoxyribose is an inhibitor of adenosine deaminase, a catabolic enzyme involved in the salvage pathway of purine nucleotide synthesis.

Normally, this enzyme is sufficient to prevent the phosphorylation of deoxyadenosine, since this enzyme converts it to deoxyinosine. However, when this enzyme is inhibited, there is an increase of the concentrations of dATP, phosphorylated deoxyadenosine. dATP at high concentrations is toxic, inhibiting ribonucleotide reductase, consequently reducing the intracellular pools of TTP, dGTP and dCTP [30].

While these effects can occur in prokaryotes, fewer reports exist studying 2AP mutagenicity in mammalian cells. It was shown that 2AP induced mutations in several mouse and hamster cancers, such as lymphoma and lymphosarcoma [31]. It was also shown that the affected cell line had an abnormally high dCTP pool and a very low TTP pool. This imbalance can lead to increased 2AP mutagenesis due to mispairing, so this mutation mechanism could also be true in mammalian cells [32]. A potential mechanism of mutation that's not present in prokaryotes is the exchange of sister-chromatids (SCE). Most mutagens induce SCEs, but no quantitative correlation between them and gene mutations. 2AP was found to be an inducer of both gene mutations and SCEs and that these two effects in mammalian cells (V79 cells) were independent from each other [32].

The mutagenic ability of 2AP is useful for a variety of studies, involving DNA repair mechanisms in both prokaryotes and mammalian cells, and being a kinase inhibitor, it can also be used to study these enzymes both *in vivo* and *in vitro*.

1.8.1. DNA repair mechanisms

Escherichia coli has a low spontaneous mutation frequency through a methyl-directed DNA mismatch repair pathway. This system scans the daughter strand for errors introduced after replication. The replication

complex can recognize incorrect bases in the daughter strand because the new strand is unmethylated. The methylation of adenine in *E. coli* is controlled by Dam methylase. Dam mutants have a 10 to 100 fold increase of their spontaneous mutation frequency, because of their inability to distinguish between the parental and daughter strands. Fluorescent nucleosides like 2AP cause mutagenesis, and Dam mutants are very sensitive to this, causing lethality in affected cells. Using 2AP as a mutagen, the methyl-directed mismatch repair complex and its corresponding genes, mutH, mutL and mutS, are active. With this method, these genes can be studied for their biochemical role in this repair mechanism [33,34].

As for mammalian cells, a similar mismatch repair mechanism was detected in a Chinese hamster ovary cell line, with 2AP being used to test this presence. It was concluded that 2AP is not an optimal probe for the elucidation of these mechanisms in mammalian cells, because of multiple secondary effects unrelated to the toxicity [30].

1.8.2. Protein kinase inhibition

Protein kinase activity is a requirement for proper control of the cell cycle. For instance, the p53 tumor suppressor gene regulates the cell cycle and apoptosis in response to genotoxic stress. 2AP suppresses p53 phosphorylation by inhibiting protein kinases. p53 activity is essential for proper gene regulation in the event of DNA injury, and a misfunction of this gene can mean life or death [35]. Another target of protein kinases is the protein kinase itself, an example is the RNA-dependent p68 protein kinase (PKR). Autophosphorylation of PKR has been suggested to be involved in double stranded RNA stimulated gene expression, and 2AP is an inhibitor of this activity [36]. 2AP can then be used as tool to study different types of kinases in *in vivo* studies.

1.8.3. Lifetime-based sensing

After the initial excitation of molecules, fluorescence emission decays over time, independently of concentration. In most fluorescent molecules, the decay rates are characterized by a single exponential function. But with fluorophores, the various interactions and dynamics cause the decay rates to shift. The complex decay profiles of the fluorophores can then be a source of information of the different conformations present in the molecules. 2AP decays in a mono-exponential way when dissolved in water, however, when

it's introduced in DNA, the rate of decay is characterized as a sum of exponential components with different lifetimes. These components reflect the various conformations and stacking effects. 2AP has been employed as a probe using this property to study transitions in DNA duplexes and the conformational changes of DNA after binding to proteins [28].

1.9. Research problem and objectives

2-aminopurine is, as mentioned earlier, a widely utilized fluorescent nucleoside. The quantum yield of 2AP is high (0,68) in neutral aqueous conditions, with a redshifted absorption spectrum, that permits excitation beyond the range of natural nucleobases and proteins. 2AP also forms a stable bond with thymine, albeit less stable than normal adenine bonds, and can maintain a stable B-DNA structure. It also can be incorporated into oligonucleotides (OND) with solid-phase synthesis, using phosphoramidite chemistry, in any position of both DNA and RNA strands [37].

These are good traits, but 2AP is not without disadvantages. 2AP can also bind, as mentioned in the previous section, to cytosine, so 2AP is not specific. Its fluorescence quantum yield is also very low when it is incorporated into single or double stranded oligonucleotides [37]. This also makes 2AP a poor probe for studies of molecular dynamics and DNA-protein interaction using techniques like FRET [10].

Many alternatives were created to offset these demerits. 8-aza-7-deazapurine-2-amine was employed to examine the thermal stability and photophysical properties of ONDs containing this nucleobase and compared to ONDs containing 2AP. It was found that ONDs containing either these bases are less stable and their emission was strongly quenched, so this base can be used as an alternative to 2AP [37].

8-vinyl-2'-deoxyadenosine (8vdA) is another fluorescent derivative, but with improved fluorescent capabilities over 2AP. 8vdA monomers have a similar quantum yield to 2AP monomers, but 8vdA can form more stable duplexes than 2AP, and its fluorescence quantum yield is significantly higher than 2AP when introduced to single-stranded ONDs and duplexes [37].

A series of 8-(1H-1,2,3-triazol-4-yl) substituted adenosine derivatives also show useful photophysical properties, such as high quantum yield and brightness factor. When these bases are incorporated into ONDs, their quantum yield is greatly affected by the type of neighboring base. Like 2AP, these triazole-adenine derivatives have lower quantum yields in single strands and duplexes, but it has a 10-50 times higher

quantum yield than 2AP. The bases have then significant potential for monitoring DNA microenvironments [37,38].

Another alternative is phosphoramidite, a menthol derivative of 2AP, and it is capable of functioning as a DNA damage mimic and as a fluorescence reporter, to elucidate DNA repair mechanisms [39].

Fluorescent nucleosides have many different properties and applications, with several of these being utilized in *in vivo* experiments, with 2AP being one of the most well-known, but due to its restrictions, many derivatives and alternatives were and are being developed to modify its properties, like improving its quantum yield or reducing its emission quenching when inserted in DNA, in order to tackle different scientific problems. To achieve this objective, new fluorescent 2AP derivatives were developed. In order to determine their potential applicability in *in vivo* experiments, *Escherichia coli* and *Schizosaccharomyces pombe* cell cultures were used in cell toxicity assays, by determining variations in growth rates and nuclei morphology in cells exposed to the derivatives. Moreover, the derivatives' bioavailability was also determined by detecting their own fluorescence inside cells. With this study, these derivatives could be applied for the production of probes targeting nucleic acids in order to study metabolism and design improved molecular techniques for research and diagnostics.

2 – Materials and experimental methods

2.1. Microorganisms, media and culture growth conditions

The *E. coli* strain used was DH5α (φ80lacZΔM15 Δ(lacZYA-argF) U169 recA1 endA1 hsdR17(rK^-, mK^+) gal^- phoA supE44 λ^- thi^-1 gyrA96 relA1). Stock cultures were made in solid LB medium (Difco™ LB Broth, Miller; 1% w/v triptone, 0.5% w/v yeast extract, 1% w/v NaCl and 1.5% agar; Liofilchem), incubated at 37 °C for 18 h and then kept at 4 °C for one week. Liquid cultures were made by inoculating liquid LB medium (without agar) with one colony and left to incubate overnight at 37 °C, with constant agitation (160 to 200 revolutions per min; rpm). Two strains of *S. pombe* were used: wild type 972h^- and a mutated *chk1* strain in 972h^- genetic background.

Both strains were maintained in solid YES medium (3% w/v glucose, 0.5% w/v yeast extract (ACROS Organics™), 0.02% w/v adenine, 0.02% w/v uracil, 0.02% w/v histidine, 0.02% w/v leucine, 0.02% w/v lysine, 2% w/v agar; Liofilchem).

Liquid cultures were made by inoculating liquid EMM medium (0.3% w/v potassium hydrogen phthalate, 0.22% w/v Na_2HPO_4, 0.5% w/v NH_4Cl, 2% w/v glucose, 20 mL/L 50x salt stock, 1 mL/L 1.000x vitamin stock, 0.1 mL/L 10.000x mineral stock (Appendix 1)) and a variant of rich EMM medium (REMM, with added 0.225% w/v adenine, 0.225% w/v leucine) with one colony of 972h^- and chk1 strains, respectively and left to incubate overnight at 30 °C, with constant agitation (160 to 200 rpm).

2.2. 2-aminopurine derivatives

A total of 7 different 2-aminopurine derivatives were tested, all of them C6-substituted with a nitrile group and N9-substituted with different substituents (Figure 12). In Table 1, the derivatives are named by their substituent at N9. All these derivatives were produced by Sofia Pêra, under Alice Dias' supervision, from the Centre of Chemistry and the Department of Chemistry, University of Minho. Stock solutions of these derivatives (100 mg/mL) were made using dimethyl sulfoxide (DMSO) as a solvent. The solutions were stored at 4 °C until use.

Table 1 - Nomenclature, substituent and abbreviations of the derivatives used.

Nomenclature	Abbreviation	Substituent in N9	Compounds in Fig.12
2-amino-6-cyano-9H-purine	H	H	(a)
2-amino-6-cyano-9-methyl-purine	CH_3	CH_3	(b)
2-amino-6-cyano-9-(1,3 butanediol)-purine	diol	$HOCH_2CH_2CH(OH)CH_3$	(c)
2-amino-6-cyano-9-cyclopentyl-purine	Cp	C_5H_{10}	(d)
2-amino-6-cyano-9-benzyl-purine	CH_2Ph	CH_2Ph	(e)
2-amino-6-cyano-9-(4-fluorophenyl)-purine	PhF	PhF	(f)
2-amino-6-cyano-9-(4-methoxylphenyl)-purine	$PhOCH_3$	$PhOCH_3$	(g)

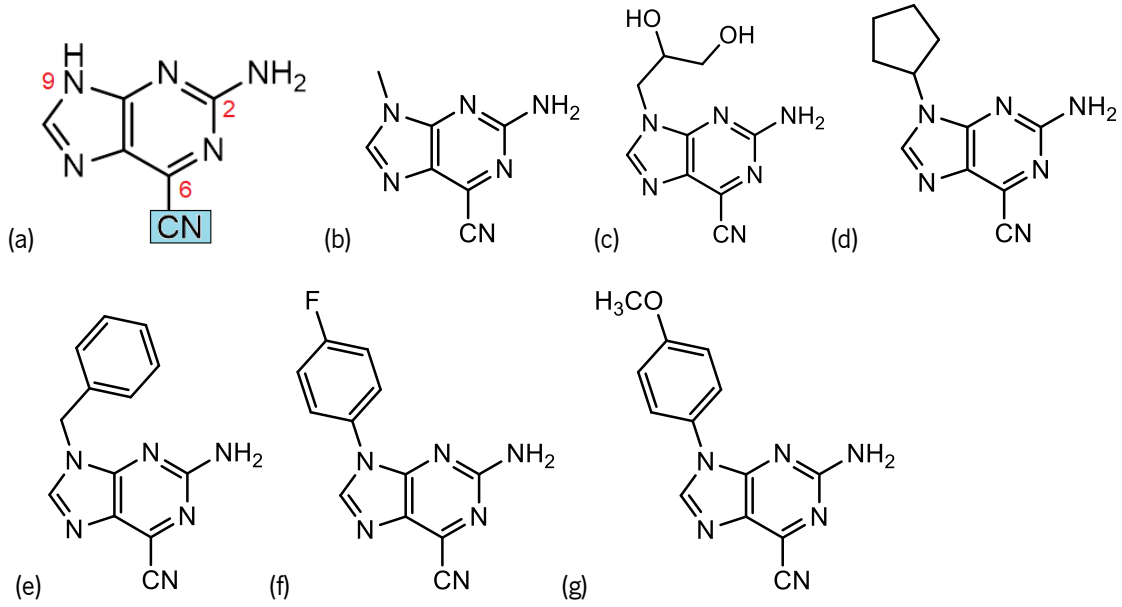

Figure 12 – Structures of the derivatives used, with no substituent on N9 (a; H) and the derivatives modified by substitution in N9, H (a), CH_3 (b), diol (c), Cp (d), CH_2Ph (e), PhF (f), $PhOCH_3$ (g). Note the nitrile group on C6 in all structures.

2.3. Determination of growth rates

Growth rates were determined by following cultures of *E. coli* and both strains of *S. pombe* treated with the different derivatives using spectrophotometry, a branch of electromagnetic spectroscopy. Three different derivatives were tested with *E. coli*, and all 7 derivatives with *S. pombe.* Overnight cultures were diluted to an optical density at 600 nm (OD_{600}) of 0.05 and then split into several cultures. Treatments were made by

adding derivatives, such that the final concentration was 200 μg/mL (with the exception of the derivative PhF, in *S. pombe* cultures, where two different concentrations were used, 40 μg/mL and 20 μg/mL). After 1 h of initial growth, 1 mL samples were removed from the different cultures and their (OD_{600}) was measured at constant intervals: 30 min for *E. coli* and 1 h for the *S. pombe* strains.

2.4. Fluorescence microscopy imaging and DAPI staining

Fluorescence microscopy was used to determine the status of the nuclei of *S. pombe* cells treated with the derivatives. While the cultures were being measured for their growth rate, at an (OD_{600}) of approximately 0.2, 100 μL of the cultures were removed and centrifuged for 2 min at 14000 *g*. The resulting pellets were resuspended in 50 μL of PBS solution (8% w/v NaCl, 0.2% w/v KCl, 1.44% w/v Na_2HPO_4, 0.24% w/v KH_2PO_4). This procedure was repeated and 20 μL of the resulting suspensions were heat-fixed in a microcopy glass slide. Four microliters of a 4',6-diamidino-2-phenylindole (DAPI) solution (10 μg/mL) in PBS was added to the preparation and visualized with a fluorescence microscope (Leica DM5000B) immediately with an excitation wavelength range of 340-380 nm (A filter cube).

2.5. Acridide orange/propidium iodide dual labeling assay

An acridine orange/propidium iodide dual fluorescence staining assay is a standard technique to detect cell viability. Acridine orange (AO) is a fluorescent dye that is permeable to live cell membranes and stain cell nuclei due its ability to intercalate into DNA. Propidium iodide (PI) is, by contrast, only capable of penetrating non-viable cells, with non-functional membranes and then stain their nuclei. When both these compounds are used in the same sample, we are capable of distinguishing between living and non-viable cells by their different fluorescent emissions, green for the former and red-orange for the latter [40]. For this assay, similar preparations for DAPI staining were made, with the final pellet resuspension being made with 50 μL of a AO/PI solution in PBS and the resulting resuspensions were placed on a microscopy glass slide without heat-fixing. The preparations were observed at an excitation wavelength ranges 450-490 nm for AO (GFP filter cube) and 515-560 nm for PI (N2,1 filter cube).

2.6. Statistical analysis

The toxicity assay samples were prepared and analyzed with 3 independent replicas, while the acridine orange/propidium iodide dual labelling assay was made with a single replica, with the results portrayed in mean±standard deviation (SD). Analysis of variance (ANOVA one-way analysis) was used to statistically analyze the data, using Graphpad Prism 7.00. Dunnett's test was used to detect any differences among mean values, with the *p*-values being 0.05, 0.005 and 0.0005, represented by the number of asterisks ($p<0.05$, *; $p<0.005$, **; $p<0.0005$, ***)

3 – Results and Discussion

3.1. Determination of growth rates

In order to investigate the toxicity of the purine derivatives, a growth rate assay was implemented, by exposing liquid cell cultures to them. Many previous experiments studied the mutagenic properties of fluorescent nucleotides, 2AP included [29,30,32], but we couldn't find studies in the literature elaborating the cell toxicity of these compounds. Liquid cell cultures with the various purine derivatives added with specific concentrations were grown and their OD_{600} was periodically measured, in order to determine growth rates. Culture growth variation indicates that the purine derivatives interact with the cells in some manner. Fluorescent nucleotides are known to interact with cells in various ways, such as DNA integration or interacting with DNA/ATP binding proteins. These interactions can cause various toxic effects to living cells, like mutagenesis or enzymatic inhibition.

As an initial toxicity screening, *E. coli* cultures were used. Analysis of the growth rates indicate that the presence of the solvent, DMSO, didn't affect the growth rate of the *E. coli* when comparing with the control without derivative or solvent (Figure 13). Among all the compounds tested, the derivative PhF is the only one that shows a significant decrease in growth rate, indicating that it is affecting *E. coli* cells and it is presumably toxic. We don't know the nature of the interaction, if it's related to some form DNA interaction or if the toxicity has some other mechanism, however this data indicates that PhF derivative has the capacity to enter and interact with living *E.coli* cells.

To analyse all the purine derivatives in a single experiment and to further analyse PhF toxicity, *S. pombe* was utilized, since it's a eukaryote, easy to manipulate and it's a commonly used experimental model for the maintenance of genome integrity, with mechanisms similar to those of higher eukaryotes [41].

The following measurements were made with two different strains of *S.pombe*, wild type and *chk1-*. The *chk1-* is a strain incapable of producing the kinase Chk1, a major component of DNA damage checkpoints, which trigger a cellular response to delay the cell cycle progression, so that DNA repairs could be made[42] and as such, this strain is more susceptible to genotoxicity than the wild type strain [43,44], so it would be more likely, to some degree, to detect genotoxicity caused by the purine derivatives.

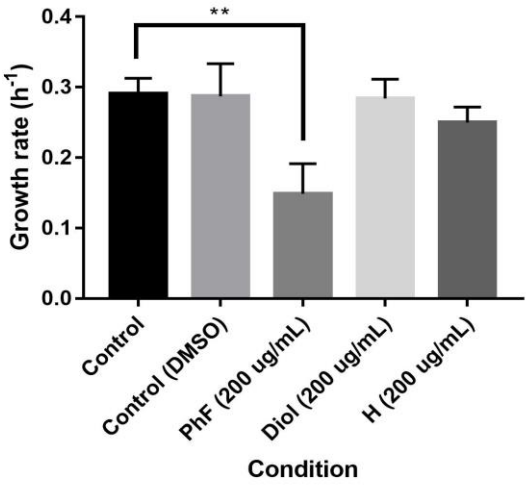

Figure 13 – Growth rate of *Escherichia coli,* strain DH5-α cultures in LB with 200 μg/mL of three purine derivatives, PhF, diol and H (abbreviations used in Table 1) and 2 controls: no derivatives or their solvent added (Control) and only the solvent added (Control (DMSO)). The cultures were incubated at 37 °C, 200 rpm and growth was monitored by optical density at 600 nm. Results are presented as mean±SD from 3 independent replicas. Significance was analyzed by Dunnet's comparison test (* - $p<0.05$; ** - $p<0.005$).

The growth rate of control wild type *S.pombe* cultures remained similar after the addition of 20 μL of DMSO, neither did the use of different media (REMM over EMM; Figure 14b). REMM medium is needed to grow the *chk1-* strain, since it's unable to produce adenine and leucine by itself [45], and needs for them to be supplemented. To control for this, REMM is also used a negative control for wild type. Similarly to the *E. coli* cultures, the presence of PhF, even at a lower concentration than the other derivatives (40 μg/mL instead of 200 μg/mL), caused a significant decrease ($p<0.005$) in the growth rate of wild type *S. pombe*. At a lower concentration of 20 μg/mL, the cultures exposed to PhF no longer show a significant decrease. In addition, we also detected other derivatives that exhibited a decreased growth rate in *S. pombe* cultures, Cp ($p<0.05$), CH_2Ph ($p<0.005$) and $PhOCH_3$ ($p<0.05$), which are derivatives with large and apolar substituents, similarly to PhF, while smaller, like CH_3, or polar substituents, like diol, didn't show significant toxicity. This correlation suggests that more apolar derivatives are more capable of permeating the apolar cellular membranes, and more likely capable of impacting growth rates. We also noticed that in cultures treated with 2AP, growth rate wasn't affected, even though it is a known mutagenic compound [29,32]. The combination of derivative exposure and medium might also be altering the growth rate of cultures, since wild type cultures treated with Cp and CH_2Ph have different growth rates between media. These derivatives might be interfering the synthesis pathways of adenine/leucine, and the supplementation of these aminoacids

allows the cultures growth to be less affected. It's important to realize that the different solubilities of the derivatives caused some of them, the most hydrophobic, to precipitate and not fully dissolve, particularly for $PhOCH_3$, which solubility in DMSO is particularly low. DMSO is a polar aprotic solvent, but water, being more polar still, is further unable to dissolve the apolar dervatives. So, it is possible that the derivative concentrations present in the cultures are underestimated, accounting for some of the variability in the growth rate measurements.

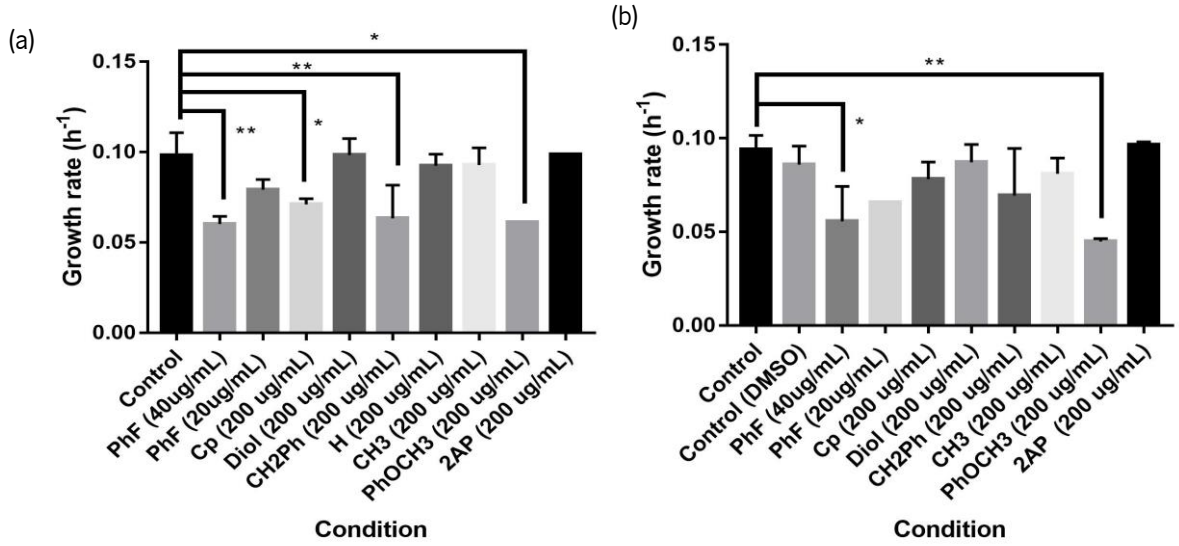

Figure 14 – Growth rate of *Schizosaccharomyces pombe* (wild type) cultures in EMM (a) and REMM (b) with 200 µg/mL of six purine derivatives (PhF, Cp, diol, CH_2Ph, H, CH_3, $PhOCH_3$) and 2AP, 40 and 20 µg/mL of PhF and 1 or 2 controls (no derivatives or their solvent added and only the solvent added (DMSO)). The cultures were incubated at 30 °C, 200 rpm and growth was monitored by measuring optical density at 600 nm. Results are presented as mean±SD from 3 independent replicas. (a) – Cultures in EMM. (b) – Cultures in REMM. Significance was analyzed by Dunnet's comparison test (* - p<0.05; ** - p<0,005)

Cultures of the mutant affected in *chk1* were also tested with most of the purine derivatives used for the wild type cultures (Figure 15). The PhF and $PhOCH_3$ derivatives showed decreased growth rates, while the addition of DMSO didn't change it, correlating with the previous results. Further, Cp and CH_2Ph derivatives didn't reveal a significant change in growth rate. This result is similar to wild type cultures grown in REMM, with similar implications, that these derivatives interact with the adenine/leucine pathways, but since this strain doesn't have a fully functioning pathway, the derivatives are unable to interfere with the cultures. Despite this, the *chk1* strain is known to be susceptible to genotoxicity [42], and as such, an increase in toxicity was expected. The previously mentioned solubility issue could partly explain these results.

Several of the tested purine derivatives seemingly are interacting with *E. coli* and *S. pombe* cells and causing decreased growth rates, however, the nature of these interactions is still unknown. If the interactions

are of a genotoxic nature, it can indicate that the cells are utilizing the derivatives as purine mimics for DNA repair and DNA replication or they are interfering with DNA-protein interactions, slowing or stopping the repair and replication pathways. If it's the former case, the derivatives' fluorescent proprieties could be used to track and follow these processes. The *chk1* strain was chosen, since the protein kinase Chk1 is an essential component to a major cell cycle checkpoint response, associated with DNA damage. The deletion of the responsible gene, *chk1*$^{+}$, causes an increased sensitivity to genotoxic effects and agents, due to defective cell cycle checkpoints failing to detect damaged DNA [42]. As such, if the tested derivatives are genotoxic, it's expected that this toxicity will be enhanced in the *chk1* strain, when compared to the wild type strain. However, the derivatives don't show significant difference in growth rate to either *S. pombe* strains (Figure 14 and 16), indicating that the toxicity is not of a genetic nature.

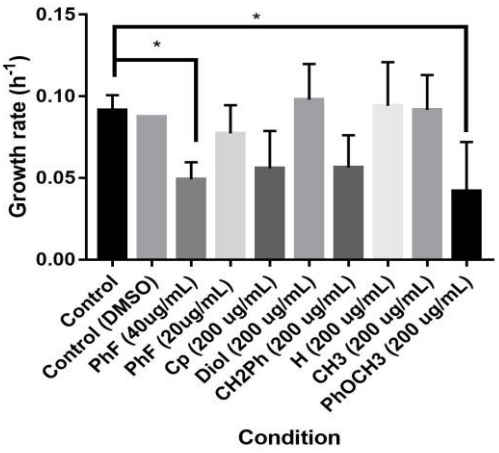

Figure 15 – Growth rate of *Schizosaccharomyces pombe* (chk1) cultures in REMM with 200 µg/mL of seven purine derivatives (Cp, diol, CH_2Ph, H, CH_3, $PhOCH_3$) and 2AP, 40 and 20 µg/mL of PhF and 2 controls: no derivatives or their solvent added (Control) and only the solvent added (Control (DMSO)). The cultures were incubated at 30 °C, 200 rpm and growth rate was monitored by optical density at 600 nm. Results are presented as mean±SD from different amounts of independent replicas. (PhOCH3, Cp, Ch_2Ph, H (2); Others (3)) and significance was analyzed by Dunnet's comparison test (* - p<(0.05)).

Finally, to determine if there is a dose-dependent response, the derivatives that caused a significant decrease in growth rate (Cp, CH_2Ph and $PhOCH_3$) were trialed with different concentrations (Figure 16). In this trial, two of the derivatives, Cp and CH_2Ph, didn't seem to show a dose-dependent response, while $PhOCH_3$ did, with larger concentrations causing a decrease in the growth rates in the cultures affected.

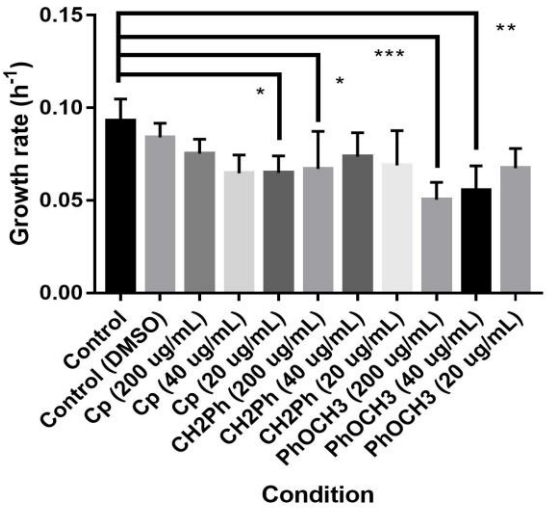

Figure 16 – Growth rate of *Schizosaccharomyces pombe* (wild type) cultures in EMM of three purine derivatives (Cp, CH_2Ph, $PhOCH_3$) with 3 different concentrations (200 μg/mL, 40 μg/mL and 20 μg/mL) and 2 controls: no derivatives or their solvent added (Control-) and only the solvent added (Control (DMSO)). The cultures were incubated at 30 °C, 200 rpm and growth rate was monitored by measuring optical density at 600 nm. Results are presented as mean±SD from 3 independent replicas. Significance was analyzed by Dunnet's comparison test (* - $p<0.05$; ** - $p<0.005$; ***- $p<0.0005$)

3.2. Fluorescence microscopy: Nuclei observation

The staining of cells with fluorochromes allows the viewing of specific cellular structures and molecules. In this case, DAPI, a stain that binds to double stranded DNA and only emits fluorescence when bound, allows the viewing of DNA inside the cells. Anomalies in the cell cycle can cause deformities on cell structure or abnormalities in the nuclei, such as elongated cells or malformed and numerous nuclei, which can be detected by using this technique. If the tested compounds are genotoxic, they could interfere with DNA replication or mitotic activity, and cause some of the mentioned abnormalities in the affected cells.

The same *S. pombe* cultures used in the toxicity assay were also stained with DAPI, and then viewed with fluorescence microscopy, in an attempt to find any genotoxic symptoms in the cells affected by the purine derivatives. In comparison with control cells, Cp, diol and 2AP didn't cause any visible abnormalities, (Figure 17b, 17c, 17d). Other compounds however, have a noticeable effect on cells, particularly PhF (Figure 17e, 17f), where its effect is visible in many cells, in the form of numerous nuclei-like structures per cell, with 3 or more in each cell, even at a lower concentration than other compounds, while in the other tested

derivatives, such as $PhOCH_3$ and CH_2Ph, the effects are sporadic and sparse, albeit at a higher rate than in control cells (not shown).

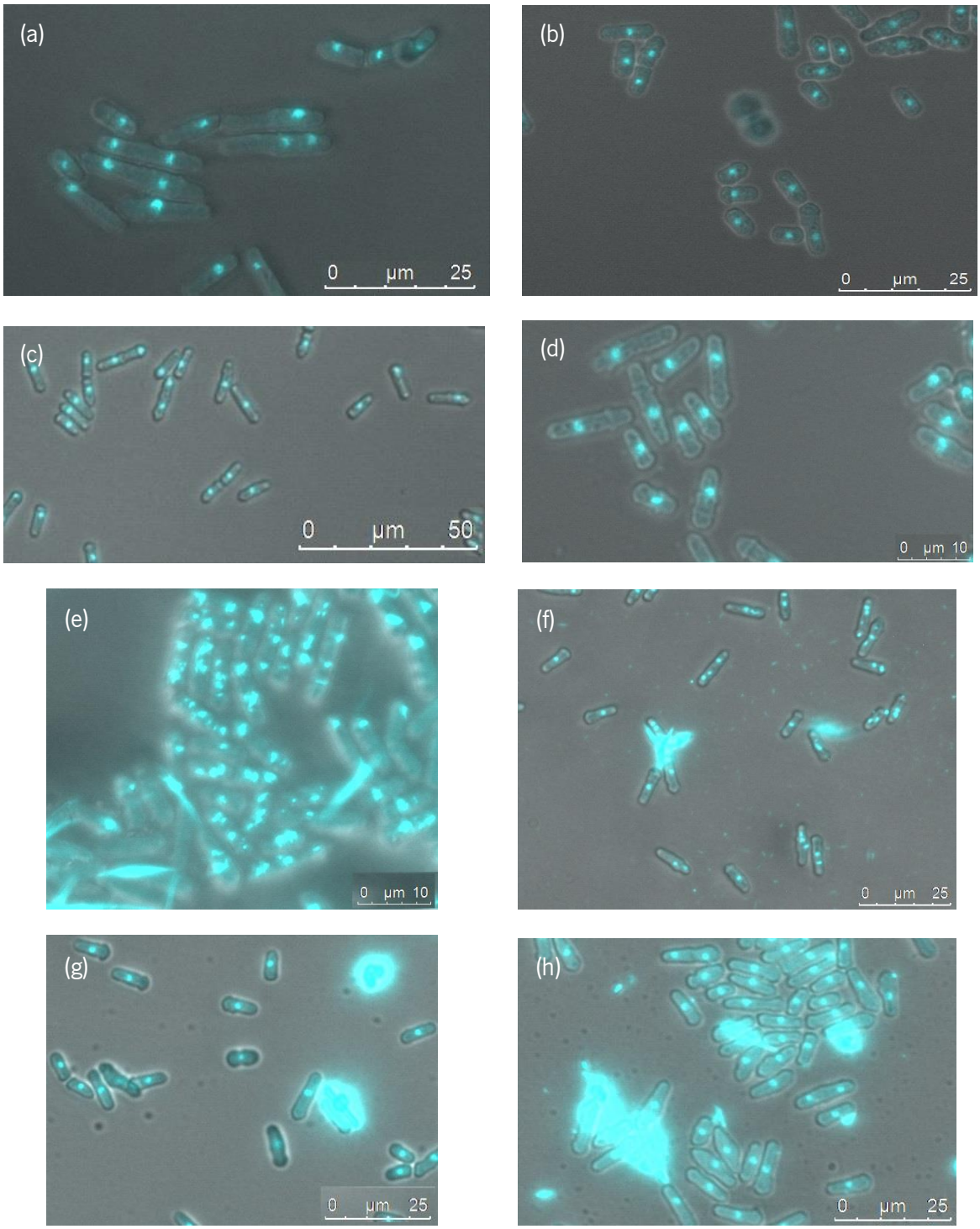

Figure 17 – Representative fluorescent microscopy images of *Schizosaccharomyces pombe* cultures with 200 µg/mL of purine derivatives and 2AP. The cultures were incubated at 30 °C, 200 rpm for approximately 4 h, then stained with DAPI. Artificial color is used. Control (a), Cp (200 µg/mL) (b), 2AP (c), diol (200 µg/mL) (d), PhF (40 µg/mL) (e), PhF (20 µg/mL) (f), PhOCH3 (200 µg/mL) (g), Ch2Ph (200 µg/mL) (h).

The inherent fluorescence of some the derivatives, such as PhF, $PhOCH_3$ and CH_2Ph was also observed and these derivates have a similar excitation and emission wavelengths to DAPI (Figure 17e, 17f, 17g, 17h). The negative control only showed the DAPI stained nuclei, while in cultures treated with the mentioned derivatives, we can see structures outside the cells that emit fluorescence (Figure 17a). Despite these interferences, we are still capable of observing the DAPI stained nuclei in the cells, allowing for analysis of their morphology.

Most derivatives did not affect cellular morphology, with no significant changes in cell shape or nuclear structure. Both the derivatives that didn't show toxicity, like diol (Figure 17d), or those that did, like $PhOCH_3$ (Figure 17g) didn't affect the cells. This suggests that their apparent toxicity is not targeting the genome or the cell-shape mechanisms.

Various unusual nuclei-like structures can be seen in each cell from the PhF derivative added cultures (Figure 17e, 17f), when compared to the nuclei of control cells (Figure 17a). This correlates with the observed, decreased growth rate of these cells (Figure 14), which could be explained with the derivative being toxic by interfering with the cell cycle. It's unclear whether or not these abnormalities are aggregates of the derivative inside the cells, or the presence of multiple nuclei, or both.

Derivatives with more apolar substituents have more potential to permeate the membrane than derivatives with polar substituents. In addition, the toxic derivatives also have the most apolar substituents, which correlates with their higher ability to permeate the plasma membrane. Despite this, derivatives with no substituent, like the H derivative, are more likely to be incorporated in DNA because this compound lacks large substituents in the position of the glycosidic bond with deoxyribose, allowing it to become a nucleotide.

3.3. Fluorescence microscopy: Derivative detection

In the previous experiment, we couldn't confirm the presence of any derivatives inside the cells themselves, just some of their effects. As such, in order to investigate this, the intracellular localization of derivatives was further studied, by using *S. pombe* cells exposed to the H and diol derivatives, and observing their intrinsic fluorescence with fluorescence microscopy in both non-fixed and heat-fixed cells. In this way, detection of fluorescence inside the cells would indicate that the compounds are permeating the plasma membranes and accumulating in their interior. After incubation of cells with the derivatives, the fluorescence

seemed to accumulate in specific structures in the cell (Figure 18a, 18c, 18f), while in heat-fixed cells, the emissions were scattered in the whole intercellular space (Figure 18b, 18d).

S. pombe is known to have a large amount of small vacuoles, that are affected by various types of stresses, leading to either their fission or fusion, in an effort to maintain cell homeostasis [46,47]. The organelles visible in our results resemble the morphology of vacuoles, suggesting to be the organelle where the derivatives are being accumulated. After heat fixation, the fluorescence remains, but it's spread across the entirety of the cell (Figure 18b, 18d).

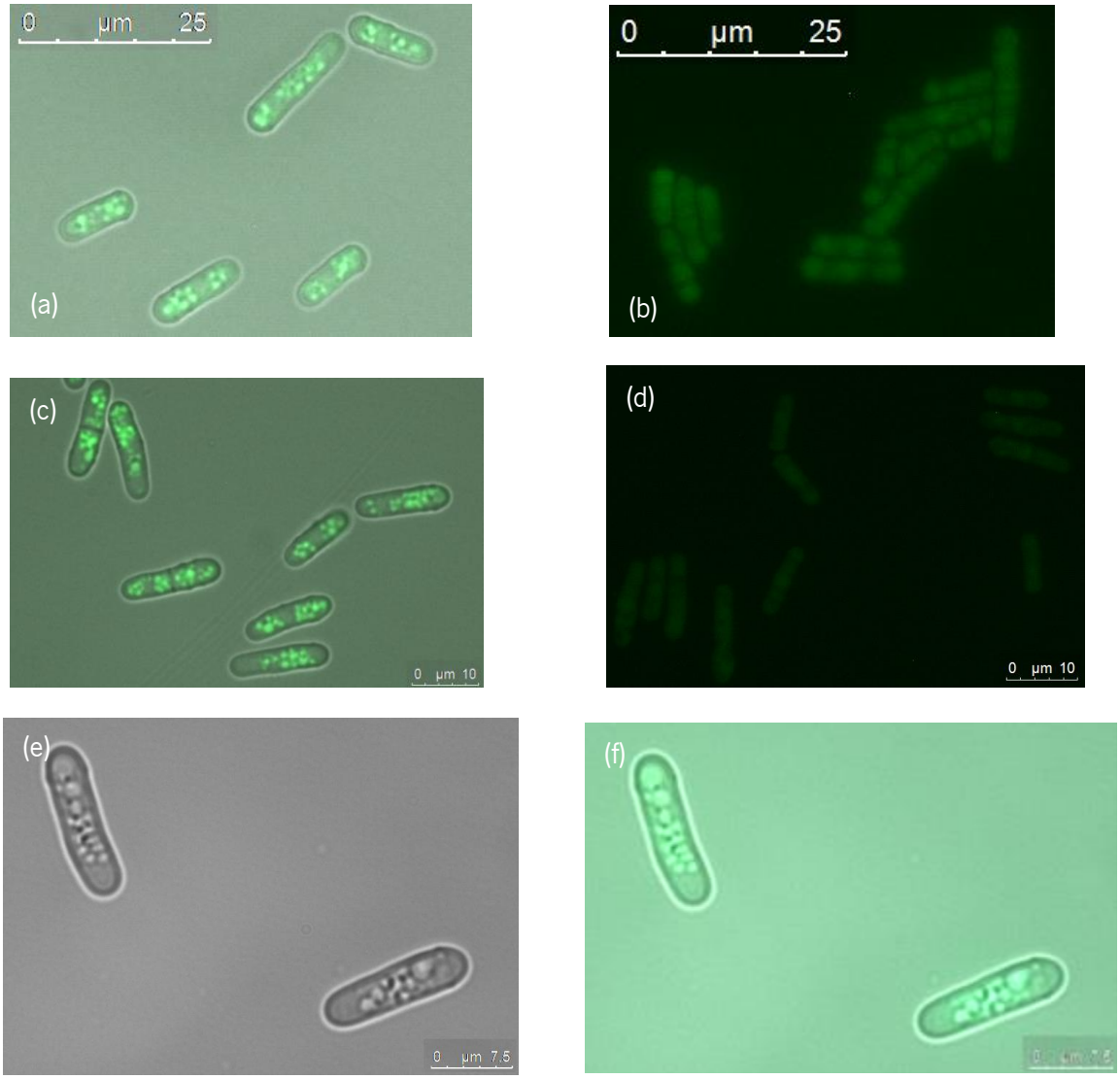

Figure 18 – Fluorescence microscopy images of *Schizosaccharomyces pombe* cultures in the presence of the H and Diol derivatives (200 µg/mL), at 30° C, 200 rpm, for 4 h. Notice that the derivatives are fluorescent when exposed to radiation with wavelength 395nm. (a) H (live cells); (b) H (heat fixed cells); (c) Diol (live cells); (d) Diol (heat fixed cells); (e) H (live cells, brightfield, 1000x); (f) H (live cells, ,1000x)

Upon heat-fixing, cellular metabolism stops, with subsequent arrest of synthesis of ATP. Therefore, cells are unable to produce ATP, which is needed to activate the ATP-dependent H^+ pumps in the vacuoles, causing the concentration of H^+ ions in the vacuoles to decrease. This can influence how the derivatives are partitioned in the cell; they may have a tendency to accumulate in the vacuoles due to the presence of H^+ ions causing protonation, making them ionized and losing their ability to permeate membranes, becoming entrapped in these organelles. In fixed cells, since the H^+ concentration is lower in the vacuoles, less protonation of the derivatives can occur and accumulation may stop, leading to them being disperse in the cytoplasm.

3.4. Fluorescence microscopy: acridine orange/propidium iodide dual assay

In an attempt to further characterize cell viability, an acridine orange/propidium iodide assay was used. With this dual labelling, we can simultaneously estimate the viability of cells since AO accumulates in all cells while PI is only able to permeate damaged, non-integral membranes, particularly those of dead cells. Therefore, cells exhibiting green (from AO) and red (from PI) fluorescence are suspected to have lost viability while cells with green fluorescence have integral and functional plasma membrane.

Fluorescence microscopy images reveal cells with two different fluorescent emissions, mostly green over orange, indicating that most cells in the cultures were viable (Figure 19). The toxicity assays shown that PhF causes culture growth to slow down, the nature of this being still unknown (Figure 14). The results obtained in this assay should correlate with the toxicity assay, with an increased amount of non-viable cells in the cultures exposed to the more toxic derivatives, like PhF, in comparison with the control cultures.

However, in the cultures exposed to PhF, even after 24h of culture growth, the number of non-viable cells didn't significantly increase in comparison with the control, with both less than 1% of cells being non-viable (Figure 19b, 19d, 20). This is not incompatible with the results of the toxicity assay, where PhF revealed significant lower growth rates, since toxicity can still be present in viable cells, with slower proliferation, without causing the cells to become non-viable. Unexpectedly, a higher number of non-viable cells was found in the diol exposed culture, close to 4% (Figure 20), even though diol didn't show a significant decrease in growth rate in the toxicity trials (Figure 14). This increase of non-viable cells could be related with the concentration of the derivatives used, with a higher concentration of diol used (200 μg/mL) than PhF (40 μg/mL). However, just a single assay was performed, and these results, to be properly considered, would require more replicas.

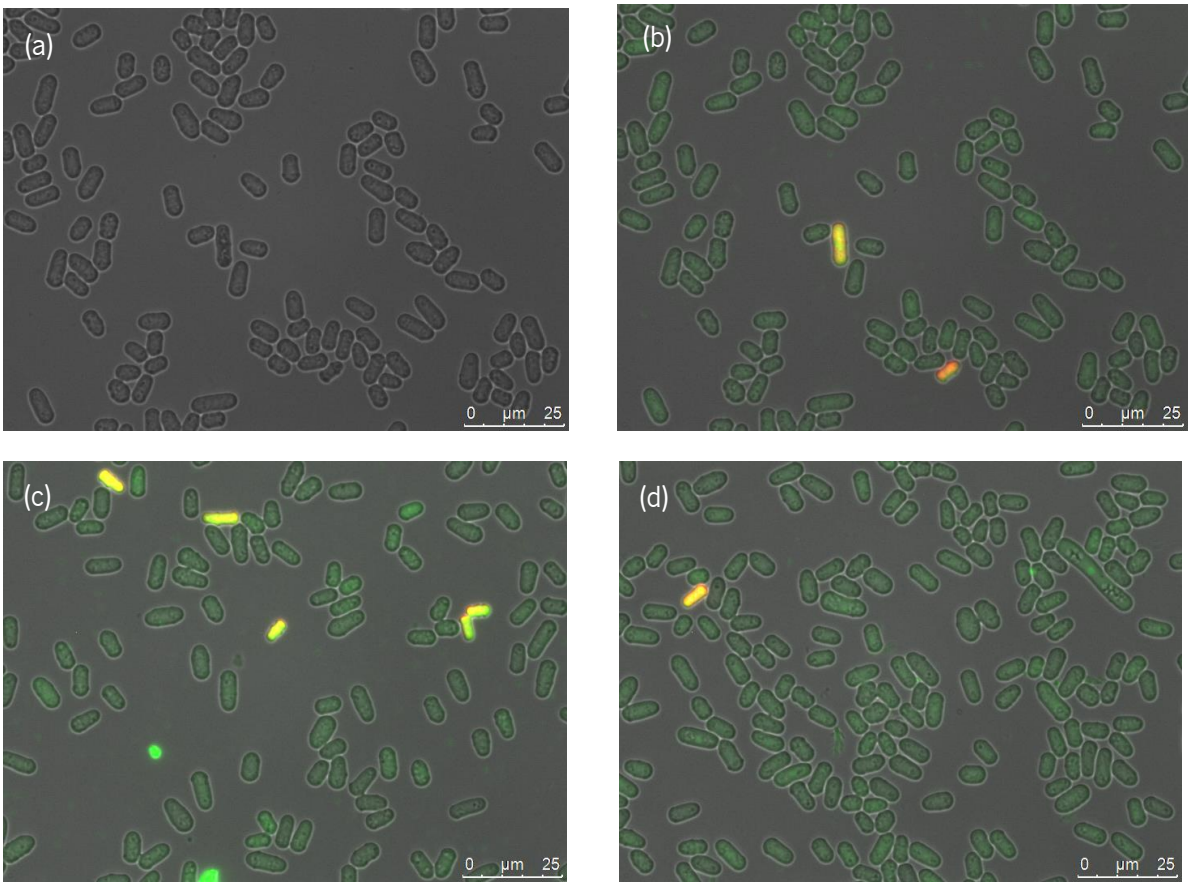

Figure 19 – Fluorescence microscopy images of *Schizosaccharomyces pombe* cultures after 24h in the presence of different derivatives and acridine orange/propidium iodide at 30° C, 200 rpm, with fluorescence images being overlays of AO and PI emissions with bright field images. (a) Control (Bright field); (b) Control (overlay); (c) Diol (200µg/mL) (overlay); (d) PhF (40µg/mL) (overlay).

Analysis of the fluorescence microscope images also revealed several structures in the interior of the cells, with AO staining the various structures differently, with brighter and darker spots (Figure 21b). AO in neutral pH emits green fluorescence, however, in lower pH environments, like in cell vacuoles, it suffers protonation, resulting in a change in emission from green to orange/red [48]. The lack of green fluorescence in some of the structures might indicate that these have a lower pH, since AO changes its emission from green to red in these conditions, and as such these structures are suspected to be the vacuoles. The brighter spots could be organelles with double-stranded DNA (dsDNA), since AO is able to bind dsDNA with emission of green fluorescence.

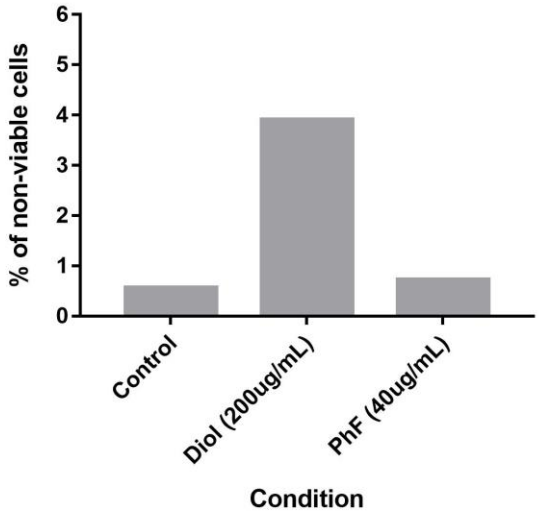

Figure 20 –Acridine orange/propidium iodide assay cell counts of *Schizosaccharomyces pombe* cultures, grown in the presence of different derivatives, diol (200 µg/mL) and PhF (40 µg/mL), and a negative control, incubated at 30 °C, 200 rpm for 24 hours. Percentage of non-viable cells was determined based on number of cells with yellow/red fluorescence in relation to the total number of cells. Results are presented from a single assay replica.

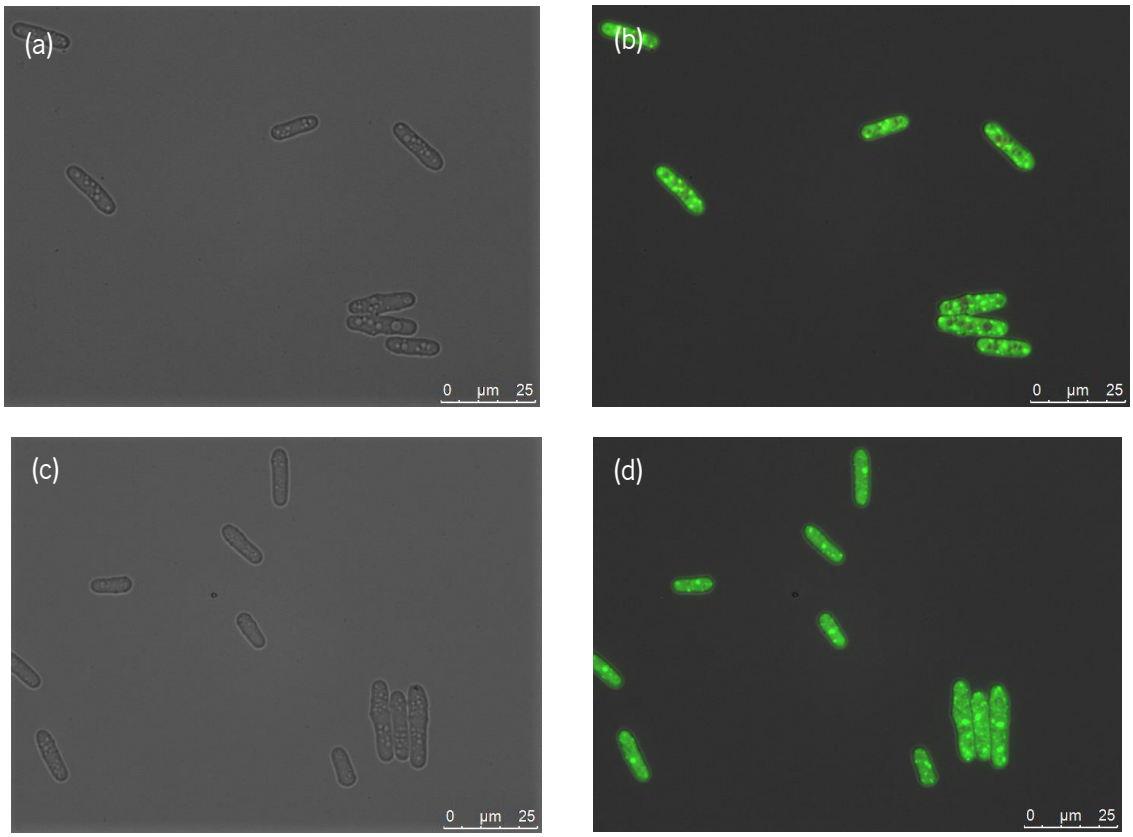

Figure 21 – Fluorescence microscopy images of *Schizosaccharomyces pombe* cultures in the presence of the Diol derivative (200 µg/mL) and stained with acridine orange/propidium iodide at 30° C, 200 rpm, for 4 h. (a) Control (bright field); (b) Control (fluorescence); (c) Diol (bright field); (d) Diol (fluorescence).

In the diol treated cells, similar structures to the control cells are also present, however, the previously mentioned vacuoles seem to have some green fluorescence (Figure 21d). This suggests that the diol presence could be arresting H+ pump activity in the vacuoles, causing their pH to increase, allowing for AO to retain its green fluorescence in the vacuoles. In addition, diol seemed to accumulate in the vacuoles (Figure 18c), and possibly its fluorescence could also cause the vacuoles to be brighter.

4 – Conclusion and future perspectives

Fluorescent nucleobases are critical for the understanding of the dynamic structures and functions of DNA and RNA. Many different fluorescent nucleobases were developed over the years, with different properties, to be used in different assays and experiments. Some of these resemble the canonical nucleobases, like 2-aminopurine, allowing for base pairing and incorporation into DNA helices, while others have different structures, such as purine ring fusions, that are too large to be incorporated by normal cellular means, but boast a broader range of fluorescent emission ranges. 2-aminopurine is one of the most used fluorescent nucleobases, and many derivatives of this nucleobase were produced, to expand or change its fluorescence and chemical proprieties. 2AP can also be used as a mutagenic agent, so its utility goes beyond its fluorescent proprieties.

In this work, several derivatives based on 2-aminopurine recently developed with potentially useful fluorescent proprieties were tested for their cellular toxicity and accumulation, but their potential to be used as probes for biological applications is to be further ascertained. A growth rates assay showed that some of these derivatives show toxicity, indicating that these derivatives are interacting with live cells. The toxicity is associated with the size and polarity of the radical attached, with larger, apolar radicals being the most toxic. Fluorescence microscopy results suggest that several derivatives are capable of entering *S. pombe* cells. 2 of the derivatives (H and diol) accumulated in *S. pombe* cellular structures resembling vacuoles by detection of their own fluorescence. Despite these conclusions, the mechanisms of integration and accumulation are not yet explored and the nature of their toxicity is still unknown. These questions serve as a starting point for further studies [49].

First, a continuation of the AO/PI cell viability double staining assay, with an increased number of replicas and statistical analysis, to further complement the toxicity assays.

To determine the nature of the toxicity, flow cytometry could be used to analyze the cell cycle in cells cultivated in the presence of the derivatives. Derivative treated cells, if the derivatives are genotoxic, should have slower or a complete stop of the cell cycle.

For further characterization of the derivatives' mutagenicity as a consequence of genotoxicity, an AMES test or red/white segregation colony assay could be used. The presence of the derivatives, if mutagenic, should cause an increase in revertant colonies in both tests, in a dose-related manner [50].

An *in vitro* approach to detect DNA incorporation of the derivatives could be made using *in vitro* polymerization reactions, utilizing the derivatives as nucleotides alongside the canonical ones. For these assays, a previous step of synthesis of the activated triphosphate form of the derivatives could be prepared by enzymes involved in nucleotide synthesis in cell free extracts.

The vacuole interactions of the tested derivatives opens potential for cellular biology studies and applications about vacuole dynamics, and the corresponding in mammalian counterpart, the lysosomes. These are related to many phenomena, and impact autophagy under cellular caloric restriction, which are related to life span in *Saccharomyces cerevisiae* [51,52], *Caenorhabditis elegans* and *Drosophila melanogaster* [52]. They are also related to several diseases, from early onset disorders, such as I-cell disease (mucolipidosis II), to neurodegenerative disorders like Parkinson's and Alzheimer's disease, among others [53].

5 - Bibliography

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Appendix 1

-50x salt stock

52.5 g/L $MgCl_2 * 6\ H_20$
0.735 g/L $CaCl_2 * 2\ H_20$
50 g/L KCl
2 g/L Na_2SO_4

-1000x vitamin stock

1 g/L pantothenic acid
10 g/L nicotinic acid
10 g/L inositol
10 mg/L biotin

-10000x mineral stock

5 g/L boric acid
4 g/L $MnSO_4$
4 g/L $ZnSO_4 * 7\ H_2O$
2 g/L $FeCl_2 * 6\ H_2O$
0.4 g/L molybdic acid
1 g/L KI
0.4 g/L $CuSO_4 * 5\ H_2O$
10 g/L citric acid